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Immunology

Affected erythrocytes of patients with paroxysmal nocturnal hemoglobinuria are deficient in the complement regulatory protein, decay accelerating factor

(complement regulation/C3 convertases)

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ABSTRACT **Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired defect of bone marrow stem cells in which the affected clones produce erythrocytes (also granulocytes and platelets) with membranes that are abnormally sensitive to complement-mediated lysis. Abnormal erythrocytes (E) from patients with PNH (PNH-E) are 3–5 times more sensitive (type II PNH-E) or 15–25 times more sensitive (type III PNH-E) to lysis *in vitro* by human complement than normal E from unaffected individuals and the functionally normal E that arise from unaffected clones in PNH patients (type I PNH-E). After complement activation by either the classical or alternative pathway, abnormal amounts of C3b are deposited on the membranes of PNH-E compared with normal E, suggesting that the PNH-E membrane cannot regulate the events responsible for C3b deposition. Two proteins that decrease the stability of the classical and alternative pathway C3 convertases on target cells have been isolated from normal human E stroma: the 70,000 M_r decay accelerating factor of stroma (DAF) and the 250,000 M_r C3b receptor (C3bR). Specific immune precipitates of solubilized membranes from ^{125}I-surface-labeled normal E demonstrate both proteins. In contrast, specific immune precipitates of PNH-E from three patients show C3bR but are deficient in DAF; type II PNH-E are relatively deficient and type III PNH-E are totally deficient in DAF. Antibody that neutralizes the activity of isolated DAF is adsorbed by intact normal E under conditions in which it is weakly adsorbed by type II PNH-E and not adsorbed by type III PNH-E. The deficiency of DAF antigen in PNH-E, as assessed by lack of immunoprecipitation and antibody adsorption, could explain the abnormal sensitivity of PNH-E to complement-mediated lysis and suggests that DAF may protect the membranes of normal E from damage resulting from autologous complement activation.**

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare acquired syndrome characterized by spontaneous episodes of intravascular hemolysis that are often more pronounced at night (1, 2) and have been related to the relative acidosis that occurs during sleep (3). The hemolysis of erythrocytes (E) from patients with PNH (PNH-E) results from an apparent abnormality of their membranes (4) that markedly increases their sensitivity to complement-mediated lysis (5). The abnormal PNH-E arise from affected stem cell clones that also produce abnormal granulocytes and platelets (6). PNH-E have been grouped into subpopulations on the basis of their susceptibility to complement-mediated lysis after sensitization by isohemagglutinins: type I PNH-E behave as normal E and are thought to arise from normal stem cells; type II PNH-E are 3–5 times more sensitive than normal E to complement-mediated lysis; and type III PNH-E are 15–25 times more sensitive (7). Complement activation that leads to the lysis of PNH-E *in vitro* may proceed by the classical pathway (8, 9), by the alternative pathway (10, 11), or by acid treatment of C5 that results in subsequent formation of the lytic complex of complement proteins, C5-9 (12, 13). The relative contribution of each of these pathways to the lysis of PNH-E *in vivo* is not known.

With equivalent amounts of isohemagglutinin sensitization, normal E, type II PNH-E, and type III PNH-E activate the same amount of C1 and bind equivalent amounts of C4b, but 6 times more membrane-bound C3b is deposited on PNH-E than on normal E (14). The classical C3 convertase, C4b,2a, loses its ability to cleave C3 enzymatically and deposit C3b when the C2a subunit "spontaneously" dissociates from the membrane-bound C4b subunit (15, 16). The increased ratio of C3b/C4b bound to PNH-E suggests that these cells may lack a regulatory mechanism for C3 convertase activity.

Two proteins that regulate complement C3 convertases on target cells have been isolated from the membranes of normal E: the 70,000 M_r decay accelerating factor of stroma (DAF) (17, 18) and the 250,000 M_r C3b receptor (C3bR) (19, 20). When DAF and the C3bR are solubilized and isolated, each can cause the accelerated decay of both membrane-bound classical (C4b,2a) and properdin-stabilized alternative complement (C3b, Bb) C3 convertases; DAF has preferential activity for the C4b,2a convertase and C3bR for the C3b,Bb convertase (18). The availability of an antiserum to each of these moieties allowed the direct determination of their presence or absence on PNH-E.

