

Increased Enzymatic Activity of the Alternative Pathway Convertase When Bound to the Erythrocytes of Paroxysmal Nocturnal Hemoglobinuria

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ABSTRACT To investigate the greater fixation of C3 to the erythrocytes of patients with paroxysmal nocturnal hemoglobinuria (PNH) upon activation of complement, we have examined the formation and the reaction of the C3 nephritic factor-stabilized alternative pathway convertase made with purified components on normal and PNH erythrocytes. Each convertase complex converts four to five times more fluid-phase C3 to C3b when affixed to a PNH cell than when affixed to a normal cell. The greater activity of the convertase on PNH cells is not due to differences in the intrinsic or extrinsic stability of the convertase complex. The excessive binding of C3 to PNH cells is due to this increased conversion of fluid-phase C3, because the efficiency of binding of nascent C3b was identical for the two cell types. This is the first instance in which the enzyme activity of a complement complex has been shown to be increased by being affixed to an abnormal surface.

INTRODUCTION

The erythrocytes (E)¹ of patients with paroxysmal nocturnal hemoglobinuria (PNH E) may be classified into three types according to their susceptibility to the lytic

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¹ *Abbreviations used in this paper:* C3NeF, C3 nephritic factor; E, erythrocytes; EC3b, erythrocytes bearing large numbers of C3b molecules after activation of fluid-phase C3 by erythrocytes bearing the stabilized convertase; EC3b_s, erythrocytes bearing small numbers of C3b molecules after activation of fluid-phase C3 by trypsin; EC3bBbC3NeF, erythrocytes bearing the alternative pathway convertase stabilized by C3 nephritic factor; ¹²⁵I-anti-C3, radiolabeled monoclonal antibody to C3; ¹²⁵I-B, radiolabeled factor B; ¹³¹I-C3NeF, radiolabeled C3 nephritic factor; GVB, veronal-buffered saline containing 0.1% gelatin; GVB⁺, GVB containing 5 mM magnesium.

action of complement: PNH I cells, which appear to be normal or nearly normal in their susceptibility; PNH II cells, which are moderately susceptible to lysis, requiring one-third to one-fifth as much complement for lysis equal to that of normal E; and PNH III cells, which are markedly susceptible to lysis, requiring 1/15 to 1/25 as much complement for lysis as normal cells (1, 2). In previous studies (3-6), it has been demonstrated that both PNH II and PNH III cells bind much more C3b per cell than do normal cells when the complement in whole serum is activated either by the classical or the alternative pathway. The increased binding of C3b to PNH II cells appears to account entirely for the increased susceptibility of these cells to lysis, inasmuch as they show the same sensitivity to the lytic effects of the C5b-9 complex as do normal E (3, 6). The greater susceptibility to lysis of PNH III cells compared with PNH II cells appears to reside in a second defect that results in greater efficiency of the C5b-9 lytic complex.

The binding of C3b to E plays a pivotal role in the lysis of E by complement by mediating the activation of C5 by the C4b2a complex. More importantly, cell-bound C3b creates a nidus for the formation of the alternative pathway convertase complexes that activate C3 and C5, markedly amplifying the formation of terminal complexes. In addition, cell-bound C3b serves to concentrate the fixation of the C5b6 complex to the cell, thereby greatly potentiating complement-mediated hemolysis (7).

The interactions of the components of the alternative pathway of complement with the microenvironment of surfaces to which they affix are complex (8-18), and the increase in the amount of C3b bound to the surface of PNH E may be the result of increased efficiency of binding of nascent C3b, enhanced activation of fluid-phase C3, or decreased degradation of bound C3b.

We have investigated the interactions of the alternative pathway convertase components with the membrane of the abnormal PNH E in an attempt to determine the reasons for the greater fixation of C3 to that cell, and thereby, to gain insight into the biochemistry of the membrane defect that accounts for this phenomenon. Our studies indicate that the abnormal PNH E does not bind nascent C3b more efficiently and that the greater fixation of C3b to PNH E is not the result of a difference in the intrinsic or extrinsic stability of the convertase when bound to PNH or normal E. When C3 is activated by the C3 nephritic factor-stabilized cell-bound alternative pathway convertase, C3bBbC3NeF, more C3b is bound to PNH E than normal E because more fluid-phase C3 is converted to nascent C3b by the stabilized convertase when fixed to PNH E. This greater conversion is due to the enhanced enzymatic activity of the convertase on PNH E. We believe this to be the first instance in which the enzymatic activity of a complement convertase has been demonstrated to be enhanced because of interactions with an abnormal surface.

METHODS

Buffers. Veronal-buffered saline, pH 7.5, veronal-buffered saline containing 0.1% gelatin (GVB), GVB containing 5 mM magnesium (GVB⁺), and Alsever's solution were prepared as outlined (19).

Human E. E from normal donors (normal E) and patients with PNH (PNH E) were prepared and stored as previously described (4). The PNH cells used for these experiments had 85% or greater markedly complement-sensitive (PNH III) erythrocytes.

