PAROXYSMAL NOCTURNAL HEMOGLOBINURIA: DEFICIENCY IN FACTOR H-LIKE FUNCTIONS OF THE ABNORMAL ERYTHROCYTES*

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Blood of patients with paroxysmal nocturnal hemoglobinuria (PNH)[1] contains variable amounts of abnormal erythrocytes that are unusually sensitive to the hemolytic action of complement (1, 2). Lysis of these cells is mediated by the alternative pathway and is enhanced in serum of reduced pH (3–6). The results of this study suggest that the increased susceptibility of PNH erythrocytes to lysis by complement is due to a deficiency in membrane-associated, Factor H-like regulatory activity.

Initiation of the alternative pathway is a spontaneous process (7, 8), resulting in the random deposition of C3b on cell surfaces (9, 10). C3b bound to activators of the alternative pathway such as certain bacteria, fungi, viruses, and virus-infected cells, is relatively resistant to inactivation by the control proteins Factors H and I. As a result, the number of bound C3b molecules rapidly increases due to the positive feedback of the pathway (11). On nonactivating particles and host cells amplification of C3b deposition is largely prevented by the action of Factors H and I. However, the present evidence suggests that at least on human erythrocytes this mechanism is not entirely sufficient to prevent cytolysis and that membrane constituents are required for efficient control of complement activation.

It is known that human erythrocytes possess at least two membrane proteins that regulate complement: the C3b receptor (12) and the decay-accelerating glycoprotein (13). These proteins have been isolated and found to have Factor H-like activities, suggesting a role in controlling activation of complement (12, 13). These activities include inhibition of Factor B binding to C3b, acceleration of the decay of the C3 convertase, C3b,Bb, and enhancement of cleavage and inactivation of C3b by Factor I. It is the purpose of this paper to demonstrate that the abnormal erythrocytes in the blood of three PNH patients lack these functions and to propose that this deficiency is a manifestation of the molecular defect underlying PNH.

1 *Abbreviations used in this paper:* E, erythrocytes; EC3b, erythrocytes bearing surface-bound C3b; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylenebis (oxyethylenenitrilo)-tetraacetic acid; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; GVB, VBS containing 0.1% gelatin; GVBE, GVB containing 10 mM EDTA; PNH, paroxysmal nocturnal hemoglobinuria; VBS, 10 mM veronal, 150 mM NaCl, pH 7.4.

Materials and Methods

Purified Proteins and Reagents. C3 (14), nephritic factor (15), cobra venom factor (16), and Factors B (17), D (18), H (19), and I (19) were prepared as described elsewhere. C3 was fluoresceinated (FITC-C3) by incubating 9.1 mg C3 with 0.2 mg fluorescein isothiocyanate in 2 ml of 50 mM Na_2CO_3 buffer at pH 9 for 50 min at 22°C. FITC-C3 was separated from free fluorescein by gel filtration on Sephadex G25. TPCK-trypsin was purchased from Worthington Biochemical Corp., Freehold, NJ. Buffers used were: VBS, 10 mM veronal, 150 mM NaCl, pH 7.4; GVB, VBS containing 0.1% gelatin; GVBE, GVB containing 10 mM EDTA. Iodination of C3 and Factor B was performed using Iodogen (Pierce Chemical Co., Rockford, IL) according to the manufacturer's directions.

Preparation of C3b-Bearing Erythrocytes. Blood was drawn into EDTA and the cells were washed first with GVBE, then with GVB. Deposition of C3b from the fluid phase was performed using the cobra venom factor-dependent C3 convertase instead of trypsin (20) in order to prevent protease-induced changes in cell surface proteins. Erythrocytes (6×10^9) were suspended in 364 µg of C3 in 200 µl and incubated with 34 µg of cobra venom factor-dependent C3 convertase (21) for 30 min at 37°C. The deposited C3b was amplified as previously described (20) using the cell-bound, nephritic factor-stabilized C3 convertase and C3 or FITC-C3.