MATERIALS AND METHODS

The following reagents were obtained as noted: acrylamide, bisacrylamide, temed, 2-mercaptoethanol, $NaDodSO_4$, hydroxyapatite, and nitrocellulose paper from Bio-Rad; Iodogen (1,3,4,6-tetrachloro-3α-6α-diphenylglycoluril) from Pierce; ^{125}I from New England Nuclear; staphylococcal protein A, silver nitrate, and diisopropyl fluorophosphate from Sigma; phenylmethylsulfonyl fluoride from Calbiochem–Behring; DEAE-Sephacel, phenyl-Sepharose, lentil lectin-Sepharose, and CH-activated Sepharose from Pharmacia; and Nonidet P-40 (NP-40) from British Drug House.

Proteins were quantitated by the method of Lowry *et al.* (21), with bovine serum albumin as the standard. Isolated DAF, a

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Abbreviations: DAF, decay accelerating factor of stroma; PNH, paroxysmal nocturnal hemoglobinuria; C3bR, C3b receptor; NP-40, Nonidet P-40; E, erythrocyte(s); PNH-E, erythrocytes from patients with paroxysmal nocturnal hemoglobinuria; type I PNH-E, functionally normal PNH-E; type II PNH-E, PNH-E that are 3–5 times more sensitive to lysis by complement than normal E; type III PNH-E, PNH-E that are 15–25 times more sensitive to lysis by complement than normal E.

mixture of molecular weight standards (Pharmacia), and intact E and PNH-E were each radioiodinated with ^{125}I by the Iodogen method (22). Rabbit anti-glycophorin A specific for the COOH terminus of the molecule (23) was provided by H. Furthmayr (Yale University School of Medicine). Two milliliters of this antiserum was absorbed sequentially with 1×10^9 human E from five different donors to remove any antibodies reacting with surface antigens. An IgG fraction was prepared from rabbit anti-DAF (18) and from rabbit anti-glycophorin A by absorption of the antibody with protein A-Sepharose and elution with a 0.1 M glycine buffer (pH 2.5). IgG fractions were stored in a 0.15 M NaCl/5 mM Na phosphate buffer/0.02% Na azide, pH 7.4, at 4°C. An affinity-purified IgG fraction of rabbit anti-C3bR (20) was provided by D. T. Fearon (Harvard Medical School).

Gradient (5–15%) polyacrylamide slab gel electrophoresis was performed with the Laemmli buffer system (24). Samples were prepared in the presence of 3% $NaDodSO_4$/5% 2-mercaptoethanol/6 mM Tris·HCl, pH 6.8/10% glycerol/0.02% bromophenol blue and were boiled for 5 min. Samples of stroma, but not the immune precipitates, were made 0.2 M with iodoacetamide and reboiled for 5 min before application to the gel. Samples were electrophoresed at 70 V until the dye front reached the end of the gel, which took about 12 hr. The ^{125}I-labeled standards included phosphorylase b (94,000 M_r), albumin (67,000 M_r), ovalbumin (43,000 M_r), carbonic anhydrase (30,000 M_r), trypsin inhibitor (20,100 M_r), and α-lactalbumin (14,400 M_r). Autoradiography of dried gels and electroblotted nitrocellulose paper was done at −70°C with an intensifying screen (Cronex, DuPont). The autoradiographs were developed with Kodak reagents after about 16 hr of exposure.