Complement components and antibody. C3 was purified to homogeneity as assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (20) according to the method of Lambris et al. (21) as modified from the method of Tack and Prah (22). Factor B was isolated by the following modification of the procedure of Götze and Müller-Eberhard (23). After the pseudoglobulins were sequentially chromatographed with DEAE-Sephacel (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) and Bio-Rex 70 (Bio-Rad Laboratories, Richmond, Calif.) as described, the factor B-containing pool was fractionated on Sephadex G-200 (Pharmacia Fine Chemicals). The final product revealed two bands in close proximity on sodium dodecyl sulfate polyacrylamide gel electrophoresis as described by Nicholson et al. (24). Functionally purified factor D was isolated by gel filtration of normal human serum on Sephadex G-75 (Pharmacia Fine Chemicals) as described by Lambris et al. (21). Partially purified C3 nephritic factor (C3NeF) was prepared from the plasma of a patient with partial lipodystrophy (kindly provided by Dr. W. Ray Gammon, University of North Carolina, Chapel Hill) by the method of Schreiber et al. (25). Monoclonal antibody to human C3 was obtained from Bethesda Research Laboratories (Gaithersburg, Md.) as mouse ascites fluid. This antibody reacts with native C3, C3b, C3bi, and C3c, but not with C3d. The IgG_{2b} fraction containing the antibody was initially isolated by 45% ammonium sulfate precipitation. After extensive dialysis against 0.01 M phosphate buffer (pH 8.0), the precipitate was ap-

plied to a 0.9 × 15-cm column (Pharmacia Fine Chemicals) of DE-52 (Whatman Inc., Clifton, N. J.) that had been equilibrated with the same buffer. The protein was then eluted with a linear phosphate gradient to a limiting concentration of 0.1 M. The final product gave a single precipitant line against anti-whole mouse serum (Meloy Laboratories, Inc. Springfield, Va.) and anti-mouse IgG_{2b} (Meloy Laboratories, Inc.) by immunodiffusion analysis and was homogeneous as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The protein concentration of C3, factor B, anti-C3, and the preparation of C3NeF were determined by Bio-Rad protein assay (Bio-Rad Laboratories).

Radiolabeling. Chloramine-T (Sigma Chemical Co., St. Louis, Mo.) at 5 M excess (26) was used with ¹²⁵I or ¹³¹I as NaI (New England Nuclear, Boston, Mass.) to trace label factor B (2.35 × 10⁵–7.40 × 10⁵ cpm/μg), C3NeF (2.2 × 10⁵ cpm/μg), C3 (2.88 × 10⁵ cpm/μg), and monoclonal anti-C3 (1.38 × 10⁶–1.76 × 10⁶ cpm/μg). Labeled protein was separated from unincorporated iodine by gel filtration through Sephadex G-25 (Pharmacia Fine Chemicals), with > 95% of the resulting radiolabel being precipitable by 10% trichloroacetic acid. After the specific activity and protein concentration had been determined by Bio-Rad protein assay, bovine serum albumin (Sigma Chemical Co.) was added to the samples to a concentration of 0.1%.

Determination of cell-bound C3b. By a double labeling procedure, it was established that ¹²⁵I-monoclonal anti-C3 (¹²⁵I-anti-C3) bound in a 1:1 M ratio with cell-bound ¹³¹I-C3. To quantitate the number of molecules of C3b bound per cell, 1 × 10⁷ erythrocytes bearing large numbers of C3b molecules were incubated (for 30 min at 37°C) with 30 μl of ¹²⁵I-anti-C3 (1.1 μg) and 20 μl GVB⁺. The cells were washed two times in 4 ml of GVB. After the suspension had been transferred to another tube, the cells were once again washed, and the radioactivity of the pellet was determined. Normal and PNH E were included as controls for nonspecific binding of ¹²⁵I-anti-C3, and this value was subtracted to define the specific binding used to calculate the cell-bound C3b.

Preparation of PNH E and normal E bearing small numbers of C3b molecules. By a modification of the method of Pangburn and Müller-Eberhard (15), erythrocytes bearing small numbers of C3b molecules (C3b_s) were prepared. Normal and PNH E were washed three times in GVB⁺ and suspended at a concentration of 1 × 10⁹ cells/ml in the same buffer. 1 × 10⁹ cells were incubated for 5 min at 37°C with 10 μl of C3 (1 mg) and 100 μl of GVB⁺. 10 μl (10 μg) of trypsin (Sigma Chemical Co.) was added, and the incubation was continued for 3 min at 37°C. The reaction was stopped by the addition of 4 ml (200 μg/ml GVB⁺) of trypsin inhibitor (Sigma Chemical Co.). The cells were washed three times in trypsin inhibitor and once in GVB⁺ and restored to 1 × 10⁹/ml GVB⁺. The amount of C3b bound was determined with ¹²⁵I-anti-C3 as described above. In the experiments indicated, the proportion of C3 and trypsin were increased to allow more C3b to be bound to normal E. To determine nonspecific binding of C3b, 10 μl of C3 (1 mg) was incubated with 10 μl of trypsin (1 mg/ml), and after 3 min at 37°C, 30 μl of trypsin inhibitor (1 mg/ml) was added. The reaction mixture was then incubated with 1 × 10⁹ normal or PNH E and 70 μl of GVB⁺ for 3 min at 37°C. The cells were washed three times with trypsin inhibitor (200 g/ml in GVB⁺) and once in GVB⁺ and restored to 1 × 10⁹/ml. The C3b bound to each cell type was determined with ¹²⁵I-anti-C3 as described above, and this value was subtracted to define specific binding of C3b to each cell type after trypsin activation of C3.

