

Relationship Between Decay Accelerating Factor Deficiency, Diminished Acetylcholinesterase Activity, and Defective Terminal Complement Pathway Restriction in Paroxysmal Nocturnal Hemoglobinuria Erythrocytes

M. E. Medof, A. Gottlieb, T. Kinoshita, S. Hall,* R. Silber, V. Nussenzweig, and W. F. Rosse*
Department of Pathology, New York University Medical Center, New York 10016; and *Department of Medicine, Duke University Medical Center, Durham, North Carolina 27710

Abstract

Paroxysmal nocturnal hemoglobinuria (PNH) erythrocytes exhibit abnormalities in decay accelerating factor (DAF), acetylcholinesterase, and resistance to autologous C5b-9 attack. To investigate the nature of the lesion underlying PNH cells, we examined the relationship of these abnormalities to one another. Analyses of DAF in acetylcholinesterase-negative erythrocytes revealed that these two abnormalities involve functionally independent molecules, coincide precisely in the same cell populations, and are similarly expressed in PNH II and more complement-sensitive PNH III erythrocytes. The DAF and acetylcholinesterase deficiencies contrast with the C3b/C4b receptor (CR1) deficit, which is less profound and similarly distributed in complement-insensitive cell populations. Hemolytic studies showed that defective resistance to autologous C5b-9 attack is mediated by another mechanism. Whereas reconstitution of PNH II erythrocytes with DAF completely corrected their complement sensitivity, DAF reconstitution of PNH III erythrocytes restored their ability to circumvent C3b uptake but had no effect on their heightened susceptibility to reactive lysis. Assays of complement-insensitive (PNH I) erythrocytes surviving after reactive lysis disclosed partial DAF and acetylcholinesterase deficits. These findings indicate that the PNH lesion involves multiple membrane components and that PNH I erythrocytes are also abnormal.

Introduction

Paroxysmal nocturnal hemoglobinuria (PNH)¹ is an acquired disorder in which variable proportions of blood elements sensitive to the lytic action of autologous complement appear in the circulation (reviewed in reference 1). It is thought that the

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Dr. Medof's current address is Department of Pathology, Case Western Reserve University, Cleveland, OH 44106.

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1. *Abbreviations used in this paper:* AChE, acetylcholinesterase; C-EDTA, C3-9 reagent; CLS, complement lysis sensitivity; CR1, C3b/C4b receptor; DAF, decay accelerating factor; E^{hu}, human erythrocyte; E^{hu}A, E^{hu}AC1, E^{hu}AC14, and E^{hu}AC1423, complement intermediates bearing respective components; FACS, fluorescence-activated cell sorter, NP40, Nonidet-P40 detergent; PNH, paroxysmal nocturnal hemoglobinuria.

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aberrant circulating cells originate from the clonal proliferation of bone marrow progenitors altered by somatic mutation. Considerable experimental data have shown that their complement sensitivity is due to intrinsic defects in their cell membranes. Two groups of investigators found that they are deficient (2-4) in a complement regulatory protein termed decay accelerating factor (DAF) (5-11), establishing a molecular basis for their abnormal interaction with the complement system.

Recently, it was observed that purified DAF could be incorporated into cell membranes *in vitro* (8) and that, once cell-associated, the exogenous DAF could move freely in the plane of the membrane and exert its activity. It was shown from studies exploiting this phenomenon that *in situ* DAF could prevent the assembly of C3 and C5 convertases of both complement pathways on cell surfaces and thereby could protect cells from complement attack. To examine the role of DAF deficiency in the lytic abnormalities of PNH cells, DAF was restored to affected PNH erythrocytes (E^{hu}) (12). Reconstitution of the DAF-deficient PNH E^{hu} with DAF corrected their excessive C3b uptake. The DAF reconstitution, however, had only a partial effect on acid lysis susceptibility of the affected cells. Although physiologic DAF levels were not achieved in these studies, this finding raised the possibility that other membrane defects that could preclude complete correction of the lytic sensitivity by DAF might exist in the PNH cells.

Several alterations have been reported in the membranes of PNH E^{hu} (1). Affected cells not only take up excessive amounts of C3b, but they are characteristically deficient in membrane acetylcholinesterase (AChE). Additionally, whereas PNH II E^{hu}, found in some patients, are three- to fivefold more sensitive to complement than normal E^{hu}, PNH III E^{hu}, present in the majority of patients, are 15- to 25-fold more sensitive. The PNH III E^{hu} are markedly more susceptible than normal E^{hu} to bystander lysis by fluid-phase terminal pathway complexes (e.g., C5b-9). There is experimental evidence suggesting that the vulnerability of PNH III E^{hu} to this passive lysis may be due to a defect in the capacity of the affected cells to restrict insertion and/or polymerization of autologous C9 (13-17) as well as to a defect in their ability to circumvent uptake of C3b.

Whether DAF and AChE deficiencies overlap in affected PNH E^{hu} and are separate abnormalities and whether either is involved in the heightened sensitivity of PNH III cells to bystander lysis are not known. The present study was undertaken to investigate the relationship of these three abnormalities in affected PNH E^{hu}.

Methods

Buffers, reagents, and labeling. Isotonic veronal contained 2.5 mM barbital, pH 7.4, 73 mM NaCl, 2.5% dextrose, 0.15 mM CaCl₂, 0.5 mM MgCl₂, and 0.1% gelatin. Isoionic veronal contained 146 mM NaCl in

place of 73 mM NaCl and 2.5% dextrose. Metal-chelating veronal was prepared as isoionic veronal but contained 10 mM EDTA in place of metals. Phosphate-buffered saline (PBS) contained 10 mM sodium phosphate, pH 7.4, and 150 mM NaCl.

Nonidet-P40 detergent (NP40) was purchased from Particle Data, Inc. (Elmhurst, IL), phenylmethylsulfonyl fluoride and Clostridium neuraminidase (type VI) from Sigma Chemical Corp. (St. Louis, MO), and the synthetic elastase inhibitor, Suc-(OMe)-Ala-Ala-Pro-Val-MCA, from Peninsula Laboratories Inc. (Belmont, CA).

Proteins and E^{hu} were labeled by Iodogen (Pierce Chemical Co., Rockford, IL) according to the manufacturer's instructions. In the case of the cells, 0.5 mCi ¹²⁵I per 10⁹ E^{hu} was used, and the procedure described in reference 18 followed.

Purified proteins and antibodies. C2 (19), C4 (20), C3 (20), C5 (20), C6-9 (21), guinea pig C1 (22), and guinea pig and human C3-9 (C-EDTA) (22) were prepared as described. Cobra venom factor was purified by the method in reference 23.

