Characterization of the Complement Sensitivity of Paroxysmal Nocturnal Hemoglobinuria Erythrocytes

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Abstract

The affected erythrocytes of paroxysmal nocturnal hemoglobinuria (PNH II and PNH III cells) are abnormally sensitive to complement-mediated lysis. Normal human erythrocytes chemically modified by treatment with 2-amino-ethylisothiouronium bromide (AET) have been used as models for PNH cells inasmuch as they also exhibit an enhanced susceptibility to complement. To investigate the bases for the greater sensitivity of these abnormal cells to complement-mediated lysis, we compared binding of C3 and constituents of the membrane attack complex to normal, PNH II, PNH III, and AET-treated cells after classical pathway activation by antibody and fluid-phase activation by cobra venom factor complexes. When whole serum complement was activated by antibody, there was increased binding of C3 and C9 to PNH II, PNH III, and AET-treated cells, although the binding of these complement components to PNH II and PNH III cells was considerably greater than their binding to the AET-treated cells. In addition, all of the abnormal cell types showed a greater degree of lysis per C9 bound than did the normal erythrocytes. PNH III and AET-treated cells were readily lysed by fluid-phase activation of complement, whereas normal and PNH II erythrocytes were not susceptible to bystander lysis. The greater hemolysis of PNH III and AET-treated cells in this reactive lysis system was due to a quantitative increase in binding of constituents of the membrane attack complex. This more efficient binding of the terminal components after fluid-phase activation of whole serum complement was not mediated by cell-bound C3 fragments. These investigations demonstrate that the molecular events that characterize the enhanced susceptibility of PNH II, PNH III, and AET-treated erythrocytes to complement-mediated lysis are heterogeneous.

Introduction

The erythrocytes of paroxysmal nocturnal hemoglobinuria (PNH)[1] have been classified into three subtypes according to their in vitro susceptibility to antibody-initiated complement-mediated lysis (1, 2): PNH I cells are normal (or nearly normal) in their susceptibility; PNH II cells are moderately sensitive to complement, requiring 25–33% as much serum (as the complement source) for an equal degree of lysis as normal erythrocytes; and PNH III cells are exquisitely sensitive to complement, requiring 4–7% as much serum for an equal degree of lysis compared to normal erythrocytes. Both PNH II and PNH III cells fix more complement (C)3b when complement is activated by either the classical or the alternative pathway (3–6). This greater binding of C3b is due to the enhanced functional activity of the C3 convertase when affixed to these abnormal cells. Enhanced convertase activity may be due to several different mechanisms inasmuch as the convertase (*a*) has greater enzymatic activity when affixed to PNH cells (7), (*b*) forms more readily on PNH erythrocytes (7, 8), and (*c*) is more stable when bound to the abnormal cell membrane (8, 9). These aberrant interactions with complement appear to be related to abnormalities in glycoproteins of the cell surface, which function as regulators of the complement system (8–11). The greater activity of the C3 convertase is primarily responsible for the enhanced susceptibility of PNH II cells to complement-mediated lysis, because unlike PNH III cells they do not display a marked susceptibility to cytolysis in reactive lysis systems (6).

Normal human erythrocytes can be made to manifest a susceptibility to complement lysis similar to that of PNH cells by in vitro modification that uses the sulfhydryl compound, 2-amino-ethylisothiouronium bromide (AET) (12). Like PNH III erythrocytes, AET-treated erythrocytes are exquisitely sensitive to reactive lysis, as well as to lysis mediated by the classical and alternative pathways (12, 13).

To define more clearly the molecular basis for the greater susceptibility of PNH II, PNH III, and AET-treated erythrocytes to complement-mediated lysis, we have compared the binding of C3, C7, C8, and C9 to these abnormal cells after classical pathway activation by antibody and fluid-phase activation by activated cobra venom factor complexes (CoFBb). These studies demonstrate that although there are areas of overlap at the phenotypic level, at the molecular level the aberrant interactions

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1. *Abbreviations used in this paper:* AET, 2-amino-ethylisothiouronium bromide; CCAD, chronic cold agglutinin disease; CoF, purified cobra venom factor from *Naja naja;* CoFBb, activated cobra venom factor complexes prepared by incubating purified cobra venom factor with factor B and factor D; CR1, complement receptor type 1; GVB, veronal-buffered saline containing 0.1% gelatin; GVB^{+}, GVB containing 5 mM magnesium; GVB^{++}, GVB containing 0.5 mM magnesium and 0.15 mM calcium; PNH, paroxysmal nocturnal hemoglobinuria; TCP, trypsin cleavage peptide; VBS, veronal-buffered saline.

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with complement are distinctive for each cell type. Abnormalities in erythrocyte membrane glycoproteins, which are regulators of the complement system, may mediate these aberrant interactions.

Methods

Human erythrocytes. Erythrocytes from normal donors and from patients with chronic cold agglutinin disease (CCAD) (14) and PNH (7) were prepared and stored as previously described. The PNH II cells were obtained from a patient with 93% type II and 7% type III cells, whereas the PNH III cells were obtained from a patient with ~88% type III and 12% type I cells (1, 2). Normal human erythrocytes treated with AET (Sigma Chemical Co., St. Louis, MO) were prepared according to the method of Sirchia et al. (12) using a 9-min incubation period.

Buffers. The following buffers were employed: Veronal-buffered saline, pH 7.5 (VBS); VBS containing 0.5 mM magnesium and 0.15 mM calcium (VBS^{++}); VBS containing 0.1% gelatin (GVB); GVB containing 5 mM magnesium (GVB^{+}); GVB containing 0.5 mM magnesium and 0.15 mM calcium (GVB^{++}); GVB containing 15 mM ethylenendiamine tetraacetic acid (GVB-EDTA); Alsever's solution (7); and phosphate-buffered saline containing 150 mM sodium chloride and 10 mM sodium phosphate, pH 7.4.

Complement components. C1q (15), C3 (7), C7 (16), C8 (17), C9 (18), cobra venom factor (CoF) (*Naja naja*) (19), factor B (7), C3 nephritic factor (7), and factor D (7) were isolated by using previously described methods. The purification of each protein was assessed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (20), and functional activities were determined by hemolytic assay (21, 22).

Complement-deficient sera. Human sera deficient in one or more of complement components C7, C8, and C9 were prepared by immunochemical depletion. Briefly, freshly drawn human serum containing 15 mM EDTA was prepared and applied to immunoaffinity columns of the appropriate goat monospecific antibody coupled to Affi-Gel 10 (Bio-Rad Laboratories, Richmond, CA). The breakthrough serum was pooled, reconstituted with C1q (70 μg/ml), dialyzed against VBS^{++}, and stored in aliquots at −85°C.

Antibodies. Monoclonal anti-C3c as ascites fluid (Bethesda Research Laboratories, Gaithersburg, MD) was purified as previously described (7). Monoclonal anti-C3d (14), anti-C3dg (14), and anti-CR1 (complement receptor type 1) (14) were gifts from Dr. Gordon D. Ross (University of North Carolina, Chapel Hill, NC). They were purified using 5% caprylic acid (Sigma Chemical Co.) (14). The anti-C3d was further purified by adsorption and elution (using 4 M $MgCl_2$) from an affinity column bearing C3d. The excess $MgCl_2$ was removed by extensive dialysis. Monospecific polyclonal anti-human C7, C8, and C9 were raised in goats. The IgG fractions were isolated by using caprylic acid (22).