Fluorescence-activated Cell Sorter (FACS) Analysis. Erythrocytes bearing specifically deposited FITC-C3b were suspended in GVBE at 10^6/ml. FACS analysis was performed on 100,000 cells of each type (normal human erythrocytes, PNH erythrocytes, and sheep erythrocytes) using a Becton-Dickinson model FACS IV (B-D FACS Systems, Becton, Dickinson & Co., Sunnyvale, CA).

Acidified Serum Assay. This test (5) was modified to exclude classical pathway participation by including EGTA (ethylenebis (oxyethylenenitrilo)-tetraacetic acid, J.T. Baker Chemical Co., Phillipsburg, NJ). Normal human serum was acidified to pH 6.4 using 1 N HCl. The percent lysis was determined after incubating 10^7 erythrocytes with varying amounts of acidified serum in GVB containing 2.5 mM $MgCl_2$ and 2.5 mM EGTA for 30 min at 37°C.

Decay Rate of C3 Convertase. ^{125}I-Factor B (0.75 µg at 2.3 µCi/µg), Factor D (0.5 µg), and 5 $\times 10^7$ EC3b were incubated for 3 min at 22°C in 60 µl GVB containing 5 mM $MgCl_2$. GVBE (440 µl) was added to stop further enzyme formation. Samples (50 µl) were removed, layered on 20% sucrose, and centrifuged 60 s in a Beckman Model 152 Microfuge to separate cells from unbound protein (20).

Cleavage of Cell-bound C3b by Factor I. In order to measure cleavage by Factor I in the absence of Factor H a modification of a previously described method (22) was used. Erythrocytes (5 $\times$ 10^8) bearing C3b deposited with the nephritic factor-stabilized C3 convertse as described above were first incubated with Factors B, D, and 5 mM $MgCl_2$ for 2 min at 37°C, then with ^{125}I-C3 in GVBE to deposit radiolabeled C3b. The cells were incubated with various concentrations of Factor I in GVBE (100 µl) for 10 min at 37°C. The cells were washed once and resuspended in 1 ml GVBE containing 2 µg trypsin. After 5 min at 37°C the supernatant was removed, combined with the first wash, and the released radioactivity determined. Background was the release in the absence of Factor I, 100% was taken as the release that occurred when 80 µg/ml of Factor H was present along with 78 µg/ml Factor I during the 10-min incubation.

Determination of the Number of C3b Receptors on Human Erythrocytes. The number of C3b receptors present on PNH erythrocytes and normal human erythrocytes and the affinity of the receptors for C3b was determined using radiolabeled dimeric C3b. The preparation of C3b dimers and the assay procedure have been previously described (23).

Results

Characterization of the PNH Erythrocyte Population by FACS Analysis. EC3b was prepared from the erythrocytes of a patient (T. J.) with PNH and from a normal individual using FITC-C3 and the C3 convertase of the alternative pathway. The resulting cells bearing FITC-C3b were washed free of unbound fluorescent protein and subjected to FACS analysis. The results indicated that the normal sample contained essentially one population of EC3b with a low concentration of C3b on
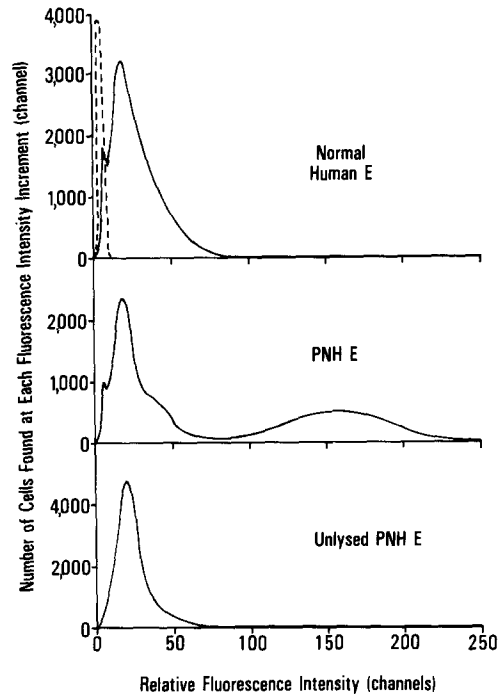
FIG. 1.   Fluorescence-activated cell sorter analysis of PNH (T. J.) and normal human erythrocytes bearing FITC-labeled C3b. The abscissa indicates the relative fluorescence intensity of the cells. The ordinate shows the number of cells found in each increment (channel) of fluorescence intensity. Erythrocytes were prepared as described in Materials and Methods utilizing fluid phase C3 convertase to deposit the initial C3b and the cell-bound nephritic factor-stabilized enzyme to deposit FITC-C3b. As a reference, the upper panel shows the distribution of normal human erythrocytes without FITC-C3b (dashed line). The results show that PNH erythrocytes (T. J.) exhibited two populations of cells (*center* panel) and that the population that acquired the highest levels of FITC-C3b was lost (*lower* panel) when the cells were treated with acidified human serum before C3b deposition.