Establishment of erythrocytes bearing a stabilized alternative pathway convertase (EC3bBbC3NeF). 1×10^9 normal and PNH EC3b, were incubated with 25 μ l of factor B (50 μ g), 20 μ l of functionally purified factor D, and 50 μ l of C3NeF (155 μ g) for 10 min at 37°C. The cells were then washed with 4 ml of GVB⁺, resuspended to 1×10^9 /ml in GVB⁺, and used immediately. To quantitate the stabilized alternative pathway convertase sites, radiolabeled factor B and/or radiolabeled C3NeF were employed as markers. Normal and PNH E were included as controls for nonspecific binding of radiolabeled factor B and C3NeF, and this value was subtracted to define the specific binding of each protein.

Determination of stabilized cell-bound alternative pathway convertase activity. 1×10^8 normal and PNH EC3bBbC3NeF were incubated for various periods of time with 100 μ l of C3 (200 μ g) and GVB⁺. The supernatant fluid was recovered, and fluid-phase C3 conversion to C3b was determined by crossed immunoelectrophoresis (27) with antiserum monospecific for C3 (Atlantic Antibody, Scarborough, Maine). The amount of C3 bound to normal and PNH E after C3 conversion by EC3bBbC3NeF was determined with ¹²⁵I-anti-C3 as described above. The amount of C3b bound to each cell type after the trypsinization step was subtracted to give the net C3b bound.

Preparation of PNH E and normal E bearing large numbers of C3b molecules. By a modification of the method of Pangburn and Müller-Eberhard (15), cells bearing large numbers of C3b molecules (EC3b) were prepared and utilized in the experiments indicated. 1×10^9 normal and PNH EC3bBbC3NeF were incubated for 30 min at 37°C with various amounts of C3 in a final volume of 200 μ l of GVB⁺. The cells were washed three times in 4 ml of GVB⁺, and the suspensions were restored to their starting volume. The amount of cell-bound C3b was determined with ¹²⁵I-anti-C3b as indicated above. The number of C3b molecules per EC3b was varied by altering the number of stabilized convertase sites per EC3bBbC3NeF and/or by varying C3 input after the formation of the stabilized convertase. The former was accomplished by increasing the amount of C3 used to form EC3b.

Binding of ¹²⁵I-factor B (¹²⁵I-B) to EC3b. These studies were performed by a modification of the method of Kazatchkine et al. (16). 1×10^7 PNH and normal EC3b were incubated (30 min at 30°C) with various amounts of ¹²⁵I-B in a final volume of 100 μ l of GVB⁺. Duplicate samples (40 μ l) were layered over 200 μ l of a mixture of phthalate (1.5 parts *n*-butyl phthalate; one part bis(2-ethylhexyl) phthalate [Sigma Chemical Co.]) in 400- μ l polyethylene microtubes (Analytical Laboratories, Rockville Centre, N. Y.). The tubes were spun for 1 min in a Beckman Microfuge (Beckman Instruments, Inc., Fullerton, Calif.) to separate bound from unbound ¹²⁵I-B. A 10- μ l sample of supernatant fluid was removed, and the radioactivity was determined and used to calculate the amount of free ligand. The tubes were then cut just above the cell pellet, and the bound ¹²⁵I-B was calculated from the radioactivity of the pellet. Nonspecific binding at each input of ¹²⁵I-B was determined at the same time with normal and PNH E bearing no C3b, and this value was subtracted to define the specific binding.

Decay of ¹²⁵I-B from normal and PNH EC3bBbC3NeF. 1×10^8 PNH and normal EC3b, 87 μ l ¹²⁵I-B (37.5 μ g), 13 μ l of functionally purified factor D, and 50 μ l C3NeF (689 μ g) in a final volume of 250 μ l of GVB⁺ were incubated for 10 min at 30°C. By the technique described above for the separation of bound from unbound ligand, duplicate samples of 5×10^6 cells were aspirated, and the amount of cell-bound ¹²⁵I-B was determined. The remaining cells were washed one

time in ice-cold GVB⁺ (4 ml) and resuspended to 500 μ l in GVB⁺. At timed intervals, 5×10^6 cells were removed, and the bound ¹²⁵I-B was determined. Controls for nonspecific binding of ¹²⁵I-B were PNH and normal E.