The DAF used as the immunogen was extracted and isolated as described (18). For the studies described in this paper, the isolation procedure was modified after the DEAE-Sephacel chromatography. The pool of active DAF fractions that eluted between 8 and 10 mS was brought to pH 7.4 with 0.1 M HCl and made 0.3 M NaCl by the addition of solid NaCl. This material was applied to an 80-ml column of phenyl-Sepharose equilibrated in a 0.3 M NaCl/40 mM Na phosphate buffer, pH 7.4/0.1% NP-40. The column was then washed with 2 bed vol of starting buffer and eluted with a gradient of 100 ml of starting buffer and 100 ml of 50 mM NaCl/50 mM Na phosphate, pH 9.5/1% NP-40. The active DAF fractions that eluted at the end of the gradient were pooled, dialyzed against 20 mM Na phosphate, pH 7.4/0.1% NP-40 and applied to a 40-ml column of hydroxyapatite equilibrated in the same buffer. The predominant DAF activity, which was found in the drop-through fractions, was pooled, dialyzed against 0.2 M NaCl/10 mM Tris buffer, pH 7.4/0.7 mM $CaCl_2$/0.7 mM $MgCl_2$/0.7 mM $MnCl_2$/0.1% NP-40 and applied to a 2-ml lentil lectin-Sepharose column equilibrated in the same buffer. The DAF activity appeared in the effluent fractions, whereas a major contaminant could be eluted with 0.2 M α-methylmannoside in the starting buffer. This purification procedure yielded 3.6 mg of protein from 670 ml of packed E, with 1 unit of DAF activity per 1.7 ng of protein. One unit of DAF activity is defined as the concentration of DAF that causes a 30% reduction in the number of C3 convertase sites as compared to their decay in buffer alone. The cellular intermediate (EAC1,4,2) was prepared with sheep E, rabbit antiserum, guinea pig C1 and C2, and human C4. The decay was determined after 15 min at 30°C by assay of the residual C3 convertase sites (18). Electrophoresis of 2 μg of DAF on a 5–15% Laemmli (24) slab gel followed by silver staining (25) showed a major band at 70,000 M_r and faint bands in the 60,000–62,000 M_r and 30,000 M_r regions.

The rabbit anti-DAF-IgG was found to be contaminated with anti-glycophorin IgG. This was recognized after electrophoresis of normal stroma on a 5–15% Laemmli gel and electroblotting (26) with a Transblot apparatus (Bio-Rad). After transfer of stromal proteins (150 mA × 20 hr) to the nitrocellulose paper, the paper was cut, and the nonspecific binding capacity of the individual strips was blocked with 2% bovine serum albumin (26). The strips were washed as described (26) and reacted for 3 hr at room temperature with mixing with either nonimmune IgG, anti-DAF-IgG, or anti-glycophorin A specific for the COOH terminus of the molecule. The strips were washed in buffer, reacted with ^{125}I-labeled protein A (3.25×10^7 cpm/μg) for 30 min at 37°C, washed again, aligned, and autoradiographed. The anti-DAF-IgG recognized monomeric glycophorin at 35,000 M_r and aggregates in the 80,000 M_r and 55,000 M_r regions. To free the anti-DAF-IgG of anti-glycophorin reactivity a glycophorin affinity column was made. Human E stroma was solubilized in a lithium diiodosalicylate buffer (27) followed by phenol and chloroform/methanol/HCl extractions (28); the starting stroma, prepared from E pooled from two donors, contained the A^+, B^+, M^+, and N^+ antigens. A sample of the glycophorin preparation (0.12 OD_{280}) was coupled to 5 ml of CH-activated Sepharose according to the manufacturer's protocol. A sample of anti-DAF-IgG (4 mg) was dialyzed against a 0.15 M NaCl/0.05 M Na phosphate buffer, pH 7.5, and applied to the glycophorin affinity column that was equilibrated in the same buffer at 4°C, and 1-ml fractions were collected. Samples of the protein-containing fractions were incubated with the ^{125}I-labeled DAF for 1 hr at 4°C and mixed with protein A-Sepharose for a second hour at 4°C. The particulate fraction was collected by centrifugation and washed, and the immune complexes were eluted with electrophoresis sample buffer. The eluate was processed, electrophoresed in 5–15% Laemmli gels, and autoradiographed. The early drop-through fractions from the glycophorin-CH-Sepharose column yielded IgG that precipitated only a 70,000 M_r band from the ^{125}I-labeled DAF. One hundred and fifty micrograms of glycophorin-absorbed anti-DAF-IgG was recovered, and 1 μg of absorbed, monospecific anti-DAF-IgG neutralized 1 unit of DAF activity.

Fresh E were obtained from normal donors. The blood, obtained by venipuncture, was diluted 1:2 in Veronal-buffered saline containing 0.1% gelatin and 0.04 M EDTA and centrifuged at 500 × *g* for 6 min. The buffy coat was removed and the residual E were washed several times in the same buffer. Human E were stored at 4°C in Alsever's solution. Frozen normal E and E from PNH patients were prepared, stored at −70°C, and thawed for use as described (29). PNH-E were obtained from the following donors: patients L and R both had >95% type III E and patient K had >90% type II E as defined by the complement lysis sensitivity test using isoimmune anti-Tj^a antiserum (30). For each experiment with PNH-E, normal E that had been frozen for approximately the same length of time as the PNH-E were used. Sheep E were stored at 4°C in Veronal-buffered saline/0.1% gelatin/0.04 M EDTA. E were enumerated with a Coulter Counter (Coulter).