DAF was isolated from butanol extracts of pooled E^{hu} stroma as described (8, 9). The purified product had characteristic mobility on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (see inset of Fig. 1, reference 8); it was devoid of glycophorin as assessed by Western blotting using antiglycophorin antibodies, and by SDS-PAGE and radioautography after radiolabeling. For some experiments, DAF was concentrated and the NP40 concentration reduced to 0.0006% by affinity chromatography employing Sepharose coupled to (IA10) monoclonal anti-DAF antibodies. The anti-DAF conjugate was cross-linked with glutaraldehyde, and the column eluted with 0.05 M diethylamine, 0.025 M Tris, 0.020 M EDTA, 0.0006% NP40, pH 11.5 buffer. Human C3b/C4b receptor (CR1) was purified from 1% NP40 extracts of pooled E^{hu} stroma by chromatography on Biorex 70 (24) followed by affinity chromatography on Sepharose coupled to (44D) monoclonal anti-CR1 antibody (25). The isolation was performed as described for DAF except that bound CR1 was eluted from the anti-CR1 conjugate with 3.0 M KSCN.

Rabbit IgM hemolysin was a gift of Dr. Eric Brown (Washington University, St. Louis, MO). Rabbit anti-glycophorin antibodies were prepared as described in reference 8; monoclonal antibodies to glycophorin A were raised from a hybridoma donated by Dr. Margaret Nichols

(New York Blood Center). Serum containing anti-I cold agglutinin was obtained from patient J.S. with chronic cold agglutinin disease (26).

Rabbit anti-DAF antibodies were prepared as in reference 8. They were monospecific as assessed by immunoblotting with crude extracts of E^{hu} stroma. Rabbit anti-(E^{hu}-associated)-AChE antibodies were a gift of Dr. Lucio Luzzatto (University of Naples, Naples, Italy). Mouse monoclonal anti-DAF antibodies (IA10, I1H6, and VIII A7) were obtained as described in reference 9. Hybridomas (AE1 and AE2) producing monoclonal anti-(E^{hu})-AChE antibodies (27) were obtained from the American Tissue Type Collection (Rockville, MD). Monoclonal anti-CR1 antibodies (44D, 57F, 31D, and 57H) (24) and control anti-malaria antibodies (3D11, 2G3, 6G2, and 2A10) of appropriate subtypes were prepared as described (28). Anti-DAF, -CR1, and -malaria monoclonals were purified by (NH₄)₂SO₄ precipitation and DEAE-Sepharose chromatography. Anti-AChE monoclonals were used as ascites fluids.

Fluorescein-isothiocyanate-conjugated goat F(ab')₂ anti-mouse Ig and rabbit anti-mouse Ig were from Cappel Laboratories (Cochranville, PA), protein-A agarose suspension from Zymed Labs Inc. (San Francisco, CA), and protein-A Sepharose CL-4B from Pharmacia, Inc. (Piscataway, NJ).

Measurements of DAF and CR1 levels. Levels of DAF and CR1 in E^{hu} were quantitated by two-site radioimmunoassays employing the respective monoclonal antibodies as described in references 9 and 25. E^{hu} samples were lysed with 1% NP40 (at 5 × 10⁹/ml in the case of DAF and 2 × 10¹⁰/ml in the case of CR1), diluted 10-fold (in PBS containing 1 mM phenylmethylsulfonyl fluoride and 50 μg/ml synthetic elastase inhibitor), and clarified by centrifugation. The amounts of DAF or CR1 in the E^{hu} samples were determined by reference to standard curves generated using increasing amounts of the respective purified membrane proteins.

Surface expression of DAF on E^{hu} was assessed by flow cytometry using a fluorescence-activated cell sorter (FACS) (Ortho Diagnostics, Westwood, MA) as described in reference 9. The E^{hu} (10⁶ cells in 25 μl of PBS containing 1% bovine serum albumin [BSA]) were incubated on ice for 30 min with pooled monoclonal anti-DAF antibodies (5 μg/ml each in 25 μl of the same buffer) or, as a control, with a mixture of nonrelevant (antimalaria) monoclonal antibodies at the same concentrations. After washing, the E^{hu} were resuspended to 25 μl in PBS containing 1% BSA, and incubated on ice for 30 min with an equal volume of a 1:50 dilution of fluorescein-isothiocyanate-goat F(ab')₂ anti-mouse Ig. The treated cells were washed again and resuspended to 1 ml in the same buffer.

Hemolytic assays. Efficiency of classical C3 convertase, C4b2a, assembly on E^{hu} was assessed as described in reference 12. E^{hu} were sensitized with rabbit IgM hemolysin to provide 50 C1 fixing sites per cell (29). After washing, the E^{hu}A (1 × 10⁸/ml in isotonic veronal buffer) were incubated at 30°C for 15 min with C1 at a concentration 10-fold higher than that required for maximum uptake of C4. Washed E^{hu}AC1 (1 × 10⁸/ml in isotonic veronal) were incubated for 15 min at 30°C with increasing concentrations of C4. The number of C4b molecules deposited at each C4 input was determined by employing ¹²⁵I-labeled C4 in parallel studies. The E^{hu}AC14 (1 × 10⁸/ml in isotonic veronal) bearing increasing numbers of C4b per cell were mixed with C2 adjusted to a concentration 10-fold higher than that giving maximum lysis of E^{hu}AC14 prepared with limited C4 and treated with anti-DAF antibodies. After incubation at 30°C for 5 min, hemolytic activity was developed by addition of 1.8 ml of C3-9 reagent (C-EDTA 1:20 in metal-chelating veronal buffer) and further incubation at 37°C for 60 min.

Complement lysis sensitivity (CLS) of E^{hu} was assessed as described by Rosse and Dacie (26, 30). E^{hu} (2 × 10⁸/ml in isotonic veronal buffer) were mixed at 37°C with an equal volume of anti-I serum (1:50 in 0.15 M NaCl). 50-μl aliquots of the E^{hu} anti-I mixtures were added to increasing volumes of 1:270, 1:90, 1:30, or 1:10 normal human serum. The resulting mixtures were preincubated at 0°C for 30 min, placed at 37°C, and after 1 h isoionic veronal buffer was added, the tubes centrifuged, and lysis read. The relative sensitivity of the cells was expressed in CLS H50 units (26).

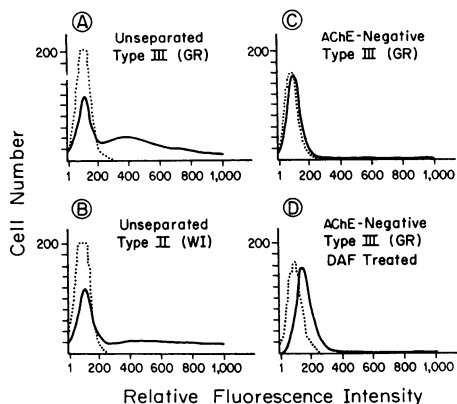


Figure 1. FACS analyses of DAF in unseparated and purified AChE-negative PNH E^{hu} before and after reconstitution of the cells with exogenous DAF. The untreated or DAF-treated E^{hu} were stained with a mixture of three monoclonal anti-DAF antibodies followed by fluorescein isothiocyanate-labeled goat F(ab')₂ anti-mouse Ig. *Dashed lines* show the background fluorescence of control cells stained with three nonrelevant antimalaria monoclonal antibodies in place of the anti-DAF monoclonals. (A) Unseparated E^{hu} from patient W.I. with PNH II E^{hu}; (B) unseparated E^{hu} from patient G.R. with PNH III E^{hu}; (C and D) purified AChE-negative E^{hu} from patient G.R. before and after DAF treatment.