Agglutination of PNH and normal EC3bBbC3NeF. 2×10^7 PNH and normal EC3b, 65 μ l of ¹³¹I-C3NeF (50 μ g), 5 μ l factor B (10 μ g), and 10 μ l of functionally purified factor D in a final volume of 120 μ l were incubated for 10 min at 30°C. With all other constituents held constant, three two-fold dilutions of ¹³¹I-C3NeF were incubated with PNH EC3b. After the incubation, the cells were washed once in 4 ml of ice-cold GVB⁺, and the suspensions were restored to their starting volume. Duplicate samples containing 5×10^6 cells were aspirated, the bound ligand was separated from the unbound ligand as described above, and the amount of ¹³¹I-C3NeF was calculated. The remaining 60 μ l containing 1×10^7 cells was allowed to remain undisturbed for 30 min at 22°C, after which time agglutination was assessed visually and scored from trace (barely discernible) to 4+ (complete).

Statistical methods. Group data were compared by Student's paired *t* test. Linear regression was determined by the method of least squares.

RESULTS

Binding of nascent C3b to PNH and normal E. To test whether nascent C3b binds more readily to PNH E than to normal E, fluid-phase C3 was activated by trypsin in the presence of normal and PNH E. When equal numbers of PNH and normal E were incubated with equal amounts of C3 and trypsin, the number of molecules of C3b bound per cell was the same for both cell types (Table I).

The efficiency of binding of nascent C3b to normal and PNH E was further determined by measuring the binding of C3 converted by cell-bound stabilized alternative pathway convertases. Equivalent numbers of PNH and normal EC3bBbC3NeF were incubated with purified C3, and at intervals, the amount of C3 converted to C3b and the amount of C3b bound were determined. The proportion of C3 converted and then bound was the same for normal and PNH E (Fig. 1).

Binding of ¹²⁵I-B to EC3b. If the amount of C3b bound to PNH and normal E were the same, greater

TABLE I
Binding of C3b to PNH E and Normal E upon Activation of C3 in Solution

Experiment	PNH	Normal
1	5,529	5,244
2	4,420	4,680
3	4,230	4,160

Equal numbers of PNH E and normal E were incubated with trypsin and C3. After washing, the amount of C3 bound to cells was determined with ¹²⁵I-anti-C3.

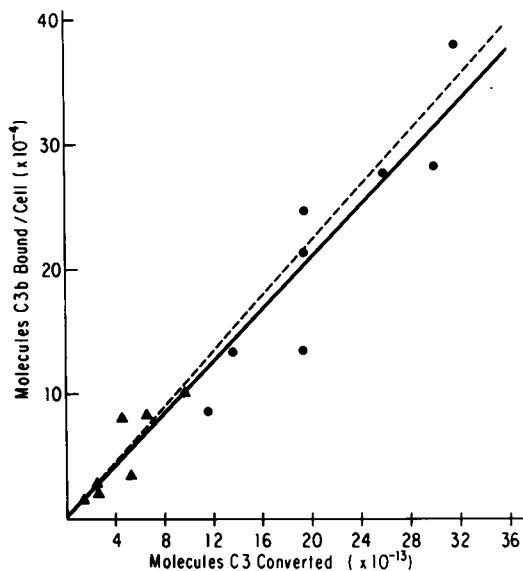


FIGURE 1 Comparison of C3 conversion and binding by PNH (●—●) and normal (▲—▲) E bearing EC3bBbC3NeF. The cells bearing the convertase were incubated with purified C3, and the amount of C3 converted (shown on the abscissa) and the number of C3b molecules subsequently bound to the cell (shown on the ordinate) was determined. The lines are derived from least squares analysis of the data points.

binding of factor B to C3b affixed to PNH E would result in a greater number of stabilized convertases on PNH E. To analyze the binding of factor B to C3b, PNH and normal EC3b were prepared, and the number of molecules of C3b bound per cell was determined. When the binding of ^{125}I -B to these cells was measured, the dose-response curve was found in each case to be concave to the abscissa (Fig. 2), indicating a saturable reaction. Analysis according to the method

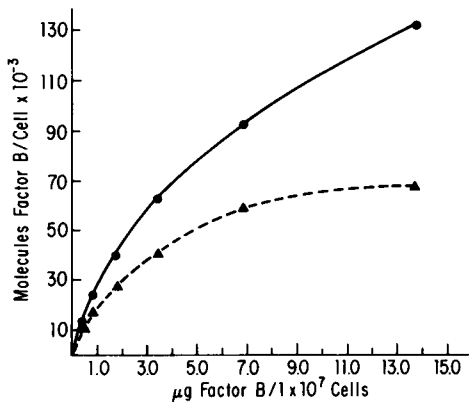


FIGURE 2 Binding of ^{125}I -B to normal (▲) and PNH (●) EC3b. Data points represent the mean of duplicate samples.