RESULTS

Specific Precipitation of DAF and C3bR from Solubilized Membranes of Surface-Labeled E. Fresh normal E, previously frozen normal E, and previously frozen type III PNH-E (patient L) were surface-labeled with 1 mCi of ^{125}I (1 Ci = 3.7×10^{10} Bq) per 4×10^8 cells in 0.5 ml of Hanks' buffer by use of 160 μg of Iodogen previously dried on the bottom of a scintillation vial. After 30 min of labeling at room temperature the cells were collected, washed, and lysed in 5 mM Na phosphate, pH 7.5/1 mM EDTA/0.1 mM phenylmethylsulfonyl fluoride,

5 mM diisopropyl fluorophosphate. The stromata, freed of hemoglobin and insoluble debris, were solubilized in 0.5 ml of 0.15 M NaCl/5 mM Na phosphate/0.1 mM phenylmethylsulfonyl fluoride/5 mM diisopropyl fluorophosphate/0.1% NP-40, pH 7.4 buffer. Each of the three solubilized stroma preparations was sequentially precipitated for 1 hr at 4°C by the addition of 1 mg of nonimmune rabbit IgG, 50 μg of absorbed anti-DAF-IgG, and 370 ng of affinity-purified rabbit anti-C3bR. After each interaction of IgG, 100 μl of a slurry of Sepharose-protein A was added and incubation was continued for 1 hr at 4°C to insolubilize the antigen–IgG complexes. The complexes were collected by centrifugation (1,000 × *g*) for 4 min, washed four times in 0.3 M NaCl/0.05 M Tris·HCl, pH 9.3/0.1% NP-40 and mixed in 100 μl of sample buffer for $NaDodSO_4$ gels. The samples were boiled for 5 min and the detergent-solubilized immune complexes were analyzed by electrophoresis and autoradiography. The anti-DAF-IgG precipitates of normal stroma, fresh or frozen, show a dark band at 70,000 M_r (Fig. 1). The comparable molecular weight band is missing in the stroma from type III PNH-E. The visible band in the 140,000 M_r range from the anti-DAF precipitates of normal stroma is not present in the nonimmune precipitates and probably represents a DAF dimer. The anti-C3bR precipitates a band of about 250,000 M_r from the stroma of both normal E and PNH-E. As the immune precipitates were done sequentially, the 70,000 M_r band in the anti-C3bR precipitate from normal E represents residual DAF-anti-DAF complexes not cleared by the Sepharose-protein A that was added before the addition of anti-C3bR IgG.

This experiment was repeated by using previously frozen E from another normal donor, type II PNH-E (patient K), and a second donor of type III PNH-E (patient R). Labeling, membrane solubilization, and immune precipitations were performed as above and the protein A-Sepharose input was doubled to 200 μl. Each protein A-Sepharose complex was washed five times in a 0.15 M NaCl/0.01 M Tris buffer, pH 9.0/0.2% $NaDodSO_4$/0.25% desoxycholate buffer in an attempt to decrease the nonspecific binding in the 94,000 M_r region. The normal stroma shows an anti-DAF precipitable band at 70,000 M_r, which is barely detectable in the stroma from type II PNH-E and undetectable in stroma from a second patient with type III PNH-E (Fig. 2). The anti-C3bR is precipitable from the stroma of normal E as well as from that of both the type II and type III PNH-E. The presence of the C3bR was also directly assessed by ^{125}I-labeled affinity-purified rabbit anti-C3bR (31) (courtesy of James Wilson, Harvard Medical School), and both the type II PNH-E (patient K) and the one type III PNH-E (patient L) tested had a normal number of antigenic sites of C3bR per E.