Susceptibility of the E^{hu} to reactive lysis was assessed using cobra venom factor as described in reference 23. Portions (100 µl) of E^{hu} (1 × 10⁸/ml in metal-chelating veronal buffer) were mixed with equal volumes of fresh human serum diluted 1:4 to 1:64 in the same buffer. Cobra venom factor:Bb complexes, prepared by incubation of cobra venom factor (1 mg/ml in isoionic veronal buffer) at 37°C for 2 h with an equal volume of (neat) serum, were diluted 1:32 in metal-chelating veronal buffer, and 50-µl aliquots of the diluted complexes were added to the various E^{hu} serum mixtures. The reaction tubes were placed at 37°C, and after 1 hr, 4 ml of metal-chelating veronal buffer was added, the tubes centrifuged, and lysis read.

Immunoprecipitation, SDS-PAGE, and autoradiography. The NP40 extract of 10⁹ ¹²⁵I-surface-labeled E^{hu} was diluted to 1 ml with PBS and cleared by rotation at 0°C for 30 min with 10% vol/vol protein A-agarose suspension in 15 mM NaCl, 9 mM EDTA, 50 mM Tris, pH 7.4, containing 0.05% NP40 and 1 mg/ml ovalbumin. After centrifugation, 250-µl aliquots of the supernatant were rotated at 0°C for 30 min with pooled monoclonal anti-DAF antibodies (IA10, I1H6, and VIII A7), 5 µg/ml each, individual anti-AChE antibodies (AE1 or AE2), ~ 5 µg/ml each, or with pooled antimalaria monoclonals (3D11, 2G3, 2A10), 5 µg/ml each. After rotation at 0°C for 30 min, immune complexes were precipitated by addition of 100 µl of protein A-agarose suspension and centrifugation. After washing twice in 15 mM NaCl, 9 mM EDTA, 50 mM Tris, pH 7.4, containing 0.05% NP40, transfer to new tubes, and washing again in the same buffer, precipitated proteins were eluted by resuspending the agarose beads in 50 µl of SDS-PAGE loading buffer containing 20% 2-mercaptoethanol, boiling for 3 min, and collection of the supernatants.

SDS-PAGE analysis of eluted proteins was carried out on 7.5% slab gels according to the method of Laemmli (31). After fixation and drying, autoradiographs of the gels were obtained at -70°C with X-Omat XAR-5 film (Eastman Kodak Co., Rochester, NY).

Purification of AChE-negative E^{hu}. AChE-negative E^{hu} from PNH patients were separated as described in reference 32. 25 µl of AE1 monoclonal anti-AChE ascites fluid was added to 2 ml of E^{hu} from the patients (1 × 10⁹ E^{hu}/ml in PBS) and the mixtures rocked at 20°C for 30 min. The cells were pelleted, washed twice in PBS containing 1% BSA, and mixed with 1 ml of rabbit anti-mouse IgG. After another incubation at

20°C for 30 min and thorough washing in the same buffer, the cells were loaded onto protein A Sepharose CL-4B (2 × 10⁸ E^{hu}/ml packed gel) equilibrated at 20°C in PBS. The column was opened and unbound cells collected by washing with 1 ml of PBS followed by PBS containing 1% BSA.

Patients. PNH patients were followed at Duke University Medical Center. All had consistent clinical presentations and characteristic abnormalities of their E^{hu} in the Ham and/or CLS assays.

Results

Relationship between deficiencies of DAF and of AChE in PNH E^{hu}. Recent analyses of PNH E^{hu} with monoclonal anti-AChE antibodies (32) have shown that diminished membrane AChE enzyme activity (33) is due to deficiency in affected cells of the membrane AChE enzyme molecule. Initial studies were performed to establish whether the AChE deficiency coincides in the same cells as DAF deficiency, whether expression of the two defects differs in PNH II and PNH III E^{hu}, and whether the two defects are separate abnormalities.

In the first series of experiments, the distributions of the two defects in PNH II and PNH III E^{hu} were examined by purifying AChE-negative E^{hu} from patients by cell-affinity chromatography (see Methods) and assaying them for DAF content by two-site radioimmunoassay employing anti-DAF monoclonals. Table I shows the results in relation to DAF levels measured in unseparated E^{hu} from the same patients. In all eight patients studied, DAF levels in the purified AChE-negative cell populations were zero, indicating that the DAF and AChE deficits in PNH coincide in the same cells. PNH II and PNH III AChE-negative E^{hu} were both totally DAF-deficient, indicating that the differences in complement sensitivity between these two classes of PNH E^{hu} do not reflect differences in DAF contents. DAF levels in the AChE-positive (adherent) E^{hu} subpopulation from each of the patients were calculated by dividing the total DAF

Table I. DAF and CR1 Levels in PNH Erythrocytes

Patient ID	% Affected cells	Unfractionated erythrocytes		Purified AChE-negative erythrocytes		AChE-positive erythrocytes
		DAF	CR1	DAF	CR1	DAF*
		<i>molecules/cell</i>	<i>ng/10⁹</i>	<i>molecules/cell</i>	<i>ng/10⁹</i>	<i>molecules/cell</i>
PNH III						
M.A.	50	482	162	0	121	964
D.I.	58	361	231	<10	205	859
P.H.	34	963	158	<10	185	1459
J.F.	60	740	165	0	198	1850
F.R.	35	568	162	0	206	873
PNH IIIb[†]						
G.R.	52	1471	244	0	253	3065
PNH II						
W.I.	42	1066	99	<10	—	1837
B.A.	90	172	180	0	130	1710
Normals						
\bar{x}		2600 [‡]	376			
SD		320	144			

* By calculation. † Comparable sensitivity to PNH III in reactive lysis but sensitivity to classical pathway lysis intermediate between PNH II and III. ‡ n = 35 (reference 9). || n = 10.

present in the unfractionated E^{hu} by the proportion of DAF-containing cells in the sample and are shown for comparison in the last column of the table. In all but one case, these levels were also reduced and, in three cases, were $< 50\%$ of normal indicating that a less pronounced DAF defect was present in the AChE-positive cells of most patients. CR1 contents of the unfractionated and the purified AChE-negative cells of the patients, as determined by a parallel two-site radioimmunoassay employing anti-CR1 monoclonals, are additionally shown. Although CR1 levels in the unfractionated E^{hu} of all cases were diminished, the extent of CR1 reduction in the AChE-negative and -positive E^{hu} populations of each patient were comparable. The CR1 abnormality thus constitutes a less severe deficiency that does not segregate with AChE or DAF deficiency.