Protein determination. Protein concentration for C1q (23), C7 (24), C8 (25), C9 (25), and IgG (14) were determined spectrophotometrically. Other protein determinations were made with the Bio-Rad protein assay (Bio-Rad Laboratories) by using bovine IgG as the standard.

Radiolabeling. C7 (2.19 and 9.30 × 10^5 cpm/μg), C8 (2.44 × 10^5 cpm/μg), and C9 (2.11 × 10^5 cpm/μg) were labeled with either ^{131}I or ^{125}I as NaI (Amersham Corp., Arlington Heights, IL) by using Enzymobeads (Bio-Rad Laboratories) without loss of functional activity, employing minor modifications of the manufacturer's suggested procedure. Anti-C3c (1.6 × 10^6 cpm/μg), anti-C3d (1.2 × 10^6 cpm/μg) anti-C3dg (1.4 × 10^6 cpm/μg), and anti-CR1 (1.1 × 10^6 cpm/μg) were radiolabeled with ^{125}I using IODO-GEN (Pierce Chemical Co., Rockford, IL) as previously described (25).

Determination of cell-bound ^{125}I-C8 and ^{131}I-C9 after classical pathway activation of complement. Normal, PNH II, PNH III, and AET-treated erythrocytes were washed three times in GVB^{++} and resuspended to 5 × 10^8/ml in the same buffer. C8/C9-depleted serum was repleted by adding ^{125}I-C8 and ^{131}I-C9 to a final concentration of 50 and 70 μg/ml respectively, and dilutions were prepared using GVB^{++}. Seven reaction mixtures were prepared for each cell type. These mixtures contained 100 μl of the appropriate cell suspension, 100 μl of various concentrations of ^{125}I-C8/^{131}I-C9-repleted serum, and 100 μl of a 1:100 dilution (in GVB^{++}) of rabbit anti-human red blood cell antiserum (United States Biochemical Corp., Cleveland, OH). To maintain consistency, dilutions of the repleted serum were prepared as a separate pool in quantity sufficient to allow use with each set of cells in duplicate experiments. As controls for nonspecific binding of ^{125}I-C8 and ^{131}I-C9, parallel reaction mixtures were prepared substituting GVB^{++} for antibody. The reaction mixtures were incubated at 37°C in a shaking water bath. After 30 min, 700 μl of cold GVB-EDTA was added to each tube, and the reaction mixtures were transferred to 1.5-ml microfuge tubes and spun for 5 min at 12,000 rpm in a Beckman model 12 microfuge (Beckman Instruments, Inc., Fullerton, CA). The supernate was recovered, and the percent lysis was determined by measuring free hemoglobin release.

The cells were then resuspended to 100 μl (5 × 10^8 cells/ml) with GVB-EDTA. Three 25-μl aliquots were aspirated from each suspension and layered onto 200 μl of 20% sucrose in 400-μl polyethylene microfuge tubes (Analytical Laboratories, Rockville Centre, NY). The bound and unbound ^{125}I-C8 and ^{131}I-C9 were separated by spinning the tubes at 12,000 rpm for 10 min. The tubes were then cut just above the pellet. After determining the radioactivity of the pellet, the amount of cell-bound ^{125}I-C8 and ^{131}I-C9 was calculated by using molecular weights of 151,000 for C8 (17) and 71,000 for C9 (18). Nonspecific binding at each serum dilution was determined, and this value was subtracted in order to define specific binding.

Determination of the cell-bound C3 and C9. Normal, PNH II, PNH III, and AET-treated erythrocytes were washed three times in GVB^{++} and resuspended to 5 × 10^8/ml. C9-depleted serum was reconstituted with ^{131}I-C9 to a final concentration of 70 μg/ml. A single dilution of the ^{131}I-C9 repleted serum was prepared (25 parts serum to 75 parts GVB^{++}). In triplicate, 100 μl of the serum dilution was incubated with 100 μl of the appropriate cell suspension and 100 μl of rabbit anti-human erythrocyte antiserum for 30 min at 37°C. To control for nonspecific binding of ^{131}I-C9 (and subsequently, nonspecific binding of ^{125}I-anti-C3d), cells were incubated with the dilution of ^{131}I-C9-repleted serum, substituting GVB^{++} for the antibody. Cold GVB-EDTA (700 μl) was added, and each reaction mixture was then transferred from 12 × 75-mm glass culture tubes to 1.5-ml microfuge tubes and spun at 12,000 rpm for 5 min. The supernate was aspirated and the cells were resuspended, washed twice, and resuspended in 200 μl of cold GVB-EDTA (2.5 × 10^8 cells/ml). In duplicate, 25 μl of each cell type was incubated with 50 μl of ^{125}I-anti-C3d (10 μg/ml) for 30 min at 37°C. 50 μl of each reaction mixture was layered onto 200 μl of 20% sucrose in a 400-μl microfuge tube and spun for 10 min at 12,000 rpm in the microfuge. The number of molecules of cell-bound ^{125}I-anti-C3d and ^{131}I-C9 were calculated as described above using 160,000 as the molecular weight of anti-C3d. Nonspecific binding was determined, and this value was subtracted in order to define specific binding.

Determination of cell-bound ^{125}I-C8 and ^{131}I-C9 after fluid-phase activation of complement. Normal, PNH II, PNH III, and AET-treated cells were washed three times in GVB-EDTA and resuspended to 3.33 × 10^9/ml. Serum depleted of C8/C9 was repleted with ^{125}I-C8 and ^{131}I-C9 as described above. The ^{125}I-C8/^{131}I-C9-repleted serum containing 20 mM EDTA was prepared and incubated for 5 min at 37°C. Four, twofold falling dilutions of the EDTA-chelated serum were prepared using GVB-EDTA as the diluent. CoFBb were prepared as previously described (14). In duplicate, 20 μl of each cell type was incubated with 100 μl of the appropriate serum dilution and 20 μl of CoFBb complexes at 37°C. Controls for nonspecific binding of ^{125}I-C8 and ^{131}I-C9 were reaction mixtures similar to those described above except that GVB-EDTA was substituted for CoFBb. After 30 min, 850 μl of GVB-EDTA was added to each tube, and the reaction mixtures were transferred to 1.5-ml microfuge tubes. After spinning for 5 min at 12,000 rpm in the microfuge, the percent lysis and the number of

molecules of ^{125}I-C9 specifically bound per cell were determined as described above.

Determination of cell-bound ^{131}I-C7 and ^{125}I-C8 after fluid-phase activation of complement. C7/C8-depleted serum was repleted by adding ^{131}I-C7 and ^{125}I-C8 to a final concentration of 50 μg/ml each. The remainder of the experiment was similar to that described above for determination of cell-bound ^{125}I-C8 and ^{131}I-C9 after fluid-phase activation of complement by CoFBb. The molecular weight of C7 used for the calculation of specifically bound molecules per cell was 92,400 (24).

