

## Abnormality of Glycophorin- $\alpha$ on Paroxysmal Nocturnal Hemoglobinuria Erythrocytes

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**A**bstract. To investigate the greater enzymatic activity of the alternative pathway convertase (and the subsequent greater fixation of C3b) on paroxysmal nocturnal hemoglobinuria (PNH) erythrocytes, we have examined the topography of binding of C3b to PNH and normal erythrocytes. Using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography, the  $\alpha$ -chain of C3b was found to bind via predominantly ester bonds to free hydroxyl groups on glycophorin- $\alpha$ , the major erythrocyte sialoglycoprotein. The pattern of binding of nascent C3b was the same for normal and PNH erythrocytes. Thus, although C3b binding to a different membrane constituent did not appear to account for the greater enzymatic activity of the alternative pathway convertase when affixed to PNH erythrocytes, it seemed possible that the glycoproteins to which C3b bound might be qualitatively abnormal on the PNH cells, and that structural differences in these molecules might impose modifications in the enzyme-substrate interactions of the alternative pathway convertase. Using methods for radiolabeling both protein and carbohydrate residues, we therefore compared the electrophoretic pattern of the cell-surface glycoproteins on PNH and normal erythrocytes. The glycophorin- $\alpha$  dimer was found to be qualitatively abnormal on the PNH cells as evidenced by its greater susceptibility to trypsin-mediated proteolysis. In addition, the abnormal erythrocytes from patients with PNH had

fewer periodate oxidizable constituents than did normal erythrocytes, indicating a relative deficiency of cell-surface sialic acid. These investigations suggest that abnormalities in membrane glycoproteins may underlie the aberrant interactions of complement with the hematopoietic elements of PNH.

### Introduction

Paroxysmal nocturnal hemoglobinuria (PNH)<sup>1</sup> is a myelodysplastic disease unique because of the aberrant interactions of the hematopoietic elements with complement. The erythrocytes from patients with PNH bind more C3b than do normal erythrocytes when whole serum complement is activated by either the classical or the alternative pathway (1–4). Using purified components, we have recently demonstrated that this greater fixation of C3b to PNH erythrocytes is due, at least in part, to the greater enzymatic activity of the alternative pathway convertase when affixed to these abnormal cells (5).

C3 is composed of an alpha (127,000 D) and a beta (75,000 D) chain. Upon enzymatic activation, a 10,000 D fragment (C3a) is cleaved from the  $\alpha$ -chain. This alters the conformation of the molecule in such a way that an internal thioester bond

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1. *Abbreviations used in this paper:* bc<sub>1-4</sub>, binding complexes involving C3b covalently linked to erythrocyte membrane glycoproteins; CR<sub>1</sub>, C3b receptor; C3NeF, C3 nephritic factor; E\*C3, radiolabeled erythrocytes bearing nonspecifically bound C3; E\*C3b, radiolabeled erythrocytes bearing C3b; E\*C3bBbC3NeF, radiolabeled erythrocytes bearing the alternative pathway convertase stabilized by C3 nephritic factor; EC3\*, erythrocytes bearing nonspecifically bound <sup>125</sup>I-C3; EC3b\*, erythrocytes bearing <sup>125</sup>I-C3b; EC3bBbC3NeF, erythrocytes bearing the alternative pathway convertase stabilized by C3 nephritic factor; GVB, veronal-buffered saline containing 0.1% gelatin; HEMPAS, hereditary erythroblastic multinuclearity with positive acidified serum test; <sup>125</sup>I-C3, C3 labeled with <sup>125</sup>I; PAGE, polyacrylamide gel electrophoresis; PNH, paroxysmal nocturnal hemoglobinuria; 5P(8)BS-PE, phosphate-buffered saline containing 150 mM sodium chloride, 5 mM sodium phosphate, 1 mM phenylmethylsulfonyl fluoride, and 1 mM ethylenediamine tetraacetic acid, pH 8.0; 5P(8)-PE, 5 mM sodium phosphate containing 1 mM phenylmethylsulfonyl fluoride and 1 mM ethylenediamine tetraacetic acid, pH 8.0; TCP, trypsin cleavage peptide; TPCK, L-(tosylamide 2-phenyl) ethyl chloromethyl ketone.

