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Immunology

# Enhanced complement-mediated lysis of type III paroxysmal nocturnal hemoglobinuria erythrocytes involves increased C9 binding and polymerization

**(reactive lysis/complement component C9 insertion/kinetics of hemolysis/membrane attack complex)**

VALERIE W. HU* AND ANNE NICHOLSON-WELLER†‡§¶

*Department of Biochemistry, Uniformed Services University of the Health Sciences, F. Edward Hébert School of Medicine, Bethesda, MD 20814-4799; and †Department of Medicine, Harvard Medical School, ‡Department of Medicine, Charles A. Dana Research Institute, §Harvard-Thorndike Laboratories, Beth Israel Hospital, and ¶Department of Rheumatology and Immunology, Brigham and Women's Hospital, Boston, MA 02215

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ABSTRACT **The interaction of terminal complement proteins (C5–C9) with normal erythrocytes and type III paroxysmal nocturnal hemoglobinuria erythrocytes (PNH-E) has been compared in terms of binding of the C5–9 complex, C9 polymerization, and C9 insertion into membranes. Complement components C5, C7, and C8 bind equally well to both types of erythrocytes, whereas the binding of C9 to PNH-E is 5–6 times greater than that to normal erythrocytes. The kinetics of C9 binding was compared with the kinetics of lysis for both types of cells under conditions leading to 100% lysis. There was a noticeable lag time between C9 binding and lysis of normal erythrocytes, but the lysis of PNH-E proceeded without a lag and the kinetics of lysis more closely paralleled C9 binding. The efficiency of C9 insertion was similar for both types of cells, but C9 polymerization was significantly enhanced on PNH-E. These data indicate that the enhanced susceptibility of type III PNH-E toward lysis by C5–9 can be correlated with abnormally high C9 binding and increased formation of poly(C9).**

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired disease characterized by an intermittent hemolytic anemia resulting from erythrocytes that are abnormally sensitive to lysis by autologous complement (1–4). PNH erythrocytes (PNH-E) can be distinguished, on the basis of the degree of their abnormal sensitivity to lysis by complement, as highly sensitive (type III PNH-E) and moderately sensitive (type II PNH-E) (5). Both types of PNH-E have abnormal amounts of C3b (a cleavage fragment of the third component of complement, C3) deposited on their surface after complement activation (6). The supranormal levels of C3b can be correlated directly with a deficiency in a membrane protein, the decay-accelerating factor, which inhibits the formation as well as accelerates the decay of the C3/C5 convertases of the alternative and classical complement pathways (7–9). However, the type III PNH-E have an additional defect, that of enhanced susceptibility to lysis by the terminal complement components, C5–9 (10). The molecular basis for the greater sensitivity of type III PNH-E to reactive lysis by complement is unknown. Recently, Rosenfeld *et al.* (11) reported that enhanced reactive lysis of type III PNH-E by C5–9 does not involve increased C7 binding or cell-bound C3b, implying that the defect could be in the mode of interaction of C8 and/or C9 with the C5–7 complex or with the membrane itself. The latter result conflicted with earlier studies by Jones *et al.* (12), which suggested that cell-bound C3b enhanced the assembly of the reactive lysis components on type III PNH-E, effectively increasing the number of active lesions per cell rather than the hemolytic efficiency of a given membrane-bound C5–9 complex.

Here we show that C8 binding (in addition to C5–7 binding) is not affected on type III PNH-E and investigate the role of C9 in the enhanced lysis by C5–9. Normal erythrocytes and type III PNH-E were compared with respect to C9 binding, C9-insertion efficiency, and tendency of cell-bound C9 to polymerize.

## MATERIALS AND METHODS

**Chemicals.** Benzidine hydrochloride was obtained from Sigma. The photoactivatable glycolipid probe, 12-(4-azido-2-nitrophenoxy)stearoyl[1-$^{14}$C]glucosamine (12-APS-GlcN), was synthesized according to published procedures (13). Its specific activity was 51.2 Ci/mol (1 Ci = 37 GBq).