their surface (Fig. 1). The PNH samples contained two populations of cells, one exhibiting low C3b fluorescence like the normal erythrocytes and the other showing approximately eightfold higher fluorescence intensity. The second population was composed of 43% of the cells and contained 83% of the cell-bound FITC-C3b. When the PNH erythrocytes were exposed to acidified (pH 6.4) normal human serum containing EGTA, 44% of the cells lysed (Fig. 2). The unlysed cells were washed and FITC-C3b was deposited on their surface as described above. FACS analysis revealed a single population of EC3b resembling normal EC3b (Fig. 1). The removal of the EC3b bearing high numbers of C3b molecules by alternative pathway mediated lysis identified these cells as the abnormal PNH erythrocytes.

Enhanced Stability of the C3 Convertase, C3b,Bb on PNH Erythrocytes Compared to Normal Human Erythrocytes.   Decay-dissociation of C3b,Bb bound to PNH erythrocytes or normal erythrocytes was measured by the release of radiolabeled Bb at 22°C (Fig. 3). Whereas the half-life of the enzyme on normal erythrocytes was 2.2 min, it was 7.6 min for the C3 convertase bound to PNH erythrocytes (T. J.). Because the majority of C3b molecules were bound to the abnormal erythrocytes in the PNH sample, as shown above, these measurements reflect the fate of the enzyme on the PNH
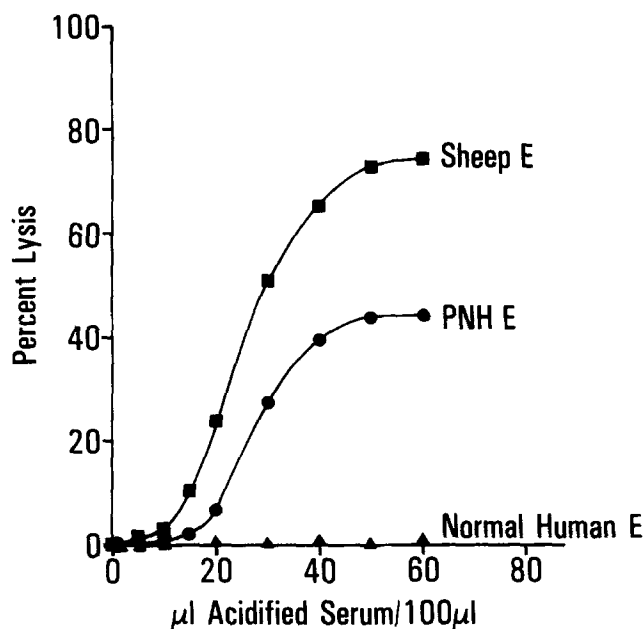
Fig. 2.   Lysis of PNH erythrocytes via the alternative pathway in acidified normal human serum. Cells were incubated for 30 min at 37°C with the indicated amounts of serum adjusted to pH 6.4. The reaction mixtures contained 2.5 mM $MgCl_2$ and 2.5 mM EGTA to prevent classical pathway activation.

erythrocytes and not on the normal erythrocyte population present in the sample. It was also noted that formation of C3b,Bb on normal erythrocytes was only one-third as efficient as on PNH erythrocytes. For these reasons the contribution of the enzyme on the normal erythrocyte population to the observed decay rate of the enzyme on PNH erythrocytes was small. For comparison, the half-life of the C3 convertase on sheep erythrocytes, which was similar to that of the unregulated enzyme in the fluid phase at 22°C, is also shown.