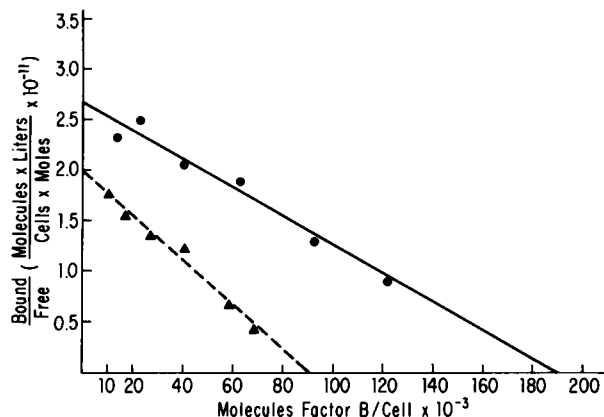


FIGURE 3 Analysis of ^{125}I -B binding to PNH (●) and normal (▲) EC3b by the method of Scatchard. Data points represent the mean of duplicate samples. The lines are derived from least squares analysis of the data points.

of Scatchard (28) (Fig. 3 and Table II) revealed that the binding of ^{125}I -B to PNH and normal E was linear, and the number of factor B binding sites calculated from the intercept of the line with the abscissa closely matched the number of C3b sites calculated with ^{125}I -anti-C3 (Table II). These data are consistent with a homogeneous binding reaction in which all molecules of C3b on the surface of both kinds of cells are equally available as binding sites for factor B. From these experiments, the affinity constant at equilibrium, K_A , for factor B binding to EC3b was calculated to be $1.96 \times 10^6 \pm 0.72 \text{ M}^{-1}$ (mean ± 1 SD) for normal EC3b and $2.49 \times 10^6 \pm 0.99$ for PNH EC3b. This difference was not statistically significant ($P > 0.2$).

Generation and activation of the stabilized alternative pathway convertase on EC3b. To explore further the binding of factor B in stabilized convertases, the number of ^{125}I -B molecules bound to PNH and normal EC3b or EC3b bearing large numbers of C3b molecules per cell in the presence of B, D, and C3NeF was determined. As shown in Table III, the ratio of factor B molecules bound:C3b bound was somewhat greater on the PNH cells. ^{125}I -B binding studies made on three separate occasions revealed the ratio of factor B bound:C3b bound to be 0.217 ± 0.128 for PNH and 0.102 ± 0.024 for normal. This difference is significant only at the $P = 0.2$ level.

The number of C3NeF molecules bound per cell can be used to define the number of stabilized convertase sites present, because C3NeF binds in a 1:1 molar ratio to the alternative pathway convertase (C3bBb) (29). PNH and normal EC3b bearing approximately equal numbers of C3b molecules per cell, (the input of C3 was increased with normal E both in the trypsinization step [500 μg for PNH E and 1,500 μg for normal E]

TABLE II
Analysis of the Binding of $^{125}\text{I-B}$ to EC3b

	C3b sites*	B binding sites†	B binding sites C3b sites	K_A ‡	Coefficient of correlation
	$\times 10^3$	$\times 10^3$		$\times 10^6 \text{ M}^{-1}$	
Normal	94.6	96.7	1.02	1.01	0.96
	64.1	67.2	1.05	1.28	0.96
	95.0	115.0	1.21	2.98	0.98
	95.0	135.0	1.42	2.00	0.99
	95.0	98.9	1.04	2.26	0.74
	99.8	89.2	0.89	2.20	0.99
			1.11 ± 0.18	1.96 ± 0.72 ¶	
PNH	41.8	43.4	1.04	1.90	0.93
	106.0	102.0	0.96	2.20	0.91
	177.7	185.4	1.04	1.58	0.99
	105.0	118.0	1.12	2.70	0.89
	105.0	88.0	0.84	4.70	0.99
	105.0	106.0	1.01	2.90	0.96
	65.0	64.7	1.00	3.00	0.93
	118.1	135.8	1.15	1.59	0.96
	182.0	205.9	0.88	1.79	0.95
			1.00 ± 0.10	2.48 ± 1.00 ¶	

* Determined by reaction with $^{125}\text{I-anti-C3}$.

† Determined by analysis by Scatchard's method.

‡ Affinity constant at equilibrium for $^{125}\text{I-B}$ binding to EC3b, determined by analysis by Scatchard's method.

^{||} Determined by least squares analysis.