**Buffers.** $GVBS^{2+}$ [Veronal-buffered saline (145 mM NaCl/5 mM sodium barbital, pH 7.4) containing 0.02% gelatin, 0.15 mM $CaCl_2$, and 1 mM $MgCl_2$] was used in all hemolytic assays. Phosphate-buffered saline ($P_i$/NaCl) contained 5 mM sodium phosphate (pH 8) and 150 mM NaCl. Tris-buffered saline (Tris/NaCl) contained 10 mM Tris Cl (pH 8) and 155 mM NaCl.

**Complement and Antisera.** Whole human serum (from Cordis, Miami, FL) was the source of complement for most experiments. In one experiment, purified C5, C6, C7, C8, and C9 were used; these were obtained from Calbiochem–Behring. Rabbit antiserum against human erythrocytes was purchased from Cappel Laboratories (Malvern, PA). Goat anti-human complement protein antisera and peroxidase-conjugated rabbit anti-goat IgG (heavy and light chains) antiserum were obtained from Miles and Kirkegaard and Perry (Gaithersburg, MD), respectively.

**Cells.** Blood was obtained from a PNH patient with a positive sugar-water-lysis test (34) and ≈50% type I (i.e., apparently normal) PNH-E and 50% type III PNH-E as determined by the complement-lysis-sensitivity assay (14) using plasma containing antibodies against the high-incidence blood group antigen P (kindly provided by R. S. Shirey, Johns Hopkins Hospital) to sensitize the cells. The patient had not been transfused in the previous 18 months and was on no medication. The PNH blood was centrifuged, the buffy

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Abbreviations: PNH, paroxysmal nocturnal hemoglobinuria; PNH-E, PNH erythrocytes; C3, C5, C7, C8, and C9 are complement proteins; C3b, a cleavage fragment of C3; C5–9, the membrane attack complex of complement; poly(C9), $NaDodSO_4$-resistant polymerized form of C9; $GVBS^{2+}$, Veronal-buffered saline/0.02% gelatin/0.15 mM $CaCl_2$/1 mM $MgCl_2$.

coat was removed, and the PNH-E were stored in the autologous plasma at 4°C until use. Blood was drawn similarly from volunteers who were not on medication. All cells were washed three times with $P_i$/NaCl before use.

**Complement Lysis of Cells.** For classical-pathway lysis, cells ($10^9$ per ml of $GVBS^{2+}$) were treated for 15 min at room temperature with an equal volume of rabbit anti-human erythrocyte antiserum at 1:200 dilution and then were washed before incubation with normal human serum. Lysis by the purified terminal components required acid-activation of C5 in the presence of C6 at 4°C (15), followed by the sequential addition of unsensitized cells, C7, C8, and C9. Hemolysis occurred at 37°C. Ghosts were prepared by washing in 5 mM sodium phosphate (pH 8.0).

**Photolabeling Procedure.** For photolabeling experiments, ghosts of $2.9 \times 10^9$ complement-treated cells were incubated under red lights with $2 \times 10^6$ cpm of the $^{14}C$-labeled photoprobe for 15 min at 37°C. Under these conditions, the ghosts took up >95% of the added probe. Crosslinking between the photoprobe and inserted or integral membrane proteins (and lipids) was accomplished by a 2-min irradiation at 366 nm using an UV mineral lamp. The membranes were washed by centrifugation with 5 mM sodium phosphate (pH 8), and the pellet was solubilized with deoxycholate and sedimented through a 4.5-ml 10–50% (wt/vol) sucrose gradient (16) in a Beckman SW 50.1 rotor spun at 37,000 rpm for 16 hr. The bottom 2.38 ml of the gradient was removed and then was dialyzed against Veronal-buffered saline, and the proteins were precipitated with trichloroacetic acid. The protein pellet then was resuspended in nonreducing sample buffer for electrophoresis.

**Electrophoretic Procedures.** Samples were electrophoresed in $NaDodSO_4$/7.5% polyacrylamide slab gels with a 2.5% acrylamide stacking gel, according to procedures described by Laemmli (17). For poly(C9) determination, the procedure described by Podack and Tschopp was followed (18). Proteins were electrophoretically transferred from gels to nitrocellulose paper by use of a Hoefer electroblot apparatus operating at 1 A for 2 hr and 0.5 A (or 0.3 A) overnight in the Tris-buffered saline described by Towbin *et al.* (19). The blots were stained for protein with amido black (19). Identification of C9 bands [monomer, dimer, and poly(C9)] was accomplished by treating the blots with goat anti-human C9 antiserum (1:100 dilution) followed by peroxidase-labeled rabbit anti-goat IgG (1:1000) and benzidine, using procedures adapted from Robb *et al.* (20).