Reduced Susceptibility of C3b on PNH Erythrocytes to Inactivation by Factor I.   Cleavage of C3b by Factor I in the absence of Factor H was determined on PNH, normal human, sheep, and rabbit erythrocytes (Fig. 4). The cells of three patients with PNH (J. H., K. W., and T. J.) were examined by this assay and the results were similar in each case. To cleave 50% of the C3b on PNH erythrocytes, 100 times more Factor I was required than for the same extent of cleavage of C3b on normal human erythrocytes. C3b on sheep erythrocytes resembled C3b on PNH erythrocytes, whereas C3b on rabbit erythrocytes was completely resistant to Factor I as is fluid phase C3b (19). C3b bound to other strong activators of the alternative pathway such as zymosan and inulin was similarly resistant. Treatment of erythrocytes with 1 mg/ml trypsin for 30 min at 37°C or with neuraminidase (20) before C3b deposition did not significantly alter the results of this assay. Trypsin treatment of normal human erythrocytes did, however, inactivate the immune adherence function.

Reduced Number of C3b Receptors on PNH Erythrocytes.   The number of C3b receptors on estimated using radiolabeled C3b-dimers as the ligand (23). Compared to four samples of normal human erythrocytes, the binding curves for PNH erythrocytes
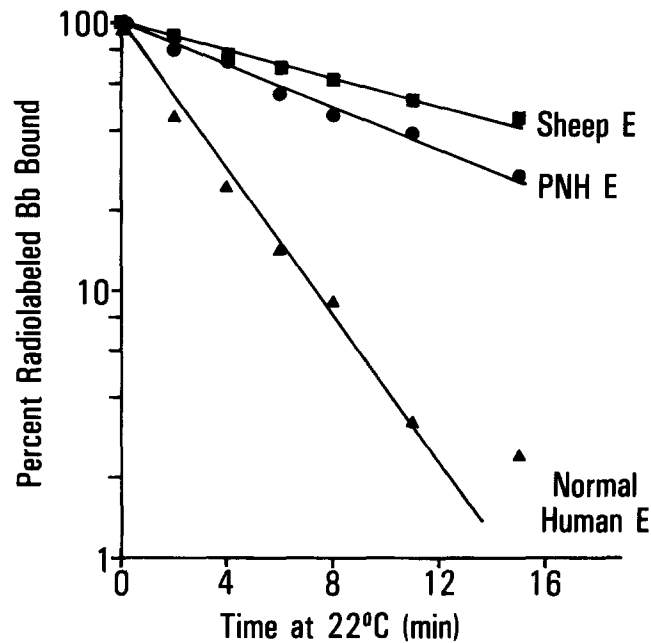
Fig. 3. Comparison of the decay rates at 22°C of the C3 convertase, C3b,Bb, bound to PNH, normal human or sheep erythrocytes. The half-life of C3b,Bb on PNH erythrocytes (7.6 min) was 3.5 times longer than the half-life on normal human erythrocytes (2.2 min). The half-life on sheep erythrocytes was 10.9 min. For reasons stated in the text the observed decay rate with PNH erythrocytes was primarily that of the population of cells which were abnormally sensitive to complement.

showed similar affinities but lower saturation levels. Receptor saturation averaged 64 ± 2% of normal for patient J. H. and 58 ± 6% of normal for patient T. J. If one of the two cell populations of patient T. J. bears a normal number of receptors then the results suggest that 42% of the cells lack C3b receptors. This percentage corresponds to the values obtained for the abnormal cells by FACS analysis (Fig. 1) and by the acidified serum lysis test (Fig. 2).