Capacity of Intact Normal E and PNH-E to Adsorb Neutralizing anti-DAF-IgG. Previously frozen normal human E, type II PNH-E, type III PNH-E, and fresh sheep E were washed and dispensed at 1×10^7, 3×10^7, and 1×10^8 cells per tube for normal E and at 5×10^7, 1×10^8, and 2×10^8 cells per tube for PNH-E and sheep E. The cells were pelleted (1,000 × *g*); the supernatants were aspirated, and 80 μl of Alsever's buffer, 10 μl (4 μg) of monospecific anti-DAF-IgG, and 10 μl of ^{125}I-labeled bovine serum albumin (1,900 cpm) were added and mixed with the cells. The reaction mixtures were kept at 4°C for 60 min and centrifuged (1,000 × *g*); duplicate 40-μl portions of each supernatant were added to separate tubes containing 40 μl of DAF (2 units). The ^{125}I cpm in the 40-μl aliquots of supernatant indicated that the antibody dilution in the cell pellets was comparable for each dose of E. The second reaction mixture was kept at 4°C for 80 min and the residual DAF activity was assayed (18). Sheep E did not adsorb any DAF neutralizing activity and these values are not plotted. Normal human E adsorbed the DAF-neutralizing antibody in a dose-de-

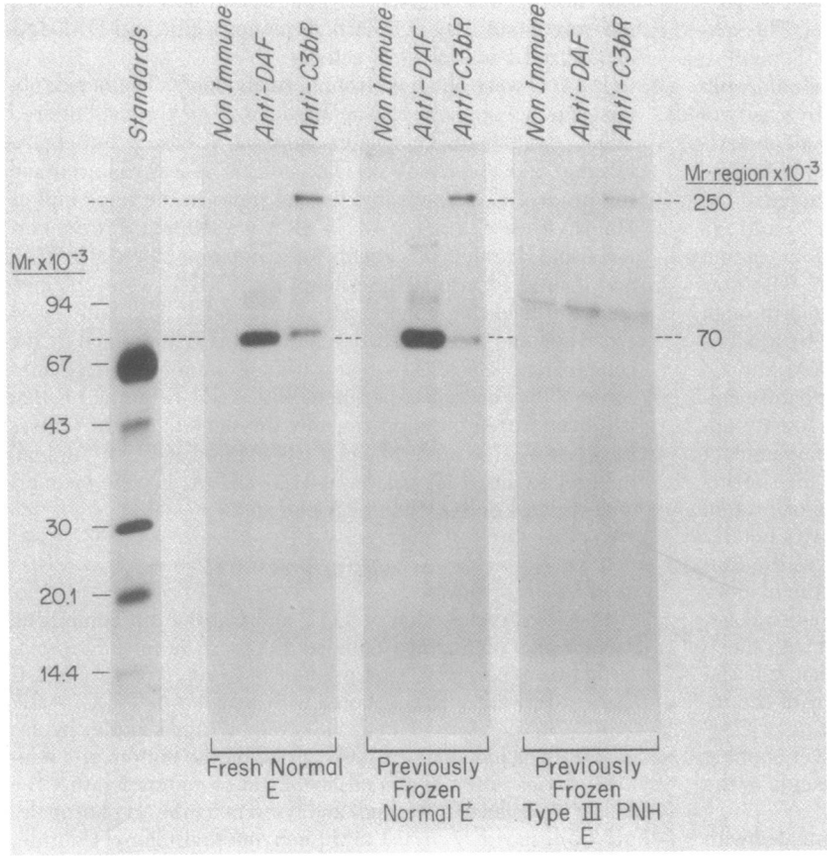

FIG. 1. Autoradiograph of immunoprecipitates made from solubilized membranes of surface-iodinated E. The precipitates were electrophoresed on a 5–15% polyacrylamide slab gel (24). Type III PNH-E were from patient L.