¶ The difference between the K_A for $^{125}\text{I-B}$ binding to normal and PNH EC3b is not statistically significant ($P > 0.2$).

and after the formation of the stabilized alternative pathway convertase [300 μg for PNH EC3bBbC3NeF and 375 μg for normal EC3bBbC3NeF] were incu-

bated with $^{125}\text{I-B}$ and $^{131}\text{I-C3NeF}$. The number of C3NeF molecules bound to PNH EC3b is somewhat greater than for normal EC3b, and the number of

TABLE III
Formation of the Alternative Pathway Convertase on Normal E and PNH E

	Exp. 1 (EC3b)*		Exp. 2 (EC3b)†	
	Normal	PNH	Normal	PNH
C3b sites/cell‡	3,110	4,820	294×10^3	324×10^3
Factor B (molecules/cell)	600	1,090	29×10^3	46×10^3
Factor B/C3b	0.19	0.23	0.09	0.14
C3NeF (molecules/cell) [¶]	—	—	11×10^3	25×10^3
C3NeF/B	—	—	0.44	0.54

* The stabilized alternative pathway convertase was formed on PNH E and normal E bearing small numbers of C3b molecules prepared by activation of C3 by trypsin.

† The stabilized alternative pathway convertase was formed on PNH and normal E bearing large numbers of C3b molecules prepared by activation of C3 by the cell-bound alternative pathway convertase.

‡ Determined with $^{125}\text{I-anti-C3}$.

^{||} Determined by $^{125}\text{I-B}$ binding.

¶ Determined by $^{131}\text{I-C3NeF}$ binding.

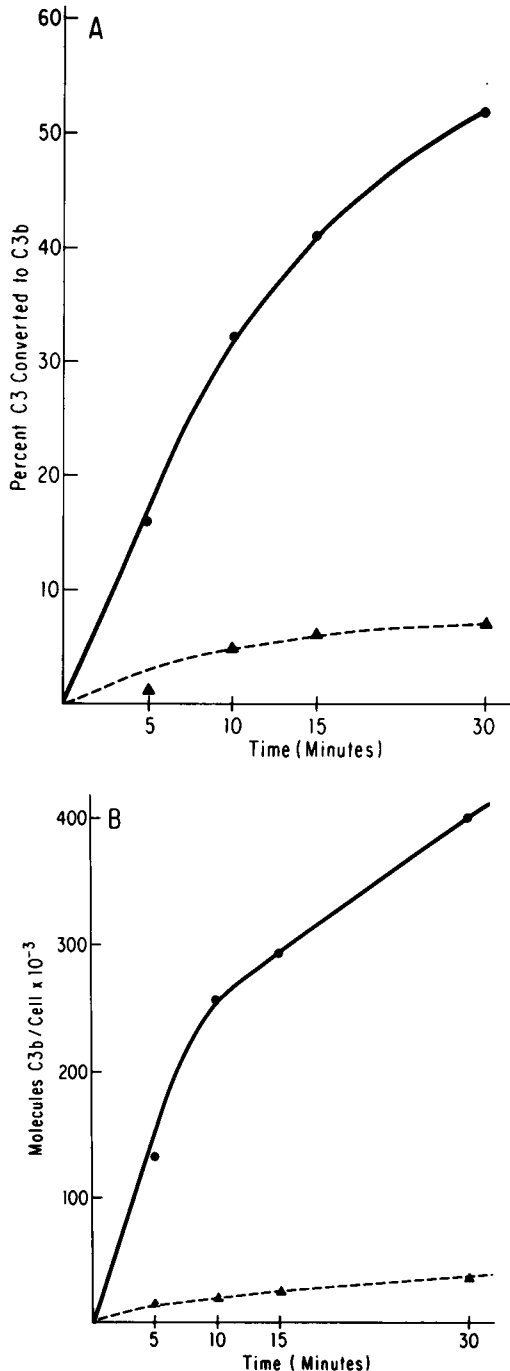


FIGURE 4 Comparison of C3 conversion (A) and of C3b binding (B) by PNH (●) and normal (▲) EC3bBbC3NeF as a function of time.

molecules bound per factor B bound is also somewhat greater for PNH cells (Table III). The ratio of ^{131}I -C3NeF molecules bound: ^{125}I -B bound, determined in three separate experiments, was 0.496 ± 0.063 for PNH

EC3b and 0.370 ± 0.070 for normal EC3b. Again, this difference is significant only at the $P = 0.2$ level.

Conversion of C3 by stabilized convertase complexes. The PNH and normal EC3bBbC3NeF prepared from cells bearing small numbers of C3b molecules described in Table III were incubated with equal quantities of purified C3, and the amount of C3 converted and bound was determined. At each interval examined, the PNH EC3bBbC3NeF had converted approximately eight times more C3 to C3b than had an equivalent number of normal EC3bBbC3NeF (Fig. 4A), and there consequently was greater binding of C3b to PNH E in direct proportion to the increase in C3 activation (Fig. 4B). Although there were more factor B molecules present upon PNH EC3bBbC3NeF than upon normal EC3bBbC3NeF, the difference (less than twofold, Table III), could not alone account for the eightfold greater conversion of C3 to C3b by PNH EC3bBbC3NeF.