Discussion

The C3b receptor of normal human erythrocytes has been identified as a 205,000-dalton glycoprotein that is functionally characterized by its ability to bind C3b, to block binding of Factor B to C3b, to dissociate the C3b,Bb enzyme and to modulate C3b and C3bi for cleavage by Factor I (12, 24-31). A second regulatory glycoprotein has been isolated from human erythrocytes (13). This protein, which has a molecular weight of 70,000 daltons, has greater decay-accelerating activity toward the C3 convertase of the classical pathway than toward the properdin-stabilized C3 convertase of the alternative pathway. In most of these functions the two membrane proteins resemble the regulatory protein Factor H, which is a 150,000-dalton serum glycoprotein. The results of the present study suggest that the abnormal PNH erythrocytes are deficient in membrane-associated Factor H-like activity. These Factor H-like functions have been implicated as a protective mechanism of human erythrocytes against hemolysis by complement (12, 13, 28). This deficiency in PNH erythrocyte membranes may represent the molecular basis of the disease.
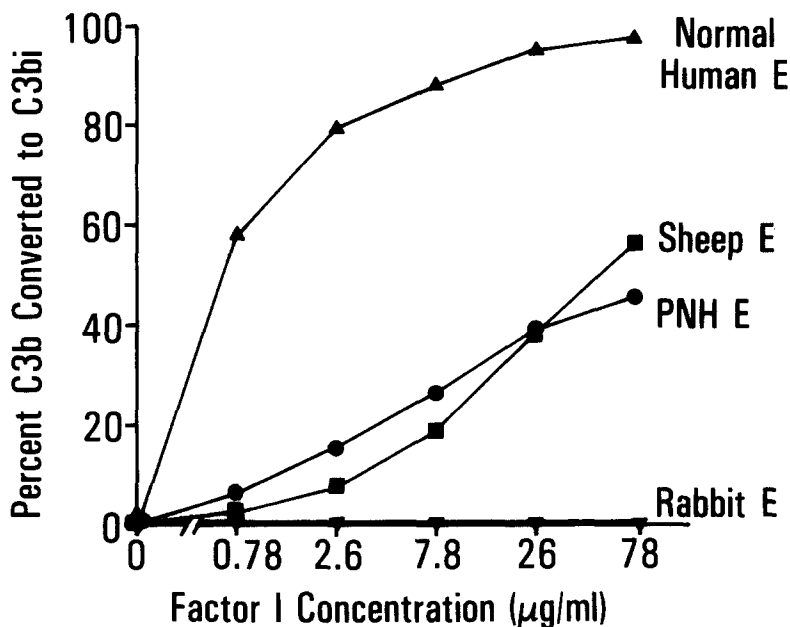
Fig. 4.   Influence of erythrocyte surface on inactivation of bound C3b by Factor I in the absence of Factor H. Radiolabeled C3b bound to normal human erythrocytes was converted to C3bi 100-fold more efficiently than C3b bound to PNH or sheep erythrocytes. C3b bound to rabbit erythrocytes, an efficient activator of the human alternative pathway, or fluid phase C3b (19) were not susceptible to Factor I without Factor H.

The abnormal PNH cells in the total erythrocyte population from patients with PNH were identified by FACS analysis. The abnormality allowed greatly increased deposition of C3b by the cell-bound C3 convertase (32). The C3b-rich cell population was shown to be highly susceptible to lysis by acidified serum, which is an established characteristic of PNH cells (1, 2).

Normal human erythrocytes are completely resistant to lysis by acidified serum. Sheep erythrocytes, like normal human erythrocytes, are considered nonactivators of the alternative pathway. However, sheep erythrocytes resemble PNH cells with respect to lysis in acidified serum. In light of the present findings it is probably significant that sheep erythrocytes lack (33) or possess only weak (Fig. 4) Factor H-like regulatory activity on their surface. The mechanism of hemolysis in acidified serum is unclear, although it has been shown that the association of C3b and Factor B is enhanced at pH 6.5, as compared with physiological pH, and that mere incubation of human serum at pH 6.5 results in enhanced spontaneous activation of the alternative pathway (34).

That PNH erythrocytes bind much more C3b than normal cells during complement activation has been demonstrated (6). This fact has been attributed to a greater apparent enzyme activity of the C3 convertase bound to PNH erythrocytes than that bound to normal human erythrocytes (32). The present study revealed two additional properties of the abnormal PNH cells that result in enhanced C3b deposition. It was found that C3b,Bb is more stable on PNH erythrocytes than on normal erythrocytes and that C3b is more resistant to inactivation by Factor I. The enhanced stability of the C3 convertase on PNH erythrocytes was not detected in a previous study because

that study employed the nephritic factor-stabilized enzyme (32). The resistance of C3b on PNH erythrocytes to inactivation affords enhanced C3 convertase formation and the increased stability of the enzyme affords increased C3b deposition.