**Liquid Scintillation Counting.** The amount of radioactivity in the C9 bound to PNH-E and to normal erythrocytes was determined by cutting the peroxidase-stained bands from the blots and placing the nitrocellulose strips in 10 ml of Beckman Ready-Solv HP liquid scintillation fluid. Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer.

**Quantitation of Bound Complement Components.** The relative amounts of complement proteins bound to the different cells were determined by electrophoresis of replicate samples of the complement-lysed ghosts in $NaDodSO_4$/7.5% polyacrylamide gels, followed by electroblotting and immunostaining, as described above, using the appropriate anti-complement-component antiserum at 1:100 dilution. Densitometric scans of the photographic negatives of the peroxidase-stained immunoblots were obtained by use of a Hoefer GS-300 densitometer operating in the transmittance mode. The relative intensities of the stained bands were obtained by cutting out and weighing the paper corresponding to the area under the densitometric peaks. The relative intensities determined by this method correspond accurately to the relative amounts of protein in a given sample (21).

## RESULTS

**PNH-E Bind 5–6 Times More C9 Than Normal Erythrocytes.** PNH-E and normal erythrocytes were lysed by purified terminal components C5–C9 upon acid activation of C5 in the presence of C6. As shown in Table 1, lysis of PNH-E was 5.6 times greater than lysis of normal erythrocytes. Analysis of the amounts of C5, C7, and C9 bound to PNH-E and the normal red cells indicated that PNH-E bound 5.6 times as much C9 as normal cells, whereas C5 and C7 were bound at comparable levels on both types of cells. These results suggest that the type III PNH-E membrane defect that is responsible for enhanced lysis by terminal components primarily affects C9 binding. It was of interest to determine whether the difference in C9 binding was related to differences in the rate of C9 binding to the cells or to differences in the number of C9 binding sites on the cells.

**Kinetics of C9 Binding to PNH-E and to Normal Erythrocytes.** Cells were treated with rabbit anti-human erythrocyte antiserum (1:200 dilution) and human serum (1:2), conditions leading to 100% lysis of both cells within 45 min. Aliquots of the cell suspensions were removed at different times and the percent of cells lysed was determined. The membrane pellets obtained at different time points then were washed and later were resolved by $NaDodSO_4$/PAGE and analyzed for bound C9. Fig. 1 shows the time course of lysis of PNH-E and normal erythrocytes as well as the time course for C9 binding to both PNH and normal cells. PNH-E were lysed faster than normal erythrocytes. However, both exhibited saturable C9 binding with similar kinetics. Although the final amount of C9 bound was higher for PNH-E (in contrast to C7 and C8, whose maximal amounts are similar on both cells), confirming the data in Table 1, the time required for half-maximal C9 binding on both cells was about 5 min. Thus, while the kinetics of lysis of PNH-E closely followed the kinetics of C9 binding, there was a lag time between C9 binding and hemolysis of normal erythrocytes. The sigmoidal shape of the lysis curve for normal erythrocytes in contrast to the apparently hyperbolic lysis curve for PNH-E suggested that an additional, perhaps cooperative, factor was involved in the lysis of the normal cells.

**More Polymerized C9 Is Found on PNH-E.** One mechanism that might be expected to exhibit cooperative behavior is the formation of poly(C9). We therefore analyzed complement-lysed PNH-E and normal erythrocytes for poly(C9). As shown in Fig. 2, a striking difference exists in the amounts of poly(C9) present on PNH and normal cells. By comparison, C9 dimer levels appear similar on both cells and the amount of C9 monomer is higher on PNH-E than on normal erythrocytes, but this difference is not as great as for poly(C9). These data imply that facilitated C9 polymerization and higher C9 binding are responsible for the more rapid hemolysis of PNH-E as well as the overall higher susceptibility of PNH-E to C5–9.