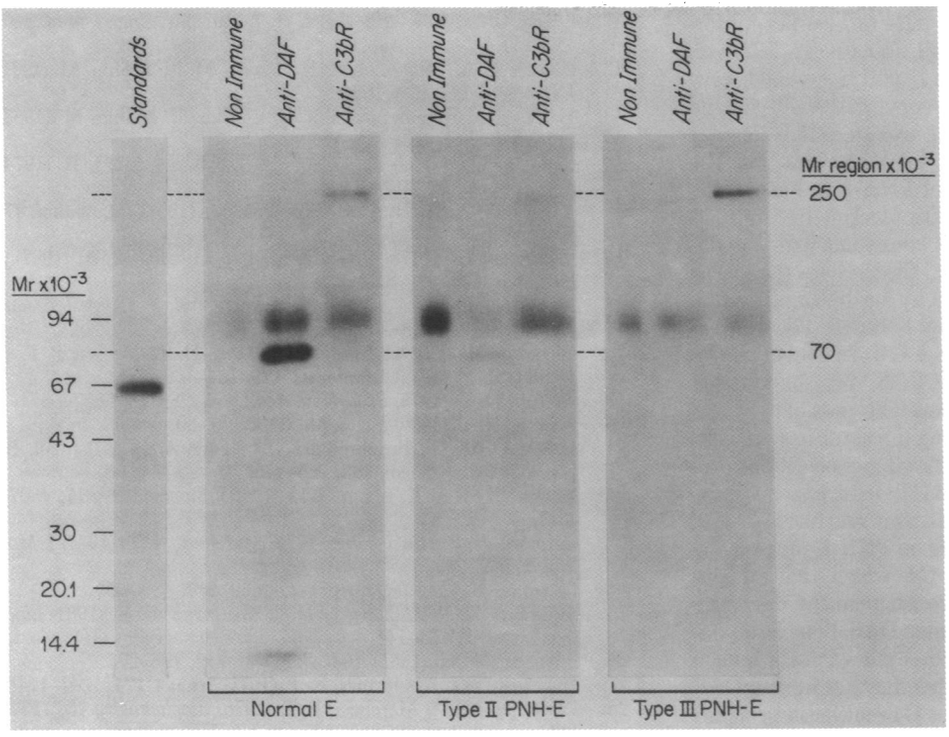

FIG. 2. Autoradiograph of immunoprecipitates made from solubilized membranes of surface-iodinated E. The precipitates were electrophoresed on a 5–15% polyacrylamide slab gel (24). Type II PNH-E were from patient K; type III PNH-E were from patient R. All cells were previously frozen.

pendent manner (Fig. 3). A larger dose of type II PNH-E had some dose-dependent neutralizing capacity, whereas type III PNH-E had none.

DISCUSSION

Although various biochemical abnormalities have been ascribed to PNH-E (32–36), these abnormalities either have not been demonstrated reproducibly or do not, as in the case of acetylcholinesterase deficiency (37), account for the susceptibility of PNH-E to complement lysis. Two proteins, DAF and the C3bR, which have the capability to limit expression of complement function, have been identified and isolated from the membranes of normal human E (18, 19). Both were initially recognized in stromal extracts by their capacity to accelerate the intrinsic decay rate of a C3 convertase formed on a sheep E with the assay for C3bR utilizing C3b,Bb and the assay for DAF utilizing C4b,2a. The identification of C3bR, extracted and isolated from the solubilized membranes, was based upon the ability of affinity-purified anti-C3bR to block the adherence function of C3bR on an E membrane (20). Neither DAF nor C3bR is present in sufficient quantities to be detected by direct staining when solubilized stromata from normal E are electrophoresed by the Laemmli technique (24). However, each can be detected in normal E after surface iodination, precipitation of relevant determinants with monospecific IgG, electrophoresis, and autoradiography. This indicates that DAF, like C3bR, is expressed on the surface of normal E (Figs. 1 and 2).

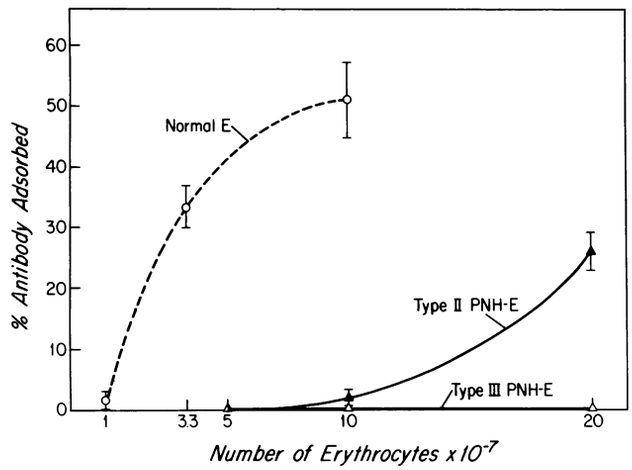

FIG. 3. Capacity of intact normal E and PNH-E to adsorb neutralizing anti-DAF-IgG. After interaction with the dose of E indicated, the nonbound anti-DAF-IgG was quantitated by its capacity to neutralize 2 units of functional DAF activity; 100% of IgG added neutralized 95% of the DAF activity. Type II PNH-E were from patient K; type III PNH-E were from patient L.