Determination of cell-bound C3 after fluid-phase activation of complement. These experiments were performed as described above with the following modifications. AB$^+$ serum from a volunteer donor was used as the complement source. After the incubation period, the cells were washed three times in GVB-EDTA and resuspended to 200 μl (2.5×10^8/ml) in GVB-EDTA. The amount of specifically bound ^{125}I-anti-C3d was determined as described above.

Determination of cell-bound C3c, C3dg, and CR1. PNH II, PNH III, and CCAD erythrocytes were washed in the appropriate buffer, and the number of molecules of cell-bound C3c, C3dg, and CR1 sites per cell were determined by using previously described methods (14).

Determination of C3 and C7 binding to PNH III and CCAD erythrocytes after fluid-phase activation of whole serum complement. PNH III and CCAD erythrocytes were washed three times in GVB-EDTA and resuspended to 3.33×10^9/ml. C7-repleted serum was prepared by adding ^{131}I-C7 to a final concentration of 50 μg/ml followed by the addition of EDTA to a final concentration of 20 mM. In triplicate, 30 μl of cells was incubated with 20 μl of CoFBb, and 100 μl of ^{131}I-C7 repleted serum. Controls for nonspecific binding of ^{131}I-C7 (and subsequently ^{125}I-C3c) were the same as those described above. After 30 min at 37°C, 850 μl of cold GVB-EDTA was added to the reaction mixtures, and they were transferred to 1.5-ml microfuge tubes and spun for 5 min at 12,000 rpm. A portion of the supernate was aspirated and used for determination of hemoglobin release by reading the OD_{541}, and these values were used to calculate the percent hemolysis. The remainder of the supernate was aspirated, and the cells were washed twice more in GVB-EDTA. In duplicate, 25 μl of each cell suspension was incubated with 50 μl (10 μg/ml) of ^{125}I-anti-C3c at 37°C. After 30 min, 50 μl of the cell suspension was aspirated, and the bound from unbound ligand was separated by spinning the cells through a 20% sucrose barrier. The amounts of specifically bound ^{125}I-C3c and ^{131}I-C7 were calculated as described above.

Determination of ^{125}I-C7 binding to normal EC3b and PNH III erythrocytes. Normal erythrocytes bearing approximately 26,000 molecules of C3b (normal EC3b) were prepared as previously described (26). Using ^{125}I-C7 repleted serum, the percent hemolysis and the amount of C7 bound to normal EC3b and PNH III erythrocytes after fluid-phase activation of complement by CoFBb were determined by using the methods described above.

SDS-PAGE of trypsinized and nontrypsinized normal, PNH II, PNH III, and AET-treated erythrocytes labeled with $NaOI_4/NaB^3H_4$. These experiments were performed by using previously described methods (11).

Results

Profile of the complement sensitivity of normal, PNH II, PNH III, and AET-treated erythrocytes when whole serum complement is activated by antibody. Because of the pivotal role of C9 in complement-mediated cytolysis (27), we compared C9 (and C8) binding to, and lysis of, normal, PNH II, PNH III, and AET-treated erythrocytes after classical pathway activation of whole serum complement.

When antibody concentration was held constant and complement concentration was varied by diluting serum, PNH II, PNH III, and AET-treated cells bound more C9 than normals (Fig. 1 *A*). The C8 binding was found to parallel the C9 binding (Fig. 1 *B*) and the ratio of ^{131}I-C9:^{125}I-C8 was ~5:1 for the normal red cells and ~6.5:1 for the abnormal cells. The greater binding of C9 was expected for PNH II and PNH III erythrocytes in that greater C3 convertase activity favors formation of C5 convertase sites and hence, formation of C5b-9 membrane attack complexes. To determine whether greater fixation of C3 (and hence a quantitative increase in C5 convertase sites) also accounted for the enhanced binding of C9 to AET-treated cells, we quantitated C3 and C9 binding to the normal and abnormal cells. For the abnormal cells, the increase in C9 binding was directly proportional to the increase in C3 binding (Fig. 2). For seven determinations that used varying concentrations of serum, PNH II, PNH III, and AET-treated cells bound 4.29±1.34, 3.48±1.09, and 1.66±0.54 (mean±1 SD) times more C3, respectively, than normal erythrocytes. Thus, for the AET-treated cells as well as for the abnormal PNH erythrocytes, the greater fixation of C9 appeared to be a consequence of greater C3/C5 convertase activity.

To determine whether there were quantitative differences in the functional activity of the membrane attack complex on the abnormal cells, we compared C9 binding to lysis for the four cell types (Fig. 3). There was a dramatic difference in the amount of C9 required for a given degree of lysis for the normal erythrocytes compared to the abnormal cells. The

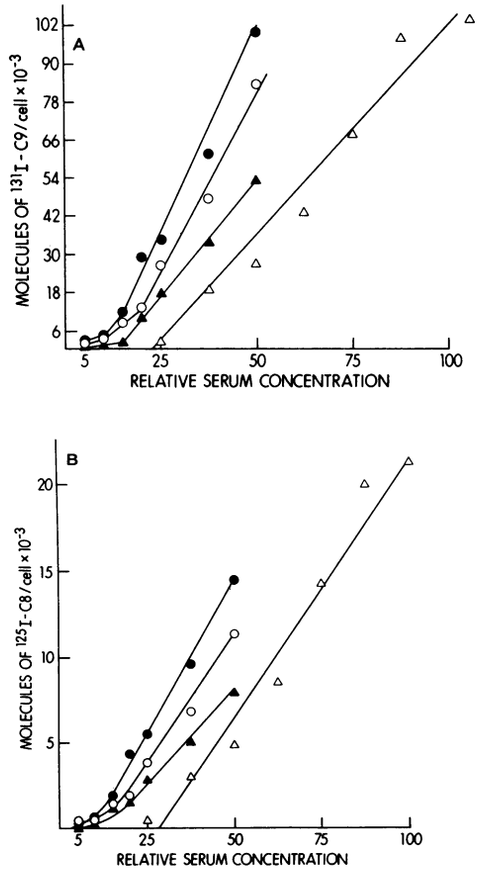

Figure 1. Binding of (*A*) ^{131}I-C9 and (*B*) ^{125}I-C8 to normal (△), AET-treated (▲), PNH III (○), and PNH II (●) erythrocytes after classical pathway activation of whole serum complement. The data points represent the mean of triplicate determinations.

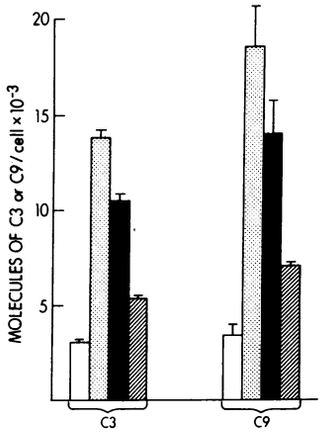

Figure 2. Comparison of the binding of C3 and C9 to normal (□), PNH II (■), PNH III (■), and AET-treated (■) erythrocytes after classical pathway activation of whole serum complement. The values depicted by the bars represent the mean±1 SD, n = 3.