Table 1. Relative amounts of late complement components bound to normal erythrocytes and PNH-E upon acid-induced lysis by C5–9

| Erythrocytes | % lysis | C5 | C7 | C9 |
|---|---|---|---|---|
| Normal | 11.8 | 1.0 | 1.0 | 1.0 |
| PNH | 65.9 | 1.1 | 0.9 | 5.6 |

The relative amounts of C5, C7, and C9 bound to normal and PNH erythrocytes were obtained by densitometric quantitation of immunoblots of electrophoresed membranes stained with the respective antibodies against human complement components. The data were normalized with respect to the amount of each component bound to normal erythrocytes, taking into consideration that ≈50% of the PNH-E were type I (apparently normal) erythrocytes.

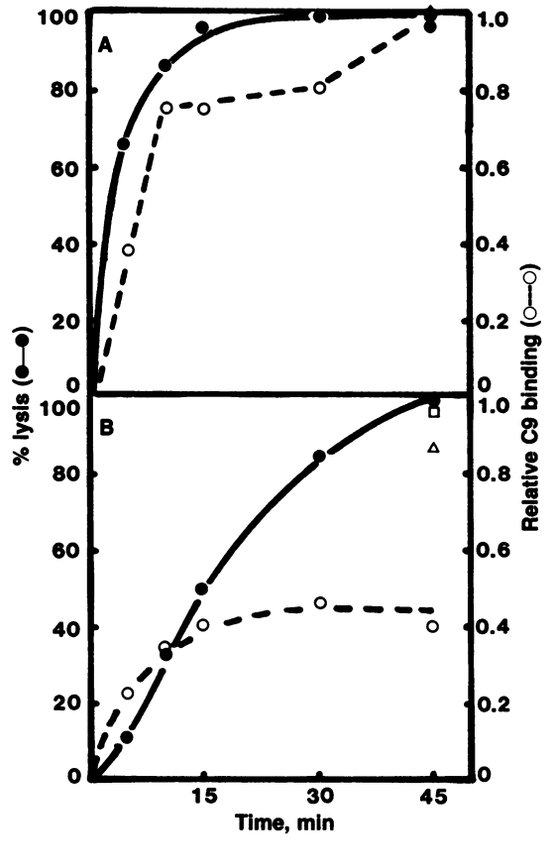


FIG. 1. Time course of lysis (○) and C9 binding (●) for PNH-E (*A*) and normal erythrocytes (*B*). Sensitized erythrocytes ($4 \times 10^8$ per ml of $GVBS^{2+}$) were incubated at 37°C with equal volumes of normal human serum. At different times, 0.5 ml of reaction mixture was removed, and the reaction was stopped by addition of 0.5 ml of ice-cold $P_i$/NaCl. The membranes and remaining intact cells were sedimented in an Eppendorf Microfuge and optical density measurements (at 412 nm) were made of the supernatants in order to determine % lysis. The pellets were washed several times with 5 mM sodium phosphate (pH 8) to lyse the remaining cells. C9 bound to the membranes was quantitated by $NaDodSO_4$/PAGE and immunoblotting. Densitometric scans of photographic negatives of the immunoblots gave relative amounts of C9 bound to the membranes at different time points. Similar procedures provided data on the relative amounts of C7 (□) and C8 (△) bound to the membranes at the lysis end point (45 min). The densitometric data (mg of paper corresponding to the area of the densitometric peak) was normalized with respect to the amount of C7, C8, or C9 bound to PNH-E after a 45-min incubation with complement.