In contrast to the presence of DAF in the radiolabeled stroma from fresh or previously frozen normal E (E from five normal donors have been tested), immunoprecipitation of labeled stroma from PNH-E revealed a marked deficiency of this 70,000 M_r band. In the stroma from two donors of type III PNH-E, the DAF deficiency appeared complete, as compared with the detection of some DAF from the radiolabeled stroma from one source of type II PNH-E. The complete lack of DAF antigen in type III PNH-E and the partial deficiency in type II PNH-E, as compared to normal E, was also evident from a comparison of the capacity of these E to adsorb monospecific anti-DAF-IgG (Fig. 3). Under conditions in which about 3.3×10^7 normal E adsorbed 30% of the antibody, type II PNH-E (patient K) required 2×10^8 E to adsorb the same amount, and type III PNH-E (patient L) had no capacity to adsorb neutralizing IgG even with 2×10^8 cells. The finding that PNH-E were normal with regard to the C3bR, as assessed both by immune precipitation and by the direct binding of ^{125}I-labeled anti-C3bR indicates the selective deficiency of DAF.

After *in vitro* classical pathway activation using isohemagglutinins (9), PNH-E have an increased ratio of membrane-bound C3b/C4b, compared with normal E (14). This ratio is consistent with a prolonged decay of the C4b,2a convertase, which would allow more C3b to be generated and potentially bound before the C2a subunit dissociates, leaving residual membrane-bound

C4b (15). Excessive C3b deposition would in turn permit formation of the amplification convertase, C3b,Bb (38, 39), with augmented C3b deposition; membrane-bound C3b would serve to convert both C3 convertases to C5 convertases (40–42) and would also function as a receptor for the activated C5,6 complex so as to facilitate formation of the lytic C5–9 complex (43). The recent finding that the susceptibility of PNH-E to lysis by the C5b,6,7,8,9 complex could be partially blocked by pretreatment of the PNH-E with anti-C3b is consistent with the deposition of C3b on PNH-E *in vivo* (44). The findings that decay factors (C3bR and H) with optimal activity for the amplification convertase facilitate C3b inactivation by I (19, 45–47), whereas the C4b binding protein that decays the C4b,2a convertase facilitates C4b and C3b inactivation by I (48, 49), suggest that DAF may have a similar cofactor function. This possibility would account for the fact that PNH-E bearing the amplification convertase express an augmented convertase function (50) or prolonged half-life (51) and protect bound C3b from enzymatic inactivation by I (44, 51). The inability to demonstrate that a classical C3 convertase had a prolonged half-life on PNH-E (52) may be attributable to the use of guinea pig C4, which yields a convertase that is 2–3 logarithms more resistant to the decay accelerating activity of solubilized human DAF than is a convertase formed with human C4b and either guinea pig or human C2a (unpublished data). This species specificity of human DAF for a convertase made with homologous C4b may also be a partial explanation for the finding that detergent extracts of PNH-E and of normal E did not differ in their decay accelerating activity for a convertase made with guinea pig C4b (53).

The possibility that each of the PNH-E populations tested was contaminated by about 5–10% of type I PNH-E cannot be excluded because of the limitations of the complement lysis sensitivity assay. Thus, trace amounts of DAF antigen in the type II PNH-E population might have been contributed by contaminating type I cells. Nonetheless, it is possible to speculate that the relative DAF deficiency expressed by type II PNH-E may be due to abnormal regulation of a gene, whereas the complete DAF deficiency expressed by type III PNH-E may result from a nonfunctional or deleted gene. The enhanced susceptibility to *in vitro* lysis by activated C5–9 of type III PNH-E as compared to type II PNH-E (30) may result from a relative lack of C3b degradation by I when DAF is totally deficient; cells bearing more C3b would be more sensitive to lysis by a source of C5–9. Alternatively, there may be another as yet unrecognized consequence of total DAF deficiency or a totally unrelated abnormality that may contribute to the lytic effectiveness of C5–9 for type III PNH-E.

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