When the normal and PNH EC3bBbC3NeF made from cells bearing large numbers of C3b molecules described in Table III were incubated with fluid-phase C3, a similar greater conversion of C3 was found (Table IV). When the cell concentration was adjusted so that the reaction mixture contained similar numbers of convertases (as measured by the binding of ^{131}I -C3NeF), the amount of C3 converted by PNH EC3bBbC3NeF was markedly greater (Table IV).

Decay of ^{125}I -B from EC3bBbC3NeF. Increased conversion of C3 to C3b by PNH E bearing the stabilized alternative pathway convertase could be related to a greater intrinsic stability of the stabilized convertase when present upon PNH E compared with normal E. Therefore, the decay kinetics of ^{125}I -B from PNH and normal E bearing the stabilized convertase were assessed (Fig. 5). The difference between the two curves is small, indicating that a difference in the intrinsic stability of the convertase does not account for the increased conversion of C3 to C3b by PNH EC3bBbC3NeF.

Agglutination of EC3bBbC3NeF. C3NeF is an antibody and is capable of agglutinating EC3bBb. PNH EC3bBbC3NeF agglutinated to a greater extent than normal EC3bBbC3NeF when the number of stabilized convertase sites was equivalent (Table V).

DISCUSSION

In previous studies (3-6), it has been demonstrated that PNH E bind more C3b than do normal E when complement in serum is activated by either the classical or the alternative pathway. The present investigations, in which purified components of the alternative pathway of complement were employed, have confirmed the greater binding of C3b to PNH E when

TABLE IV
Conversion of Fluid-phase C3 by Normal E and PNH E Bearing EC3bBbC3NeF

	Exp. 1		Exp. 2*	
	Normal	PNH	Normal	PNH
Convertase sites†	1.07×10^{11}	2.5×10^{11}	1.07×10^{11}	1.25×10^{11}
Percent C3 conversion‡	5	41	5	21

* The PNH EC3bBbC3NeF from experiment 1 were diluted to equalize the concentration of convertase sites in the reaction mixture employed in experiment 2.

† Determined by binding of ^{131}I -C3NeF.

‡ Determined by crossed immunoelectrophoresis.

C3 is activated by this pathway. This increased fixation of C3b was found to be due to an increase in the enzymatic activity of the alternative pathway convertase when sited upon the membrane of the PNH E.

Other systems in which there is a relatively greater fixation of C3b to a particular surface have been described (8, 12-17). This greater binding could be attributed to difference in the biochemical composition of that surface that either favored binding of nascent C3b (8-10) or modified interactions of surface-bound C3b with the endogeneous control proteins (12-17). The latter, termed activator surfaces, favor the binding

of factor B to C3b relative to the binding of $\beta 1\text{H}$, thereby enhancing the formation and relative stability of the alternative pathway convertase.

Neither of these known causes of excessive C3b binding pertain to PNH E. The same proportion of activated C3 is bound by normal and PNH cells (Table I and Fig. 1), showing that the PNH membrane does not more efficiently bind activated C3b. The increased conversion and fixation of C3 by PNH E bearing the alternative pathway convertase occurred in the absence of the control proteins, and further, the convertase complexes were stabilized with C3NeF, which renders them resistant to extrinsic decay by endogenous control proteins when they are present. However, it is possible that the endogenous control proteins may, in fact, interact aberrantly with elements of the alternative pathway convertase when present on PNH E in such a way as to contribute to the greater fixation of C3b to PNH E observed in whole-serum systems. For this reason, we are currently investigating the interactions of $\beta 1\text{H}$ and C3bINA with the alternative pathway convertase on PNH and normal E.

In the absence of the endogenous control proteins, the duration of activity of the alternative pathway

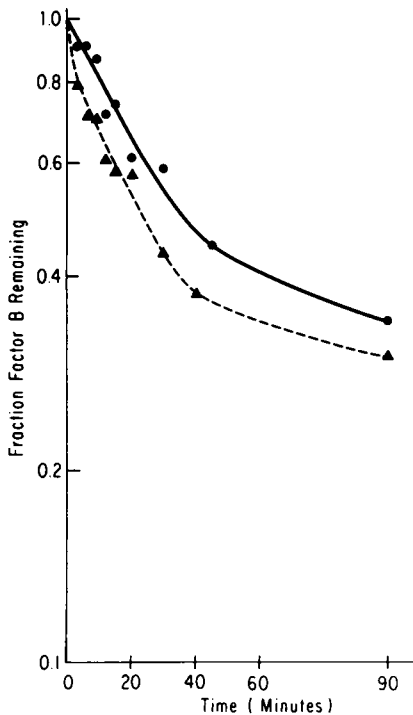


FIGURE 5 The kinetics of decay of ^{125}I -B from PNH (●) and normal (▲) EC3bBbC3NeF. The data points represent the mean of duplicate samples.