**C9 Insertion Efficiency Is Unaltered on PNH-E.** Since membrane insertion of terminal components, C9 in particular, can be correlated with functional membrane damage by complement (22), we analyzed complement-lysed PNH-E and normal erythrocytes for the amount of C9 inserted. To measure insertion, we used a glycolipid photoprobe that is restricted in reactivity to segments of proteins within the hydrophobic core of membranes (23). Complement-lysed cells were incubated with the photo-reagent and irradiated to effect crosslinking between inserted proteins and the radioactive photoprobe. The membrane attack complex was isolated, resolved by $NaDodSO_4$/PAGE, and analyzed for radioactivity in C9. The C9 band, identified by immunoblotting procedures, was cut from the blot and the radioactivity was measured by liquid scintillation spectrometry. Results are presented in Table 2. The insertion index is the ratio of the radioactivity in the C9 band to the intensity of the immunostained C9 for a given sample. Its numerical value is an arbitrary number that depends on the units used to measure

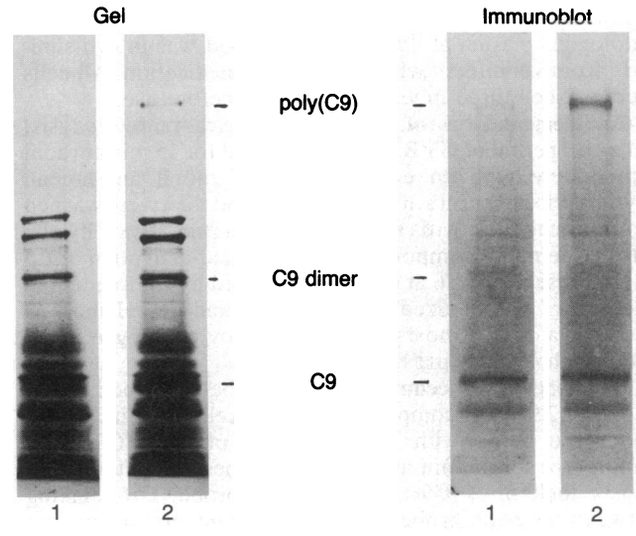


FIG. 2. Coomassie blue-stained gel and corresponding anti-C9-stained immunoblot of normal (lanes 1) and PNH (lanes 2) erythrocytes after reaction with complement. Duplicate samples of complement-treated normal and PNH erythrocytes were resolved by $NaDodSO_4$/2.5–10% gradient PAGE. One set was electrophoretically transferred to nitrocellulose paper, and the blots were stained for C9 with goat anti-human C9 antiserum, peroxidase-labeled rabbit anti-goat IgG antiserum, and benzidine. Several specific C9 bands were stained, but some nonspecific but weaker staining of erythrocyte bands 1 and 2 was also seen above the C9 dimer. The relatively heavily stained band below the C9 monomer is a breakdown fragment of C9 that also has been seen in a purified C9 sample.

radioactivity and binding (in this case, cpm and weight of the densitometric peaks in mg). As discussed previously (24), the insertion indices are meaningful in that they reflect the relative efficiency of insertion of a given molecule on different membranes or under different conditions. The data in Table 2 indicate that the efficiency of C9 insertion into PNH-E is comparable to that for normal erythrocytes. Thus, C9 insertion is not the limiting factor in the lysis of erythrocytes by complement.

## DISCUSSION

We have studied the mechanism through which type III PNH-E exhibit higher sensitivity to the terminal complement components. With purified acid-activated C5–C9, an almost 6-fold higher percentage of PNH-E were lysed compared with normal erythrocytes, which corresponded with an ≈6-fold increase in C9 binding to type III PNH-E (Table 1). In contrast, no differences in the binding of C5 and C7 were noted. Using conditions to activate the classical pathway, we compared the kinetics of C9 binding with the kinetics of lysis. Interestingly, both PNH-E and normal erythrocytes achieved half-maximal binding of C9 in about 5 min, indicating that the measured differences in C9 bound to PNH-E and normal

Table 2. Comparison of efficiency of C9 insertion into normal and PNH erythrocytes

| Erythrocytes | Radioactivity in C9, cpm | Weight of densitometric peak, mg | Insertion index* |
|---|---|---|---|
| Normal | 480 ± 24 | 19.5 ± 0.1 | 24.6 |
| PNH | 379 ± 19 | 15.5 ± 0.1 | 24.4 |

*The insertion index as shown here is defined as the ratio of cpm to the weight of the paper in mg.