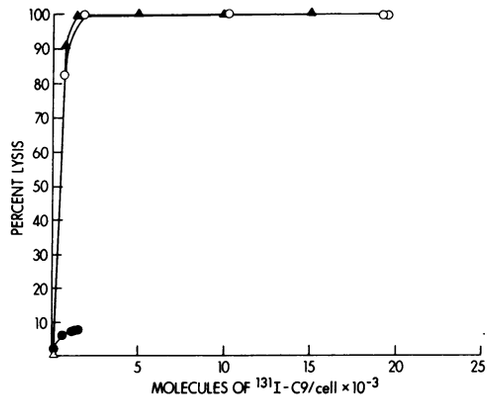

Figure 4. The relationship between C9 bound to, and hemolysis of, normal (△), PNH II (●), AET-treated (▲), and PNH III (○) erythrocytes after fluid-phase activation of whole serum complement by CoFBb. The data points represent the mean of triplicate determinations.

PNH III cells were the most sensitive with <2,500 C9 molecules being cell-bound at the 50% lysis point, whereas >70,000 molecules of C9 were associated with normal erythrocytes for the same degree of lysis. The curve of C9 bound versus lysis was similar for PNH II and AET-treated cells.

Profile of the complement sensitivity of normal, PNH II, PNH III, and AET-treated erythrocytes when whole serum complement is activated by CoF. PNH III and AET-treated erythrocytes are very susceptible to hemolysis in reactive lysis systems, whereas PNH II and normal cells are insusceptible. To determine the molecular basis for this greater sensitivity to bystander lysis, we examined the binding of C9 to the four cell-types when complement was activated by CoFBb complexes. PNH III and AET-treated cells were exquisitely sensitive to hemolysis in this reactive lysis system (Fig. 4). For both cell types, 100% lysis was associated with <2,000 molecules of membrane-bound C9. A very small portion of the cells from the patient with predominately PNH II cells were lysed. This was probably due to the presence of the small percentage (~7%) of PNH III cells (see Methods). Normal cells were insusceptible to reactive lysis and bound no C9. PNH III cells bound more C9 than the AET-treated cells at high serum concentrations (Fig. 5). The binding of C8 to the two cell types was essentially the same (Fig. 6); therefore, the C9 to C8 ratio was higher for PNH III cells (~3.3:1) than for AET-treated cells (~2.5:1). These ratios were considerably different from those observed when complement was activated by antibody (see above). The erythrocytes from the patient with predominantly PNH II cells bound very modest amounts of C8 and C9 (Figs. 5 and 6) (again due to the small population of PNH III cells present).

Fluid-phase activation of complement results in the formation of C5b6 complexes. The subsequent binding of C7 to this bimolecular complex produces conformational changes within the constituents of this newly formed trimolecular complex such that a membrane binding site (associated with the C7 component) becomes exposed. Because of the extreme lability of this binding site, it must bind quickly or the trimolecular complex loses its capacity to integrate into the membrane's lipid bilayer. If C5b-7 complexes with the membrane, then C8 and C9 binding follows spontaneously and in sequence. Packman et al. (28) have suggested that the interactions of C8 and C9 with C5b-7 on PNH III cells are aberrant. Further, Rosenfeld et al. (29), in using a reactive lysis system

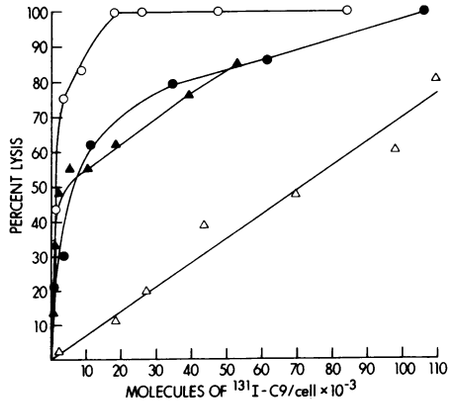

Figure 3. The relationship between C9 bound to, and hemolysis of, normal (△), AET-treated (▲), PNH II (●), and PNH III (○) erythrocytes after classical pathway activation of whole serum complement. The data points represent the mean of triplicate determinations.

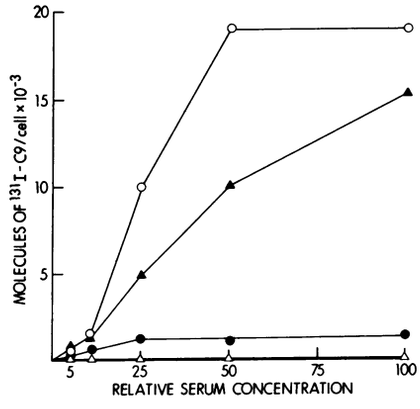

Figure 5. Binding of C9 to normal (△), PNH II (●), AET-treated (▲), and PNH III (○) erythrocytes as a function of C9 concentration in whole serum after complement activation by CoFBb. The data points represent the mean of triplicate determinations.

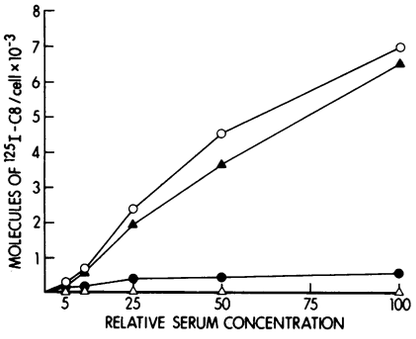

Figure 6. Binding of C8 to normal (△), PNH II (●), AET-treated (▲), and PNH III (○) erythrocytes as a function of C8 concentration in whole serum after complement activation by CoFBb. The data points represent the mean of triplicate determination.

which employed purified components, have recently reported that C5b-7 binding to PNH III and normal erythrocytes is quantitatively equivalent. To determine whether the greater binding of C8 and C9 to PNH III and AET-treated erythrocytes is the consequence of more efficient fixation of C5b-7 or of aberrant stoichiometric interactions of C8 and/or C9 with the trimolecular C5b-7 complex, we compared the binding of radiolabeled C7 to PNH III, AET-treated, PNH II, and normal erythrocytes after fluid-phase activation of whole serum complement by CoFBb.

For PNH III and AET-treated cells, the binding of C7 was associated with a marked degree of cytolysis (Fig. 7). As was the case with C9 binding, a very modest amount of C7 binding was associated with the lysis of a minor portion of the cells from the patient with predominantly PNH II erythrocytes. Normal erythrocytes bound no C7. The PNH III and AET-treated cells bound C7 with equal efficiency over the range of serum concentrations tested (Fig. 8 *A*). Although C8 binding (Fig. 8 *B*) paralleled C7 binding, the ratio of ^{125}I-C8 to ^{131}I-C7 was consistently less than unity, and this was particularly evident at higher serum concentrations.