TABLE V
Comparison of Agglutination of PNH E and Normal E Bearing the Stabilized Alternative Pathway Convertase

	Molecules C3NeF/cell*	Agglutination*
PNH‡	23,780	3+
	17,420	2+
	12,425	1+
	8,145	trace
Normal	17,220	trace

‡ PNH E bearing the stabilized convertase were prepared using twofold dilutions of ^{131}I -C3NeF beginning with the concentration used to prepare the normal EC3bBbC3NeF.

* The extent of agglutination was assessed visually, and the number of ^{131}I -C3NeF molecules bound per cell was determined.

convertase (C3bBb) when bound to human E is determined by the intrinsic lability of binding of Bb within this complex. The rate of decay of ^{125}I -Bb from the C3bBb complex is the same on normal and PNH E (data not shown). On human E the binding of factor B to cell-bound C3b is modified by an intrinsic protein of the E membrane, which also functions as the receptor for C3b (30, 31). The number of C3b receptor sites on PNH and normal E appears to be the same.²

C3NeF prolongs the activity of the alternative pathway convertase by specifically binding to neoantigens exposed upon the formation of the convertase complex (32, 33). This antibody retards the intrinsic decay of the catalytic Bb subunit from the convertase complex. Using ^{125}I -B, we found no significant difference in the rate of decay of Bb from the stabilized convertase complex (EC3bBbC3NeF) when bound to PNH or normal E (Fig. 5). Thus, in these experiments in which C3NeF was used to stabilize the cell-bound alternative pathway convertase, the increased conversion and concomitant increase in C3b bound by PNH E cannot be attributed to a difference in the intrinsic stability of the convertase when present upon PNH E compared with normal E.

The greater activation of C3 by convertase complexes appears to be due to a markedly greater enzyme activity of the convertase when it is fixed to PNH E. Each C3NeF-stabilized convertase complex fixed to PNH E converts four to five times more fluid-phase C3 to C3b than one fixed to normal E (Table IV and Fig. 4A). This appears to be the first instance in which the kinetics of an enzyme of the complement system have been altered because of fixation of the enzyme complex to an abnormal surface. Detailed studies of the enzyme kinetics of the convertase when bound to normal and PNH E are underway to elucidate the nature of the difference in the enzyme-substrate interactions.

There are precedents to suggest that the biochemical properties of a membrane can influence the activity of membrane-associated enzymes (34). This phenomenon may explain why PNH E lack acetylcholinesterase activity (35, 36), inasmuch as the enzyme molecule is said to be present but its activity diminished (37, 38) due to changes in enzyme kinetics imposed by an abnormality of the lipid environment of the PNH membrane (38). It is possible that the kinetics of the membrane-associated enzyme examined in the present study (the stabilized C3 convertase of the alternative pathway) may also be influenced by the lipid environment of the PNH E membrane.

Interactions between C3b and/or C3bB and the abnormal PNH E membrane may result in a spatial orientation of the C3b molecule or the C3bB complex that differs from that on normal E membrane. Although the difference in the ratio of ^{131}I -C3NeF: ^{125}I -B binding to PNH E was not significantly different from that of normal E ($P = 0.2$), in general, higher numbers of C3NeF molecules were bound to PNH E. This suggests that the abnormal PNH E membrane may alter interactions between factor D and C3bB and/or between C3NeF and cell-bound C3bBb in such a way as to favor the formation of the stabilized alternative pathway convertase. The greater agglutination of PNH EC3bBbC3NeF (Table V) for a given amount of bound C3NeF suggests that the conformation of the alternative pathway convertase when bound to PNH E is aberrant. The cross-linking of cells by C3NeF reacting with C3 convertases may occur more readily on PNH E and reflect a difference in the accessibility of the surface-bound convertase to the antibody, and, perhaps, of the catalytic site for its substrate, C3.

The present investigations have been concerned with the C3 convertase complex of the alternative pathway. Activation of the classical pathway in the presence of whole serum (and hence of the components of the alternative pathway) also results in the fixation of more C3 to PNH E (3-6). In previous studies, we have shown that the formation of the convertase of the classical pathway (C4b2a) occurs equally on normal and PNH E, that the rate of degradation of that complex is the same (39), and that the amount of a specific protein in the membrane that facilitates that degradation is the same on the two types of cells (40). We are currently investigating the reactions of the C4b2a complex to determine whether the enzymatic activity of this complex likewise is increased when it is attached to the PNH E and the role activation of the alternative pathway by the classical pathway plays in the increased binding of C3 to PNH E when complement is activated by antibody.

The three known abnormalities of the PNH E membrane (increased activity of the alternative pathway convertase, decreased activity of acetylcholinesterase, and increased efficiency of the C5b-9 complex) could all be plausibly explained as the result of an abnormality or abnormalities in the lipids of the membrane. However, no consistent abnormalities in lipid composition of the PNH E membrane have been demonstrated (41). Perhaps qualitative rather than quantitative differences in the lipid environment underlie the membrane abnormality. Further, a lipid abnormality, if present, may not be primary but rather secondary to a more basic molecular defect.

² Personal communication, Dr. Gordon Ross, University of North Carolina.

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