erythrocytes after 45 min reflect differences in the number of C9 molecules bound to equivalent numbers of C5–8 sites on the two types of cells rather than differences in the rate of C9 binding or of C5–9 formation. However, the rate of lysis of normal erythrocytes lagged behind the rate of C9 binding (Fig. 1*B*), whereas the rate of lysis of PNH-E more closely paralleled the rate of C9 binding (Fig. 1*A*), suggesting a mechanism for more efficient channel formation on PNH-E by bound C5–9 complexes. In addition, the total amount of C9 bound was greater for the PNH-E, whereas the amounts of C7 and C8 bound were not significantly affected, thereby confirming as well as extending the data in Table 1. In this case, the data were not adjusted to account for the fact that the PNH-E population contained about 50% apparently normal erythrocytes (type I PNH-E) and 50% type III PNH-E. Thus, the observed differences in C9 binding between the normal control erythrocytes and the PNH-E would undoubtedly be greater if the total PNH-E population were pure type III cells.

The experiments discussed above indicated that the specific defect of type III PNH-E is manifested at the level of C9. These data are consistent with previous observations that guinea pig C9, in contrast to human C9, failed to produce enhanced lysis of type III PNH-E (25). The species source of C8 appeared to be irrelevant. Consequently, two forms of C9 interaction with/on membranes were studied, namely the efficiency of C9 insertion and the tendency of C9 to form $NaDodSO_4$-resistant polymers. C9 insertion has been functionally correlated with marker release from liposomes (22) and lysis of erythrocytes (21, 26), and the degree of C9 polymerization has been correlated with channel size (27). To measure C9 insertion after membrane attack by complement, the proteins within the membrane were selectively tagged with a $^{14}C$-labeled photoactivatable, membrane-restricted probe. Solubilization of the cell membranes and resolution of the C9 antigen on an immunoblot made it possible to quantitate the inserted (and hence radiolabeled) C9 within the membrane. The ratio of the radioactive counts associated with inserted C9 to the relative amount of C9 bound provided a measure of relative insertion efficiency. Bound C9 was inserted as efficiently into the membrane of normal erythrocytes as into that of PNH-E (Table 2). Thus, the defect of type III PNH-E responsible for enhanced hemolytic efficiency of C5–9 is not specifically related to C9 insertion efficiency, although the total amount of inserted C9 is higher on PNH-E as a result of increased C9 binding.

Although it has been reported that one C9 molecule per C5–8 is sufficient to generate a functional lesion (28), multiple C9 molecules may bind per C5–8 complex (27, 29–32). The presence of larger channels can be correlated with $NaDodSO_4$-resistant poly(C9), which suggests that the polymerized form of C9 has functional significance (27). The analysis of normal and PNH erythrocyte membranes after the respective cells were exposed to membrane attack by complement revealed that there was much more poly(C9) on PNH-E membranes. In addition, the amount of poly(C9) formed on different samples of normal erythrocytes seemed to relate to the respective degree of lysis (unpublished data). These data suggest that the enhanced C9 binding on PNH-E might accelerate C9 polymerization and, in so doing, directly or indirectly enhance the rate of lysis as well as the overall lytic efficiency of the terminal complex. A recent study described the production of fully functional complement lesions by a modified form of C9 that is incapable of poly(C9) formation (33). The authors suggest that, although poly(C9) formation is not an obligatory event in lysis by complement, C9-mediated aggregation of C5b–8 complexes may be of major importance for lytic function, with poly(C9) formation being incidental to this primary event. However, poly(C9) formation by native C9 may still be symptomatic of dynamic processes leading to functional complement channels and therefore relevant when considering relative susceptibilities of cells to complement.

In summary, the abnormal sensitivity of type III PNH-E to lysis by C5–9 has been investigated by comparing the interactions of the complement components with these cells and normal erythrocytes. Significantly more C9 was bound to PNH-E, and the kinetics of lysis indicated that the C9 bound was more efficient at inducing lysis of the PNH-E. Moreover, the efficiency of C9-induced lysis could be correlated with the formation of poly(C9). It is not clear whether enhanced C9 polymerization represents the critical defect or whether it is a consequence of enhanced C9 binding. In any event, the enhanced C9 binding and polymerization on PNH-E in comparison to normal erythrocytes imply that normal cells have a mechanism for regulating these processes. At present, the membrane component(s) controlling C9 binding and polymerization is unknown.

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