Role of cell-bound C3 in the modulation of reactive lysis of normal and abnormal human erythrocytes. For sheep erythrocytes, the presence of cell-bound guinea pig C3b potentiates cytolysis in reactive lysis systems in which cytolysis is effected by guinea pig complement components (30, 31). To determine whether cell-bound C3 contributes to the enhanced lysis of PNH III and AET-treated cells, we determined the amount of C3 fragments present on the four cell-types after fluid-phase activation of whole serum complement. Although PNH III cells fixed slightly more C3 than the other cell types, the actual

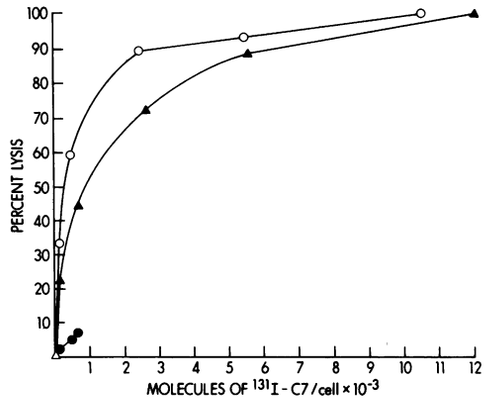

Figure 7. The relationship between C7 bound and hemolysis of normal (△), PNH II (●), AET-treated (▲), and PNH III (○) after fluid-phase activation of whole serum complement. The data points represent the mean of triplicate determinations.

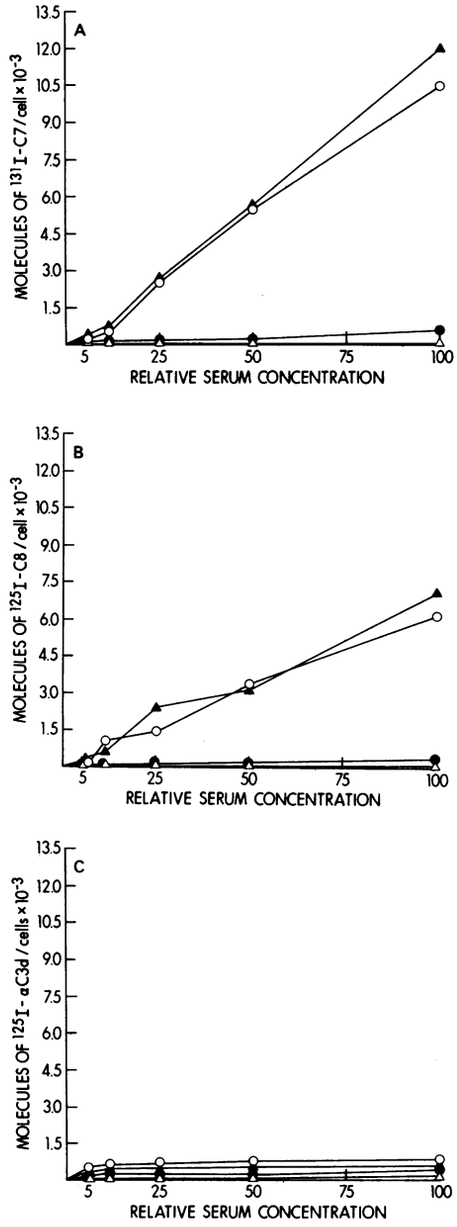

Figure 8. Binding of (*A*) ^{131}I-C7, (*B*) ^{125}I-C8, and (*C*) C3 to normal (△), PNH II (●), AET-treated (▲), and PNH III (○) erythrocytes as a function of C7, C8, or C3 concentration in whole serum after fluid-phase activation of complement by CoFBb. The data points represent the mean of triplicate determinations. Cell-bound C3 was quantitated using a monoclonal antibody, which recognizes an epitope in the C3d portion of the molecule and is expressed by C3b, iC3b, C3dg, and C3d. The same scale is used in all panels to emphasize that relatively little C3 is bound to the PNH III and AET-treated cells compared to the amount of C7 and C8 bound.

amount of C3 bound was very small compared to the amount of C7 bound (cf. Fig. 8 *C* vs. *A*). For example, when neat serum was employed in the experiments, the amount of C7 bound to PNH III erythrocytes was ~10,500 molecules/cell whereas the amount of C3 fragments bound was ~1,300 molecules/cell. Further, there was little difference between the amount of C3 fragments present on the PNH II erythrocytes (~400 molecules/cell when neat serum was used) compared to AET-treated cells (~650 molecules/cell); yet the chemically modified cells bound much more C7 (~12,000 molecules/cell) and were readily lysed, whereas the PNH II cells bound little C7 (~700 molecules/cell) and were not susceptible to bystander lysis (Figs. 7 and 8, *A* and *C*).

Erythrocytes from chronic cold agglutinin disease bind fluid-phase activated C3b more efficiently than normal erythrocytes (14). To examine further whether cell-bound C3 plays a functional role in the cytolysis of human erythrocytes in a reactive lysis system, we quantified cell-bound C3c, C7, and percent lysis for CCAD and PNH III cells when whole serum complement was activated by CoFBb (Fig. 9). Although the CCAD cells had three times more membrane bound C3c than the PNH III cells, the PNH III erythrocytes bound much more C7 than the CCAD erythrocytes. There was little lysis of the CCAD erythrocytes, whereas all of the abnormal PNH III cells were lysed under these experimental conditions.

Jones et al. (32) have suggested that PNH erythrocytes bear functionally active C3 fragments, which are responsible for their enhanced cytolysis in reactive lysis systems. For the PNH III erythrocytes, we found no cell-associated C3 fragments (Table I). C3 fragments were associated with the PNH II cells (Table I), yet these cells were not hemolyzed when whole serum complement was activated by cobra venom factor complexes (Fig. 4). The CCAD erythrocytes bore large numbers of C3 fragments (Table I), yet, as with the PNH II cells, they were not susceptible to reactive lysis (Fig. 9). The low number of CR1 sites on PNH II and CCAD erythrocytes is an epiphenomenon associated with the activation and binding of C3 to the membrane (33). This complement regulatory protein

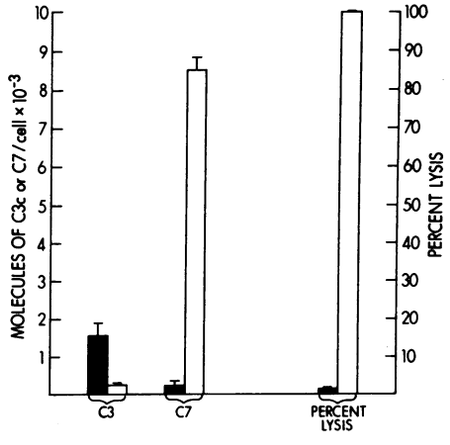

Figure 9. Comparison of binding of C3 and C7 to, and hemolysis of, CCAD (■) and PNH III (□) erythrocytes after fluid-phase activation of whole serum complement by CoFBb. Bound C3 was quantified using a monoclonal antibody, which recognizes an epitope on the C3c portion of the molecule and is expressed by C3b and iC3b. The values depicted by the bars represent the mean±1 SD, n = 3.