In the next series of experiments, the overlap of DAF deficiency in the AChE-negative E^{hu} population of each patient was verified in FACS analyses. The results of a representative study are shown in Fig. 1. The distributions of DAF in unfractionated E^{hu} from two of the patients and in AChE-negative cells from one of them are shown. Consistent with the previous findings, DAF-deficient PNH II E^{hu} (Fig. 1 A) appeared identical to DAF-deficient PNH III E^{hu} (Fig. 1 B) with respect to extent of DAF deficiency, both coinciding with the negative controls. Purified AChE-negative cells from the latter patient (Fig. 1 C) comprised a single population containing no DAF-bearing cells. Control studies with anti-AChE monoclonals revealed no AChE-positive E^{hu} . Identical results were obtained in FACS analyses of AChE-negative E^{hu} from all other patients studied.

The relationship between the proteins responsible for AChE and DAF activities was next examined in studies employing anti-DAF and anti-AChE monoclonals. First, structural studies were performed. Normal E^{hu} were surface-labeled with ^{125}I , and the washed stroma extracted with 1% NP40. Aliquots of the extract were immunoprecipitated with pooled monoclonal anti-AChE, anti-DAF, or nonrelevant (antimalaria) antibodies, and immunoprecipitated proteins were analyzed by SDS-PAGE followed by radioautography. As shown in Fig. 2, the anti-AChE and anti-DAF monoclonals precipitated distinct proteins that differed slightly in apparent M_r . Parallel studies with neuraminidase-pretreated E^{hu} revealed a decrease in the apparent M_r of DAF but not of AChE. In a second set of experiments functional analyses were carried out. Preincubation of the normal E^{hu} AC14 with polyclonal (100 $\mu\text{g}/\text{ml}$ IgG) or pooled monoclonal (100 $\mu\text{g}/\text{ml}$ each) anti-AChE antibodies had no effect on the ability of the cells to restrict C4b2a assembly and subsequent lysis upon addition of human C2 and heterologous guinea pig C3-9 (a source of terminal pathway components not inhibited by human cell factors which can be used to assay uptake of autologous C3b [13, 14]). In contrast, under the same conditions, $< 5 \mu\text{g}/\text{ml}$ each of pooled monoclonal anti-DAF antibodies completely overcame membrane DAF activity and rendered the cells susceptible to lysis (not shown). Alternatively, pretreatment of normal E^{hu} with 50 $\mu\text{g}/\text{ml}$ polyclonal anti-DAF antibodies rendered them PNH-like in acid lysis (12) but had no effect on membrane AChE enzyme activity, whereas treatment with 5 $\mu\text{g}/\text{ml}$ of anti-AChE antibodies completely abrogated enzyme function (32). Conversely, reconstitution of AChE-deficient PNH E^{hu} with DAF did not restore AChE enzyme activity to the cells. The deficiencies of DAF and of AChE in affected PNH E^{hu} thus constitute defects in two separate entities with independently mediated activities.

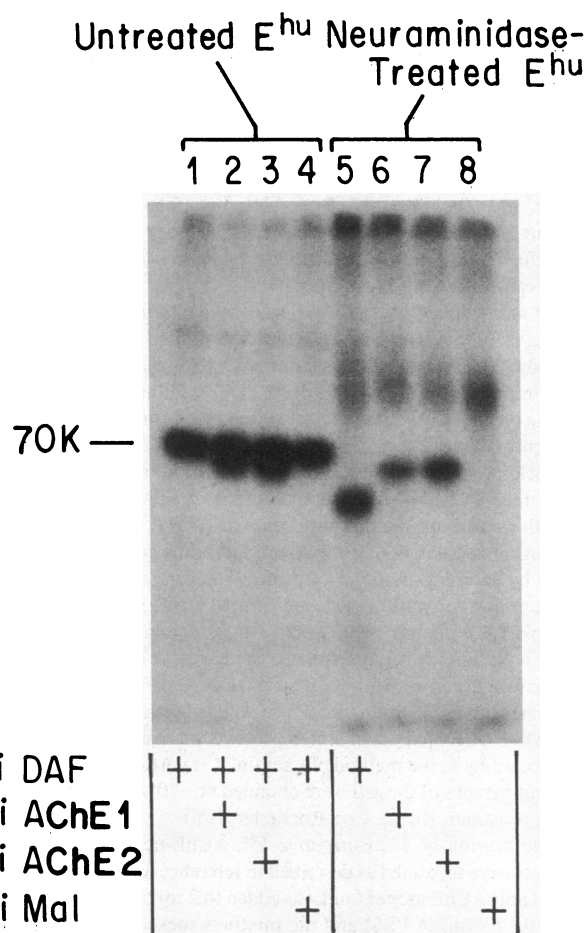


Figure 2. Differential immunoprecipitation of membrane DAF and AChE. E^{hu} were incubated at 37°C for 2 h with 3 U of type VI neuraminidase. E^{hu} untreated or pretreated with neuraminidase were surface labeled with ^{125}I , and after hypotonic lysis the membranes were extracted with 1% NP40. Extracts of untreated E^{hu} were incubated with either pooled monoclonal anti-DAF antibodies (lane 1), anti-DAF and anti-AChE monoclonal AE1 together (lane 2), anti-DAF and anti-AChE monoclonal AE2 together (lane 3), or pooled anti-DAF and antimalaria monoclonals (lane 4). Extracts of neuraminidase-treated cells were incubated with either pooled anti-DAF monoclonals (lane 5), anti-AChE AE1 alone (lane 6), anti-AChE AE2 alone (lane 7), or pooled antimalaria monoclonals (6G2, 2A10) (lane 8). After addition of protein A-agarose, the immunoprecipitated proteins were extracted with SDS, subjected to SDS-PAGE on 7.5% gels, and radioautographs prepared. The anti-DAF and anti-AChE monoclonals precipitated different proteins and, unlike DAF, membrane AChE did not decrease in size following pretreatment of the cells with neuraminidase.

Relationship between DAF and AChE deficiencies and defective terminal pathway restriction in PNH E^{hu} . Increased sensitivity to lytic terminal pathway complexes (e.g., C5b-9) distinguishes PNH III from PNH II E^{hu} (1, 15, 16) and is thought to play a role in the greater susceptibility of the PNH III cells to lysis in vivo. This sensitivity is demonstrable in vitro by markedly greater sensitivity of PNH III than of PNH II E^{hu} in the CLS assay (15, 16, 25) and by susceptibility of PNH III but not PNH II E^{hu} (or normal E^{hu}) to lysis in zymosan- (15) or cobra venom factor-treated (21) serum (a source of C5b-9). The intrinsic

membrane defect of PNH III E^{hu} responsible for this abnormality is not known. Studies were next conducted to investigate the relationship between this functional defect and the DAF and AChE deficiencies of the cells.