The C3b receptor on normal human erythrocytes enters into a receptor-ligand interaction with C3b covalently linked to other particles or cells, thereby mediating immune adherence. It has been shown that the isolated C3b receptor of human erythrocytes accelerates decay-dissociation of the C3 convertases C3b,Bb (12) and C4b,2a (24, 35) and that it renders C3b susceptible to inactivation by Factor I. Inactivated C3b has lost the ability to bind Factor B, C5, or C5b-6. Since the results indicated the absence of the Factor H-like activities on PNH erythrocytes, the question arose as to the presence of C3b receptor function on the abnormal cells. In both patients examined the total number of erythrocyte receptors was depressed. However, due to the large variation among normal individuals of the number of C3b receptors on erythrocytes (13, 23, 36) the lower values observed are inconclusive. The finding that trypsin inactivates receptor function, but not the C3b,Bb decay-accelerating activity on normal human erythrocytes, also suggests caution in assigning the defect to the C3b receptor. It will be necessary to isolate the abnormal PNH erythrocyte population in order to evaluate the presence or absence of C3b receptor function, and if absent to determine whether the receptor protein is lacking or is present in inactive form.

On the basis of the data presented and the known regulatory functions associated with the membrane of normal human erythrocytes, a defect in one or more regulatory factors would be expected to have the following consequences. C3b randomly deposited on these cells from the fluid phase, would be less susceptible to inactivation by Factor I, would be more likely to form a C3 convertase, and the C3 convertase would be more stable and more likely to form a C5 convertase. Because of the increased stability of the C3b subunit of the C5 convertase, C5, C5b, and C5b-6 would be handled more effectively in the initiation of membrane attack (37). The net result would be that initially deposited C3b molecules would be more likely to succeed in producing a lytic event on PNH cells than would C3b on normal human erythrocytes.

Summary

Erythrocytes from patients with paroxysmal nocturnal hemoglobinuria (PNH) contained a subpopulation that lacked membrane-associated Factor H-like activity present on normal human erythrocytes. Initial deposition of C3b on the erythrocytes was effected using a fluid phase C3 convertase. The cells were then treated with fluorescein-labeled C3 and the cell-bound C3 convertase. Analysis utilizing the fluorescence-activated cell sorter revealed two distinct cell populations, one of which was highly fluorescent, indicating a large number of C3b molecules per cell. Only this population (43%) was susceptible to lysis (44%) when exposed to acidified serum before C3b deposition. The less fluorescent population resembled normal human erythrocytes and was not affected by prior treatment with acidified serum.

Since C3b deposition occurred almost exclusively on the complement-sensitive cells in the PNH erythrocyte population, these cells could be examined for the Factor H-like regulatory activities without prior isolation. These functions include enhancement of inactivation of erythrocyte-bound C3b by Factor I and acceleration of the decay of erythrocyte-bound C3 convertase, C3b,Bb. It was found that C3b on PNH erythro-

cytes was 100-fold less susceptible to inactivation by Factor I than C3b on normal human erythrocytes. The half-life at 22°C of C3b,Bb on PNH erythrocytes was threefold greater than on normal human erythrocytes and similar to that of the enzyme bound to particles that do not possess Factor H-like activity. These observations suggest that the abnormal susceptibility of PNH erythrocytes to lysis by complement is due to a functional deficiency in one or more of the Factor H-like proteins present on normal human erythrocytes.

Note added in proof: The IgG fraction of antiserum to purified DAF inhibited the decay-accelerating activity on normal human erythrocytes. It did not affect cleavage of C3b by Factor I. Antiserum to purified CR1 did not affect the decay-accelerating activity on normal human erythrocytes, while completely inhibiting cleavage of C3b by Factor I. These results suggest that the two activities examined in this paper are properties of two distinct molecules on normal human erythrocytes that may be deficient on PNH erythrocytes.

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