Table I. C3 Fragments on the Abnormal Cells

Erythrocyte type	C3c $\left(\frac{\text{molecules}}{\text{cell}}\right)^*$	C3dg $\left(\frac{\text{molecules}}{\text{cell}}\right)^‡$	CR1 $\left(\frac{\text{sites}}{\text{cell}}\right)^§$
PHN II	70	578	70
PNH III	33	56	521
CCAD	212	7,769	89

* Cell-bound C3c was determined using ^{125}I-monoclonal anti-C3c; <85 molecules/cell is within the normal range.
‡ Cell-bound C3dg was determined using ^{125}I-monoclonal anti-C3dg; <85 molecules/cells is within the normal range.
§ The number of CR1 sites was determined using monoclonal ^{125}I-anti-CR1; the range for normals is 145–1214 sites/cell and the mean is 643 (n = 125).

apparently plays no functional role in the modulation of reactive lysis, in that it is present in a normal number of copies on the PNH III cells (which are susceptible to reactive lysis) and deficient on PNH II and CCAD erythrocytes (which are insusceptible).

We also compared the binding of C7 to normal EC3b and to PNH III erythrocytes (Fig. 10). There was extremely modest binding of C7 to the normal EC3b, and these cells were virtually resistant to reactive lysis.

These data demonstrate that the greater binding of the components of the membrane attack complex to PNH III and AET-treated cells (and the consequent greater hemolysis of these abnormal cells) after fluid-phase activation of whole serum complement is not modulated by cell-bound C3 fragments.

Trypsin-mediated proteolysis of membrane glycoproteins on normal, PNH II, PNH III, and AET-treated erythrocytes. We have recently demonstrated a qualitative abnormality in glycophorin-α (the major erythrocyte sialoglycoprotein) on PNH III erythrocytes (11). To determine whether glycophorin-α is abnormal on PNH II and AET-treated cells, as well as on PNH III erythrocytes, we compared the electrophoretic pattern of trypsinized and nontrypsinized membrane proteins from normal, PNH II, PNH III, and AET-treated erythrocytes after radiolabeling of the terminal sialic acid residues with $NaIO_4$/NaB^3H_4. The Coomassie Blue staining pattern among the four cell types was the same (data not shown). There were very subtle differences in the radiolabeling pattern of the untrypsin-

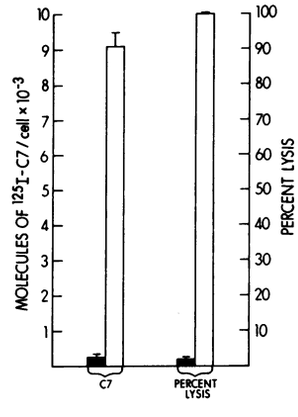

Figure 10. Comparison of C7 binding to, and hemolysis of, normal erythrocytes bearing ~26,000 molecules of C3b (normal EC3b) (■) and PNH III erythrocytes (□) after fluid-phase activation of whole serum complement by CoFBb. The values depicted by the bars represent the mean±1 SD, n = 3.

ized PNH II cells (the glycophorin-α_2 appeared more diffuse and had a slightly slower electrophoretic mobility (Fig. 11, lane *2* compared with lane *1*). After trypsinization, there was a modest decrease in the radioactivity of the trypsin cleavage protein (TCP) (the residual portion of glycophorin-α_2) for the PNH II cells compared with their normal counterparts (Fig. 11, lane *4* compared with lane *3*). For the PNH III cells, the labeling pattern of the untrypsinized cells showed a decrease in the radioactivity of the glycophorin-α_2. In addition, there were subtle decreases in the radioactive intensity of the glycophorin-γ homodimer, the glycophorin-α monomer, and perhaps the glycophorin-β monomer (Fig. 11, lane *6* compared with lane *5*). After trypsin treatment, there was a marked decrease in radioactivity in the area of the TCP for the PNH III cells (Fig. 11, lane *8* compared with lane *7*). The radiolabeling pattern for the untrypsinized AET-treated cells was indistinguishable from that of the normal erythrocytes (Fig. 11, lane *10* compared with lane *9*). However, after trypsin treatment, the AET-treated cells had a distinctively different fluorographic pattern (Fig. 11, lane *12* compared with lane *11*). There was incomplete cleavage of the glycophorin-α_2, and for the AET-treated cells, a band was visible just above the TCP. In addition, for the AET-treated cells the glycophorin-β and glycophorin-γ monomers were not cleaved by trypsin.

These experiments demonstrate the marked heterogeneity among the four cell types in the labeling pattern of their glycophorin molecules and in the susceptibility of these sialoglycoproteins to trypsin-mediated proteolysis.

Discussion

These studies demonstrate that diverse mechanisms underlie the greater susceptibility to complement-mediated lysis of PNH II, PNH III, and AET-treated erythrocytes.

Previous studies have shown that both PNH II and PNH III erythrocytes bind more C3b than normal cells when complement is activated by antibody (5, 6). Because C5 convertase activity depends upon the presence of cell-bound C3b (34), it follows that greater C3b binding would enhance C5 convertase activity, thereby favoring membrane attack complex formation. This is in keeping with our finding that the greater binding of C9 to PNH II and PNH III cells (Fig. 1) paralleled the greater binding of C3b (Fig. 2). In these experiments in which whole serum complement was activated by antibody, PNH II cells bound slightly more C3b (~4.3 times more than normals) than did the PNH III cells (~3.5 times more than normals). This may be in part due to the fact that the PNH III cells were from a patient with 88% abnormal cells, whereas the PNH II cells were from a patient with 100% abnormal cells (93% type II and 7% type III). In addition, the PNH II cells were relatively deficient in CR1 whereas the PNH III cells were not (Table I). The AET-treated cells consistently bound more C3b than untreated normal cells (~1.7 times more) but considerably less than the abnormal PNH cells. The molecular basis for this greater binding of C3b to AET-treated cells is not delineated by these experiments. Both PNH III and AET-treated cells are susceptible to reactive lysis whereas PNH II cells (and normal erythrocytes) are not (6, 13). These findings (along with others which will be discussed below) suggest that factors other than greater C3/C5 convertase activity mediate the enhanced susceptibility of PNH III and AET-treated cells to complement-mediated hemolysis. The greater lysis of these cells per C9 molecule bound (Fig. 3) could then be explained on the basis of some other aberrant interaction with constituents of the membrane attack complex. However, because PNH II cells are not abnormally hemolyzed in reactive lysis systems, this reasoning cannot be used to explain the greater efficiency of C9 at inducing lysis of the PNH II cells