The effects of anti-DAF and anti-AChE antibodies on the capacity of normal cells to restrict membrane damage by autologous C5-9 were first investigated. Normal E^{hu}AC1423 bearing ~ 10,000 C3b per cell were prepared by incubating E^{hu}AC14 with pooled anti-DAF monoclonals, excess human C2, and human C3 (50 µg/ml). Upon incubation of the intermediates with guinea pig C5-9 which develops autologous C3b sites, immediate lysis was observed. In contrast (despite reincubation with > 1,000 SFU of C2 or oxidized C2), when the intermediates were incubated with autologous human C5-9 (in place of heterologous guinea pig C3-9) no lysis occurred demonstrating the capacity of normal E^{hu} to restrict lysis by autologous (as opposed to heterologous) terminal pathway components. Retreatment of the E^{hu}AC1423 with pooled anti-DAF monoclonals (10–100 µg/ml each), anti-AChE monoclonals (10–100 µg/ml each), or anti-DAF and anti-AChE monoclonals together, or with polyclonal anti-DAF or anti-AChE antibodies (10–100 µg/ml IgG), separately or in combination, followed by addition of human C5-9 did not induce lysis, indicating that the resistance of the C3b-bearing normal E^{hu} in human C5-9 (13, 14) is mediated by another entity. In accordance with previous findings (15, 16) that PNH E^{hu} lack this terminal pathway resistance, E^{hu}AC1423 prepared with PNH III E^{hu} and bearing < 2,000 autologous C3b per cell rapidly lysed upon exposure to the human C5-9 (not shown).

The effects of DAF reconstitution on the relative sensitivities of PNH II and III cells in the CLS assay and on the susceptibility of PNH III cells to passive lysis in the presence of cobra venom factor-treated serum were next examined. Unfractionated or affinity-purified AChE-negative PNH E^{hu} were reconstituted with DAF or incubated with NP40 detergent as control. Fig. 1, C and D show FACS profiles of AChE-negative cells from one of the patients (G.R.), before and after the DAF reconstitution. In this case employing purified AChE-negative cells, an increase in anti-DAF fluorescence of the entire DAF-negative cell population occurred. With unfractionated E^{hu}, the uptakes of DAF into DAF-deficient and into DAF-containing cell populations were comparable (not shown) suggesting that affected PNH E^{hu} are not abnormal with respect to their ability to accept DAF.

To establish that DAF molecules incorporated after DAF reconstitution of the PNH cells were functional, the DAF-treated PNH E^{hu} were tested for their ability to restrict assembly of autologous C4b2a (12). The results of two representative experiments are shown in Fig. 3. In studies with normal E^{hu} (Fig. 3 A), despite > 14,000 C4b per cell, no lysis occurred upon incubation of the cells with C2 followed by guinea pig C3-9 (the developing reagent used to assay C4b2a assembly and function). In contrast, significant lysis of the control PNH cells was observed with < 1,000 C4b per cell, whether AChE-negative (Fig. 3, A and B) or unfractionated E^{hu} (Fig. 3 B) were used. After treatment with DAF, the capacity of the PNH cells to circumvent C4b2a assembly was almost completely restored. Measurements with the two-site immunoradiometric assay showed that 344 DAF (Fig. 3 A) and 600 DAF (Fig. 3 B) molecules per cell had incorporated, respectively. The efficiency of the incorporated DAF in inhibiting C4b2a assembly was comparable to that observed

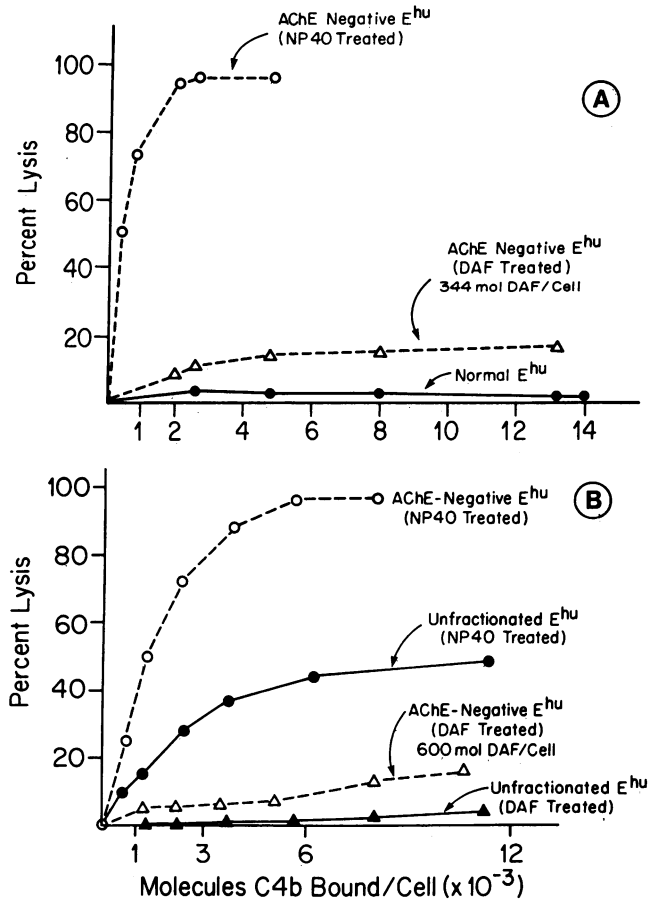


Figure 3. Inhibitory effect of incorporated DAF on C4b2a assembly on PNH E^{hu}. E^{hu}AC1 were incubated with increasing concentrations of C4. Aliquots of the resulting E^{hu}AC14, bearing increasing numbers of C4b per cell, were incubated at 30°C for 4–6 h with DAF or with NP40-containing buffer as control. After thorough washing, the DAF-treated or control cells were incubated with excess human C2 and guinea pig C3-9 (see Methods) and lysis quantitated. Percent lysis of the cells is shown as a function of the number of deposited C4b per E^{hu}. The results of studies with normal and AChE-negative E^{hu} from PNH patient G.R. are shown in (A) and with unseparated and AChE-negative E^{hu} from patient M.A. in (B). The incorporation of 344 and 600 molecules of DAF per cell, respectively, as determined by two-site radioimmunoassay, markedly reduced the abnormal C4b2a assembly by the PNH cells.

in previous studies (8, 12). The incorporated DAF was thus fully functional.