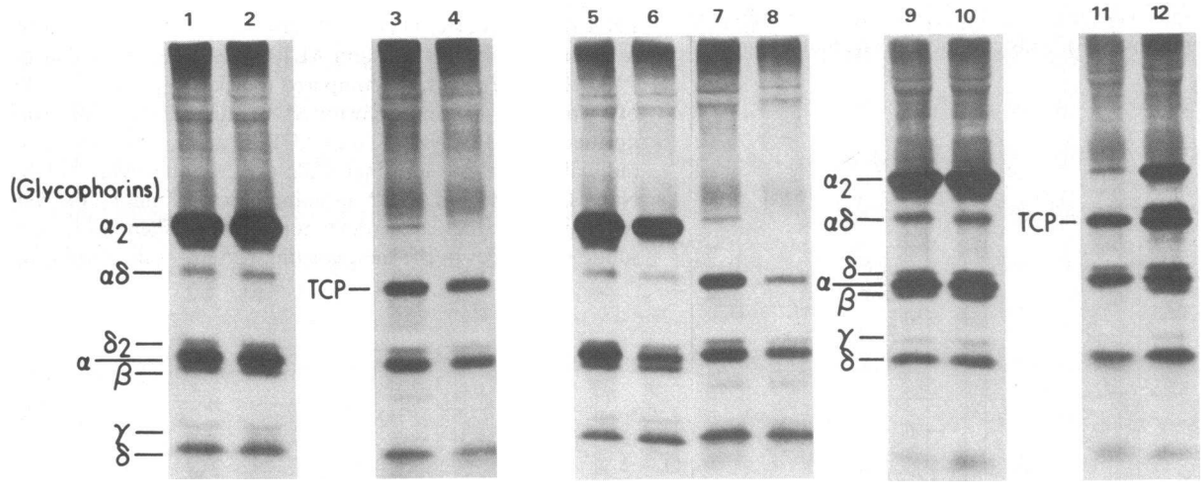

Figure 11. Fluorograph of trypsinized and nontrypsinized normal, PNH II, PNH III, and AET-treated erythrocytes labeled with $NaIO_4$/NaB^3H_4. The trypsinized cells were treated with 125 μg/ml of *n*-tosyl-L-phenylalanine chloromethyl ketone-trypsin prior to radiolabeling. 25 μg of solubilized protein were loaded onto each track. Lane *1*, untrypsinized normal erythrocytes; lane *2*, untrypsinized PNH II erythrocytes; lane *3*, trypsinized normal erythrocytes; lane *4*, trypsinized PNH II erythrocytes; lane *5*, untrypsinized normal erythrocytes; lane *6*, untrypsinized PNH III erythrocytes; lane *7*, trypsinized normal erythrocytes; lane *8*, trypsinized PNH III erythrocytes; lane *9*, untrypsinized normal erythrocytes; lane *10*, untrypsinized AET-treated erythrocytes; lane *11*, trypsinized normal erythrocytes; lane *12*, trypsinized AET-treated erythrocytes. The glycophorin monomers, and homo- and heterodimers are denoted by Greek letters using a modification of the nomenclature of Anstee (11). TCP, trypsin cleavage peptide. For each cell type, the glycophorin molecules show a different susceptibility to trypsin-mediated proteolysis.

(Fig. 3). It seems likely that increased C3/C5 convertase activity not only results in a quantitative increase in C9 binding to the abnormal cell (Fig. 1), but also favors the formation of cytolytically effective complement lesions (Fig. 3). The latter may be due to clustering of membrane attack complexes around the superactive convertase site, thus allowing individual complexes to coalesce or otherwise act in concert.

At the molecular level, the resistance of normal and PNH II cells to reactive lysis is due, at least in part, to inhibition of binding of components of the membrane attack complex (Figs. 4–8). However, the mechanism by which normal and PNH II cells resist binding of fluid-phase activated C5b-7 (and subsequent binding of C8 and C9) is not clear. In whole serum, fluid-phase inactivation of this trimolecular complex is accomplished by interactions of the labile binding site (associated with the C7 constituent [35–37]) with the S protein, the C5b-7 inhibitor protein, and with other less well-characterized lipoproteins (38, 39). One of the difficulties in speculating about how normal and PNH II cells inhibit binding of membrane attack complex components (whereas PNH III and AET-treated cells do not) is that little is known about the interactions of the labile binding site of C5b-7 with membrane constituents. The binding of the trimolecular complex is noncovalent, being mediated by exposure of the hydrophobic regions within the three molecules which allows for their insertion into the lipid bilayer of the cell. The role of the interactions of the labile binding site with membrane constituents in mediating this insertion is not clear. It is possible that normal (and PNH II) cells have a membrane component that actively inhibits binding of C5b-7, and that this membrane inhibitor is absent (or functionally inactive) on PNH III and AET-treated cells. Alternatively, membrane constituents may serve as cofactors or receptors for serum inhibitors of C5b-7 binding to the cell surface. Against this latter hypothesis, however, are the studies of Götze and Müller-Eberhard (40) and Jones et al. (32), who have reported quantitative increases in C5 and C5b6 binding, respectively, to PNH erythrocytes; these workers used systems that employed purified (or partially purified) complement components.

At least two other explanations for the greater binding of membrane attack complex constituents to PNH III and AET-treated cells seem plausible: (*a*) The initial binding of C5b-7 is equal for all the cell types, but membrane surface constituents prevent insertion of the trimolecular complex into the lipid bilayer of normal (and PNH II) erythrocytes. The failure to insert renders the complex unstable, and it is consequently lost from the cell surface (41). (*b*) Changes in membrane surface properties on PNH III and AET-treated erythrocytes could increase the affinity of the abnormal cells for the CoFBb. This would result in generation of the labile binding site of the C5b-7 complex closer to the cell surface and thereby favor insertion of the complex into the membrane bilayer.

Regardless of the exact mechanism, normal human erythrocytes have evolved a very effective system for resistance to reactive lysis. The need for such protection is obvious, because the cells are exposed to continuous (albeit low-grade) fluid-phase activation of complement (42). Failure to inhibit reactive lysis is apparently responsible for most of the hemolytic component of the PNH syndrome, inasmuch as patients with very high proportions of exclusively PNH II cells (which have greater C3/C5 convertase activity but are relatively resistant to bystander-mediated lysis) show little evidence of intravascular hemolysis, whereas patients with even a small population of PNH III cells have clear signs and symptoms of hemolytic disease. Further, circumstances that result in fluid-phase activation of complement (e.g., surgery, trauma, infections) are associated with hemolytic paroxysms in patients with PNH III erythrocytes (43).

The quantitative increase in binding of membrane attack complex components to PNH III and AET-treated cells after fluid-phase activation of complement by CoFBb complexes is not mediated by cell-bound C3 fragments (Figs. 8 *C*–10 and Table I). Although PNH III cells bind slightly more C3 than PNH II, AET-treated, or normal cells, they bind very much greater amounts of C7 (and C8 and C9) than the normal or PNH II erythrocytes (Figs. 5, 6, 8 *A* and *B*). Further, PNH II and AET-treated cells bind essentially the same amount of C3 after fluid-phase activation of complement (Fig. 8 *C*), yet the AET-treated cells bind much more C7-9 (Figs. 5, 6, 8 *A* and *B*). Our experiments that use CCAD erythrocytes, which bind fluid-phase-activated C3 more efficiently than normal erythrocytes (14) (Fig. 9), and those that use normal EC3b (Fig. 10) also suggest that cell-bound C3 does not contribute significantly to hemolysis of human erythrocytes in reactive lysis systems.

We also quantified the presence of C3 fragments and CR1 receptor sites on PNH II, PNH III, and CCAD cells ex vivo (Table I). The quantity of membrane-bound C3 fragments was within the normal range for PNH III cells whereas both PNH II and, to a much greater extent, CCAD erythrocytes bore abnormally large amounts of C3 fragments. It has been reported that inactivation of cell-bound C3b is impaired on PNH erythrocytes, due to deficient factor I cofactor activity on the abnormal cells (8). For human erythrocytes, CR1 serves as the cofactor for the enzymatic cleavage of cell-bound C3b to iC3b and C3dg by factor I (44–47). The number of CR1 sites on PNH III cells was well within the normal range, whereas the PNH II and CCAD erythrocytes had extremely low numbers of CR1 sites (Table I). These experiments demonstrate that the greater binding of the components of the membrane attack complex to PNH III cells (and their consequent greater hemolysis) is not mediated by cell-bound C3 fragments nor by a quantitative deficiency of CR1.

The inordinate binding of components of the membrane attack complex to PNH III cells apparently does not occur when complement is activated on the cell surface by antibody, in that PNH II cells (which do not bind membrane attack complex constituents after fluid-phase activation of complement) bind more C9 than PNH III (or AET-treated) cells after classical pathway initiation of the complement cascade. In the case of antibody activation of complement, binding of the terminal components (C5b-9) appears to be directly related to C3/C5 convertase activity (Fig. 2). There may be other fundamental differences between cytolysis mediated by a reactive lysis system and that initiated by classical pathway activation. For instance, the ratio of C9 to C8 per cell is different for the two systems. For PNH III cells, the ratio is ~6.5:1 for antibody-mediated activation and approximately ~3.5:1 for fluid-phase activation. In addition, cell type may also influence C9 to C8 ratios, as the normal cells, which were relatively resistant to antibody-mediated lysis, had a ratio of ~5:1 (compared with ~6.5:1 for sensitive cells [PNH II, PNH III, and AET-treated]). Thus mechanisms of complement activation, cell type, and concentration of components appear to influence C9 multiplicity within the membrane attack complex.

This may account for some of the apparent discrepancies in reports of size and molecular composition of the membrane attack complex (27).

The greater susceptibility of PNH III and AET-treated cells to reactive lysis appears to be due to a quantitative increase in cell-bound membrane attack complexes. However, because normal cells did not bind the terminal components, it is not possible to determine if qualitative differences in membrane attack complex efficiency exist. The studies noted above by Packman et al. (28) and by Rosenfeld et al. (29) suggest that the greater susceptibility of PNH III cells to reactive lysis may be in part mediated by qualitatively aberrant interaction of constituents of the membrane attack complex with the abnormal membrane of PNH III erythrocytes.

The relationship of the pathologic basis of the aberrant interactions with complement between PNH II and PNH III cells is unclear, but two explanations appear plausible: (*a*) The two cell types have the same basic membrane defect with the PNH III cells being more severely affected (10). (*b*) PNH III cells have two distinctive membrane abnormalities. One accounts for the greater binding of the components of the membrane attack complex after fluid-phase activation of whole serum complement. The other, which is shared by PNH II cells, mediates the greater functional activity of the C3/C5 convertase.

Either hypothesis could be supported by the results of our experiments with glycophorin digests (Fig. 11). For both PNH II and PNH III cells, there is less radioactivity in the area of the TCP compared to normals (the effects of trypsin-mediated proteolysis on glycophorin are detailed in reference 11). However, the radioactive intensity of the TCP is clearly less for the PNH III cells than for the PNH II cells. In addition, the labeling pattern of the untrypsinized cells is more distinctively abnormal for the PNH III cells. Thus, the glycophorin molecules appear to be more defective on PNH III cells. By virtue of its being the predominant binding site for nascent C3b, glycophorin-α_2 may be regarded as a subunit constituent of the alternative pathway convertase and, hence, may play a regulatory role in the functional activity of the convertase (11). Recent studies have suggested that glycophorin modulates both sensitivity of cells to reactive lysis (48) and alternative pathway activity (49). If glycophorin regulates both C3 convertase activity and susceptibility to reactive lysis, then one could speculate that greater susceptibility to reactive lysis requires a more severe derangement of glycophorin structure than regulation of convertase functions, thus accounting for the differences between PNH II and PNH III cells.

In support of the hypothesis that PNH III cells have two membrane abnormalities, one could speculate that the abnormality in glycophorin is responsible for the greater susceptibility of PNH III cells to reactive lysis and that regulation of C3/C5 convertase activity is mediated by another membrane constituent (e.g., decay-accelerating factor [9, 10]), which is dysfunctional on both PNH II and PNH III cells. The abnormality in glycophorin on PNH III cells appears to be related to the carbohydrate moiety of the molecule (11). Because glycophorin is the major erythrocyte sialoglycoprotein, the abnormalities in glycophorin may reflect a more global disturbance in glycosylation of membrane constituents, some of which may be important regulators of the functional activity of complement, whereas others (e.g., acetylcholinesterase [43]) may not.

The chemical process by which AET transforms normal erythrocytes into complement-sensitive cells remains speculative, but disruption of membrane structural properties by reduction of disulphide bonds appears to be the most likely mechanism (50). Although the fluorographic patterns of the radiolabeled AET-treated cells was the same as that of normals (Fig. 11, lane *10* compared with lane *9*), the glycophorin molecules of the chemically modified cells were relatively resistant to trypsin-mediated proteolysis (Fig. 11, lane *12* compared with lane *11*). These data suggest that AET modifies cellular constituents that are important for the maintenance of the three-dimensional orientation of these integral membrane glycoproteins, thereby modifying their susceptibility to trypsin.

The electrophoretic pattern of trypsinized AET-treated cells contrast sharply with that of the trypsinized PNH III cells (Fig. 11, lane *8* compared with lane *12*), yet the two-cell types share a common mechanism for their markedly abnormal sensitivity to reactive lysis (they both bind large quantities of membrane attack complex constituents). There appear to be two plausible explanations for these seemingly disparate observations: (*a*) The abnormalities in glycophorin are an epiphenomenon, and the greater binding of membrane attack complex components to the two cell types is due to dysfunction of other membrane constituents. Different mechanisms for mediating this enhanced binding of the terminal components may exist and a unique mechanism could be operative for each cell-type. (*b*) The abnormalities in glycophorin do mediate the greater binding of membrane attack complex constituents. Abnormalities in glycosylation (PNH III cells) and treatment of cells with AET result in similar changes in the three-dimensional orientation of glycophorin. This may be the common molecular abnormality underlying the enhanced binding of the terminal components.

AET-treated cells (and other types of chemically modified cells [43]) have been used as models for investigating the membrane defects in PNH. Our studies demonstrate that AET-treated erythrocytes resemble PNH III cells in that they are more susceptible to reactive lysis (as a result of greater binding of the terminal components). AET-treated cells, however, fix less C3b than PNH III (or PNH II) cells after complement activation by antibody. Further, the marked difference in the trypsin-cleavage pattern of the glycophorin molecules for the AET-treated cells compared to the PNH cells raises the possibility that the molecular basis for the aberrant interactions of these chemically modified cells with complement may be different than for the PNH cells. Although AET-treated cells do provide an important model for studying complement interactions with human erythrocytes, they can no longer be considered a paradigm for the investigation of the molecular basis of the aberrant interactions of complement with PNH erythrocytes.

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