The effect of the incorporated DAF on the sensitivity of the PNH cells in the CLS assay was next examined. The results of one study with PNH II cells are illustrated in Fig. 4. Restoration of DAF activity to PNH II E^{hu} (Fig. 4 A) reduced their sensitivity in CLS to near normal levels. Conversely (Fig. 4 B), treatment of normal E^{hu} with polyclonal anti-DAF antibodies (30 µg/ml IgG) increased their sensitivity in CLS to the level of PNH II cells. Augmenting the concentration of anti-DAF antibody 3- or 10-fold further (to 300 µg/ml IgG) had no additional effect. In contrast, in multiple studies (see Table II), DAF reconstitution had only a minimal effect (< 30% reduction) on the sensitivity of PNH III cells in CLS (Fig. 5). Augmenting the incorporated

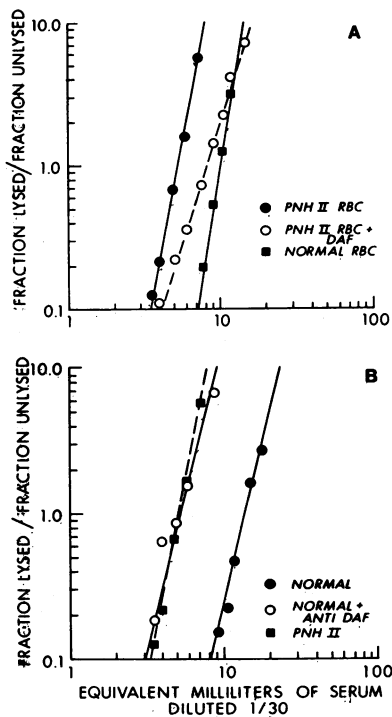


Figure 4. Effect of DAF and of anti-DAF antibodies on CLS of PNH II E^{hu} and normal E^{hu}. Anti-I-treated E^{hu} mixtures prepared at 37°C were added to increasing amounts of human serum, and after sensitization at 0°C and incubation at 37°C, as described in the Methods, lysis was quantitated. The ratio of lysed to unlysed cells is shown as a function of the equivalent milliliters serum diluted 1:30 in the reaction tubes. In (A), purified AChE-negative (PNH II) E^{hu} from patient W.I. were pretreated with DAF or with NP40 detergent as control. The sensitivities of the reconstituted and control cells were then compared with normal

cells. In this and other experiments the DAF treatment diminished the sensitivity of the PNH II cells to near normal levels. Analyses with the two-site radioimmunoassay for DAF showed that 645 molecules per E^{hu} had incorporated. In (B), normal E^{hu} were pretreated with polyclonal anti-DAF antibodies (300 µg/ml) or with buffer as control. The sensitivities of the antibody-treated and control cells were then compared with untreated PNH II E^{hu} from patient W.I. The anti-DAF treatment of the normal E^{hu} increased their sensitivity to that of the PNH II E^{hu}.

DAF levels (Table III) had little additional effect. Even after incorporation of 573 DAF molecules per E^{hu}, sensitivity of the DAF-treated PNH III cells was still 10 times that of normal cells (Table III).

Table II. Incorporation of DAF into PNH Erythrocyte Membranes

Patient ID	% Affected cells	Unfractionated erythrocytes		Purified AChE-negative erythrocytes	
		NP40-treated	DAF-treated	NP40-treated	DAF-treated
		molecules DAF/cell		molecules DAF/cell	
PNH III					
D.I.	58	361	430	<10	275
P.H.	34	963	1187	<10	223
F.R.	52	1471	2804	0	585
G.R. (IIIb)*	35	774	1376	0	573
PNH II					
W.I.	42	1505	2150	<10	—
B.A.	90	172	1118	0	722

Normal erythrocytes have ~2000 mol DAF/cell. * Comparable sensitivity to PNH III in reactive lysis but sensitivity to classical pathway lysis intermediate between PNH II and III.

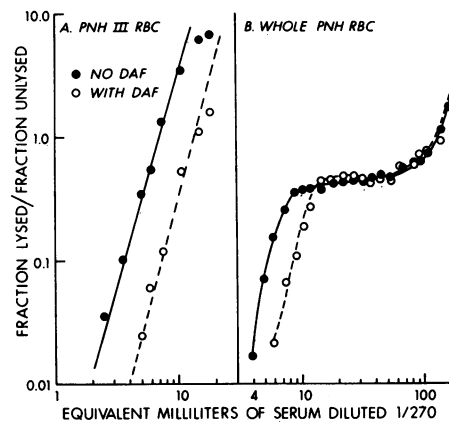


Figure 5. Effect of DAF on CLS of PNH III E^{hu}. Cells were treated with DAF or NP40 detergent containing buffer and then tested for their complement sensitivity as described in the legend of Fig. 4. (A) shows the results with separated AChE-negative PNH III E^{hu} and (B) the results with unseparated PNH III E^{hu}.

The effect of DAF reconstitution of PNH III E^{hu} on the sensitivity of PNH III cells to passive lysis in cobra venom factor-treated human serum was next evaluated. The results of a typical study are shown in Fig. 6. Significant lysis of PNH III E^{hu} occurred in 1/64 cobra venom factor-treated serum, whereas minimal lysis of normal E^{hu} occurred in 1/8 cobra venom factor-treated serum. Reconstitution of the PNH cells with DAF did not diminish their sensitivity to lysis in this assay. Conversely, prior treatment of the normal cells with polyclonal anti-DAF antibodies (30–300 µg/ml IgG) did not abrogate their inherent resistance. In three other studies with both unfractionated and purified AChE-negative cells (Table II), incorporation of up to 585 DAF molecules per cell (the maximal attainable DAF level with the reagents used) had no effect. The inability of DAF to influence this terminal pathway defect under conditions in which inhibition of C3 convertase assembly was restored argues that a third abnormality of the membrane must exist in PNH III cells.

The evaluation of DAF levels in AChE-positive E^{hu} of the PNH patients by calculation from immunoradiometric data had indicated that complement-insensitive (PNH I) E^{hu} are partially DAF-deficient (Table I). In the final series of studies, comple-

Table III. Effect of Incorporated DAF Molecules on CLS of PNH Erythrocytes

DAF Concentration µg/ml	PNH III E ^{hu} **		PNH I E ^{hu} **	
	DAF-treated	NP40-treated	DAF-treated	NP40-treated
12 [‡]	35.5 [§]	41.0	3.1	3.1
24	33.7	45.0	2.6	3.3
48	30.0	43.5	1.7	3.0

* CLS H50 units.

[‡] E^{hu} (from patient G.R.) were incubated at 30°C for 10 h with decreasing dilutions of either DAF-NP40 or NP40 alone in isoionic veronal buffer. After washing, the treated cells were assayed for CLS as described in Methods.

[§] Normal range ≤2 U.

^{||} 573 mol DAF incorporated/cell.

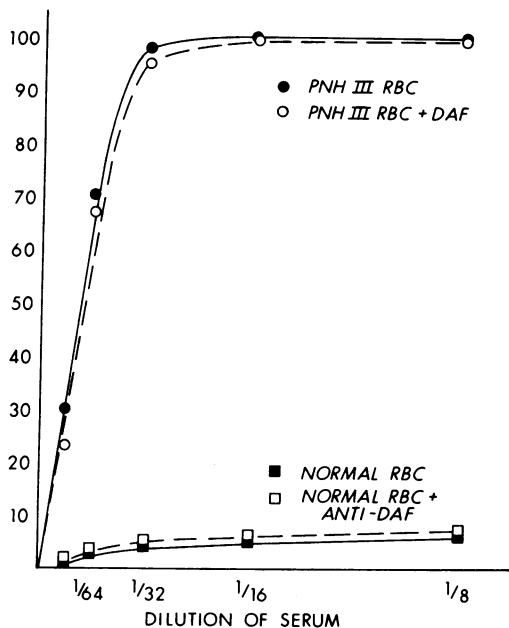


Figure 6. Effect of DAF and of anti-DAF antibodies on the susceptibility of PNH and of normal E^{hu} to reactive lysis. Normal E^{hu} were pretreated with polyclonal anti-DAF antibodies (30–300 $\mu\text{g/ml}$ IgG) or with buffer as control, and purified AChE-negative PNH E^{hu} from patient G.R. were pretreated with DAF or with NP40 detergent as control. Cobra venom factor:Bb complexes, prepared as described in Methods, were added to mixtures of the E^{hu} in decreasing dilutions (1:64 to 1:4) of normal human serum in metal-chelating veronal buffer. After incubation at 37°C for 2 h, additional metal-chelating veronal buffer was added, the tubes centrifuged, and lysis quantitated. Percent lysis of the cells is shown as a function of the serum dilution. The anti-DAF treatment did not impair the natural resistance of the normal E^{hu} to lysis, and the DAF treatment did not reduce the abnormal lytic sensitivity of the PNH III E^{hu} . Analyses with the two-site radioimmunometric assay for DAF showed that in the case of the PNH E^{hu} 573 DAF molecules had incorporated per cell.

ment-sensitive E^{hu} from two of the patients with PNH III E^{hu} were selectively lysed with cobra venom factor-treated serum, and the remaining complement-insensitive PNH I E^{hu} examined directly for DAF and AChE expression by flow cytometry. The results are shown in Fig. 7. Fig. 7, A and B show the broad distribution of DAF and AChE in normal E^{hu} . Fig. 7, C–F show the distributions of the two proteins in unseparated E^{hu} of patients M.A. and F.R., respectively, and in purified PNH I E^{hu} from each surviving after reactive lysis. In the left hand panels, the numbers under the peaks give the measured DAF contents as determined by radioimmunometric assay. As can be seen, levels of both DAF and AChE in PNH I cells were markedly reduced compared with normal, demonstrating that PNH I cells in these patients are abnormal.

Discussion

Previous studies have shown that DAF is deficient in complement-sensitive E^{hu} of PNH patients (2–4) and that the DAF deficiency is causally related to the heightened sensitivity of the cells to lysis (12). The main finding of the present study is that although the DAF defect accounts for excessive C3b uptake by

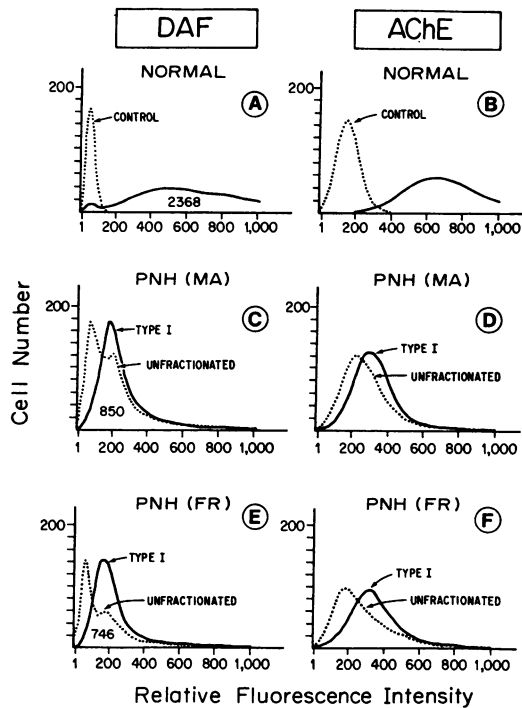


Figure 7. FACS analyses of DAF and AChE in purified PNH I E^{hu} from two patients. E^{hu} were stained with pooled anti-DAF, anti-AChE, or nonrelevant antimalaria monoclonals as control followed by fluorescein-isothiocyanate-labeled goat anti-mouse Ig as in Fig. 1. Analyses with anti-DAF monoclonals are shown in the left hand panels and with anti-AChE monoclonals in the right hand panels. Control studies using normal E^{hu} are shown in (A) and (B). Solid lines show the results with anti-DAF and dashed lines the results with nonrelevant antimalaria monoclonals. Studies with PNH patients M.A. and F.R. are shown in (C) and (D), and (E) and (F), respectively. In these cases, solid lines show the results with purified complement-insensitive PNH I E^{hu} and dashed lines the results with unfractionated E^{hu} . The numerical values in the left hand panels are the measured DAF contents in the DAF-positive cells as determined by radioimmunometric assay.

the E^{hu} , the marked lytic susceptibility of PNH III E^{hu} and other functional abnormalities of the cells, e.g. AChE deficiency, do not derive from DAF deficiency alone. Rather, these characteristic defects of PNH occur as a result of alterations in multiple membrane components.

One problem that has complicated the characterization of these and other membrane alterations that have been described in PNH (1) has been the mixture in the blood of PNH patients of complement-sensitive and complement-insensitive cell populations. Separation of AChE-negative E^{hu} subpopulations by cell-affinity chromatography in the present study, and examination of the purified cells by radioimmunometric assays (Table I) and by FACS analyses (Fig. 1) revealed that the DAF and AChE deficiencies completely overlap in the same cells. Evaluation of the cells in functional assays with whole human serum and cobra venom factor-treated human serum (Figs. 5 and 6) showed that in PNH III E^{hu} , the defect in terminal pathway restriction additionally resides in these same cells. The findings that the DAF, AChE, and terminal pathway restriction deficits each constitute profound abnormalities and precisely coincide with the complement-sensitive E^{hu} population suggest that they represent underlying defects that arise primarily in the cells.

The results of our measurements of CR1 levels in purified and unfractionated cells may help to clarify previous reports by others that CR1 expression in PNH E^{hu} can fall within the broad normal range of values (34) or can be reduced (4, 35). In contrast to the abnormalities of the above three factors, the reduction in CR1 expression was only partial. Moreover, differently from the defects in the above factors, it was similarly distributed between complement-sensitive and -insensitive E^{hu} populations. These findings raise the possibility that the CR1 defect may arise secondarily. Levels of E^{hu}-associated CR1 are diminished in conditions associated with heightened activity of the complement system and the extent of the reduction is reciprocally related to acquisition of C3 activation fragments on the cells (35, 36). Increased levels of such fragments have been detected on PNH E^{hu} (35), suggesting that the CR1 defect in PNH might likewise arise as a consequence of increased rates of complement activation in PNH blood. Such a mechanism could account for the presence of the defect in both complement-sensitive and -insensitive E^{hu} populations.

Although the use of the anti-DAF (2–4) and anti-AChE (32) antibodies has demonstrated that the ~70-kD proteins associated with these two functional activities are deficient in PNH E^{hu}, it has not been previously established whether the two defects are due to different abnormalities. The selective immunoprecipitation of DAF and AChE by the respective monoclonals (Fig. 2) unequivocally demonstrates that they are in fact different proteins (37) and avoids uncertainties arising from interference by anti-glycophorin antibodies within polyclonal anti-DAF antisera (38). Although association of the two proteins in the membrane of intact E^{hu} cannot be excluded, their lack of coprecipitation argues that they are not anchored in the membrane covalently by a common linking molecule. The limitation of the inhibitory effects of anti-AChE and anti-DAF antibodies to the activities associated with their respective antigens on normal E^{hu} and the failure of DAF reconstitution to augment AChE activity in PNH E^{hu} indicate that the functional activities of AChE and DAF in E^{hu} membranes are independently mediated.

PNH III E^{hu} are characterized by their abnormal susceptibility to reactive lysis in addition to their exaggerated uptake of C3b (1, 15, 16). This functional defect is due to an inability of their membranes to circumvent damage by the membrane attack complex, according to current evidence, at the level of C9 (13, 14). The relationship of this defect, however, to DAF deficiency previously was not clarified. It has been suggested that the greater passive lysis susceptibility of PNH III than PNH II E^{hu} could be due to more profound DAF-deficiency in the PNH III cells (3, 11). Of relevance, it has been reported that cell-bound C3b might enhance C9 insertion, providing a potential mechanism for a DAF effect (14). Several findings in the present study argue against this possibility and provide strong support for the alternative suggestion (39) that the two phenomena are mediated by different mechanisms. Analysis of purified AChE-negative PNH II and PNH III E^{hu} by radioimmunometric assay and flow cytometry revealed that both are totally DAF-deficient (Table I, Fig. 1) suggesting that an additional defect must be present in the PNH III cells. Despite abrogation of DAF activity as evidenced by uptake of 10,000 C3b molecules by the cells and brisk lysis in heterologous guinea pig C5-9, anti-DAF treatment of normal E^{hu}AC1423 did not render the intermediates susceptible to lysis in homologous C5-9. Similarly, exposure of normal E^{hu} to 10-fold higher concentrations of anti-DAF antibodies than necessary to overcome resistance to C3 convertase assembly (12)

increased the sensitivity of the normal cells in the CLS assay only three- to fivefold (to that of PNH II E^{hu}) (Fig. 4), and did not render the cells susceptible to passive lysis in cobra venom factor-treated human serum (Fig. 6). Finally, reconstitution of PNH III E^{hu} with DAF in amounts sufficient to restore their ability to restrict C4b2a assembly (12) reduced their sensitivity in CLS by < 30% (Fig. 5) and had no discernable effect on their vulnerability to passive lysis (Fig. 6). The finding that polyclonal or pooled monoclonal anti-AChE antibodies, alone or in combination with the anti-DAF antibodies, also did not enhance the sensitivity of the C3b-bearing normal E^{hu} intermediates to homologous C5-9 argues that the entity responsible for resistance to reactive lysis is not DAF or AChE, and therefore that PNH III E^{hu} are abnormal with respect to a third activity.

Membrane factors that can inhibit C9 polymerization and thereby limit the activity of the membrane attack complex have been reported in extracts of E^{hu} stroma by E. Podack, New York Medical College, Valhalla, NY (personal communication) and by Zalman and Müller-Eberhard (40). The activity detected by Podack elutes from DEAE-Sepharose at higher ionic strength than DAF and migrates as an ~80-kD molecule on SDS-PAGE, whereas that described by Zalman and Müller-Eberhard has an apparent *M_r* of 65 K. Another terminal pathway inhibitory factor with the ability to bind to C8 and block its interaction with C9 has been isolated from extracts of E^{hu} stroma by Schonermark et al. (41). The relationship, however, of this factor to the above inhibitors of C9 polymerization has not yet been investigated.

The demonstration in this study that the characteristic abnormalities of PNH are a result of deficiencies in multiple membrane components rather than in DAF alone has important implications concerning the mechanism of the PNH defect. Previous findings that small amounts of DAF antigen are detectable in affected leukocytes of patients (9) and that, when cultured in vitro, DAF-positive erythroid precursors in patients' marrow give rise to DAF-negative erythroid colonies (42) suggest that the diminished DAF expression in PNH is not due to absence or nonfunction of the DAF structural gene. That deficient expression of the factors does not arise from the failure of PNH membranes to hold the factors or to support their function is indicated by the ability of exogenous DAF to incorporate into PNH E^{hu} and to restore the resistance of the affected cells to C3 convertase assembly (12). The simultaneous deficiency of the three entities could arise from a biosynthetic defect in a structural element shared by the three factors. Structural analyses of E^{hu} AChE and DAF have revealed the presence of a covalently linked glycolipid at the COOH-terminus of each molecule rather than hydrophobic amino acids typical of conventional membrane polypeptide anchors (43–47). This non-amino acid anchor is comprised of fatty acids, inositol, and an oligosaccharide and is closely similar in both proteins (48). In AChE, this anchoring structure has been shown to be linked through an ethanolamine to the COOH-terminal amino acid of AChE polypeptide and in studies of DAF, there is evidence that it is incorporated post-translationally before addition of *O*-linked sugars to DAF in the Golgi. If the putative terminal pathway inhibitory molecule shares the same glycolipid anchoring structure, a defect in the assembly of this structure or its attachment to the respective proteins could lead to deficient expression of all three molecules.

A biosynthetic defect in a structure common to the proteins, however, would not account for the sporadic occurrence of defects in only two of the factors and thereby would not explain the findings in the present study that PNH II and PNH III E^{hu}

differ with respect to the absence or presence of a defect in terminal pathway restriction (Figs. 5 and 6). It is possible that different biosynthetic defects could give rise to the two classes of PNH cells. It is alternatively possible that PNH involves a regulatory rather than a biosynthetic defect and that expression of DAF and AChE and expression of the entity responsible for terminal pathway restriction are independently controlled. Recent findings that normal platelets manifest DAF activity (9, 10, 49) but not terminal pathway restriction (49) support this possibility. In either case, it would not be surprising if more than one alteration could lead to DAF deficiency and the consequent clinical expression of PNH and if the changes that characterize PNH II and III cells could arise by different mechanisms.

Current evidence indicates that PNH is a clonal disorder (1). For this and other reasons, it has been assumed that the complement-insensitive (PNH I) E^{hu} in PNH blood are products of natural hematopoiesis and remain entirely normal. Our observation that DAF and AChE levels are reduced in the PNH I cells of most patients (Table I and Fig. 7) raises the possibility that PNH I cells could also derive from abnormal progenitors. Alternatively, it is possible that their DAF alterations could arise secondarily as a consequence of effects of the DAF-deficient E^{hu} population on them in the circulation (or on their precursors in the marrow) or as a result of the complement dysregulation that occurs in PNH blood.

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