within the  $\alpha$ -chain is exposed (6–8). This exposed but intact thioester constitutes the labile binding site of C3b. It has a half-life measured in milliseconds (9), and can either become inactive by undergoing hydrolysis in the fluid phase or form an ester or imidoester bond with a nearby reactive surface (10–12). Because cell-bound C3b serves as the nidus for the formation of the C3 convertase of the alternative pathway (13), we examined the topography of binding of C3b to PNH and normal erythrocytes. These investigations demonstrated that C3b does not bind to a different membrane component on the PNH erythrocytes, but rather that the cell-surface glycoprotein to which nascent C3b predominately binds (glycophorin- $\alpha^2$ ) is qualitatively abnormal. Thus, it seems likely that C3b binding to an abnormal membrane constituent results in a modification of the enzyme-substrate interactions of the cell-bound alternative pathway convertase, thereby accounting for its greater activity when affixed to PNH erythrocytes.

## Methods

**Human erythrocytes.** Erythrocytes from normal donors and from patients with PNH were prepared and stored as previously described (5). The PNH cells were from patients with 88% or greater type III erythrocytes (17, 18).

**Buffers.** The following buffers were employed: veronal-buffered saline, pH 7.5; veronal-buffered saline containing 0.1% gelatin (GVB); GVB containing 5 mM magnesium (GVB<sup>+</sup>); GVB containing 15 mM ethylenediamine tetraacetic acid (Sigma Chemical Co., St. Louis, MO) (GVB-EDTA); Alsever's solution (5); phosphate-buffered saline containing 150 mM sodium chloride and 10 mM sodium phosphate, pH 7.4 (PBS); PBS containing 150 mM sodium chloride, 5 mM sodium phosphate, 1 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co.), and 1 mM EDTA, pH 8.0 [5P(8)BS-PE]; 5 mM sodium phosphate containing 1 mM phenylmethylsulfonyl fluoride and 1 mM EDTA, pH 8.0, [5P(8)-PE].

**Protein assay.** Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA) was used to determine protein concentrations using bovine  $\gamma$ -globulin as the standard.

**Complement components.** C3 (5), factor B (5), and cobra venom factor (19) were purified to apparent homogeneity as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (20). Functionally purified factor D, and partially purified C3 nephritic factor (C3NeF) were prepared as previously described (5).

**Radiolabeling using  $^{125}\text{I}$ .** C3 ( $1.15\text{--}1.25 \times 10^5$  cpm/ $\mu\text{g}$ ) was trace-labeled with  $^{125}\text{I}$  using Iodo-Gen (Pierce Chemical Co., Rockford, IL) by a modification of the method of Fraker and Speck (21) as previously described (22).

2. A modification of the nomenclature of Anstee (14) and Furthmayr (15) is used to describe the major erythrocyte sialoglycoproteins. Glycophorin (from Furthmayr's nomenclature) is the general term used in referring to these sialoglycoproteins, whereas the Greek letters (from Anstee's nomenclature) are used to denote the specific glycophorin subspecies (e.g., glycophorin- $\alpha$ ). The following is a comparison of Anstee's designations for the glycophorin subspecies with that of Furthmayr:  $\alpha = \text{A}$ ;  $\beta = \text{C}$ ;  $\delta = \text{B}$ ;  $\gamma = \text{C}$ . In comparing the nomenclature of Steck (16) with that of Anstee: PAS 1 =  $\alpha_2$  (homodimer of  $\alpha$ ); PAS 2 =  $\alpha$ ; PAS 2' =  $\beta$ ; PAS 3 =  $\delta$ ; PAS 4 =  $\alpha\delta$  (heterodimer of  $\alpha$  and  $\delta$ ).

Surface labeling of PNH and normal erythrocytes with  $^{125}\text{I}$  was accomplished using Iodo-Gen exactly as described by Markwell and Fox (23).

**Preparation of PNH erythrocytes and normal erythrocytes bearing  $^{125}\text{I}$ -C3b (PNH and normal EC3b\*).** 1 vol of whole blood from normal volunteers and from patients with PNH, which had been stored in Alsever's solution, was sedimented at 4°C using 2 vol of 1% Dextran T-5000 (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, NJ) in 5P(8)BS-PE. The red cells were washed twice in 5P(8)BS-PE and twice in GVB-EDTA, aspirating the top one-tenth of the cell pellet after each wash. PNH and normal erythrocytes bearing the nephritic factor-stabilized alternative pathway convertase (EC3bBbC3NeF) were subsequently prepared as previously described (22). PNH and normal EC3b\* were then generated by incubating the EC3bBbC3NeF with 100  $\mu\text{g}$  of  $^{125}\text{I}$ -C3 at 37°C. After 30 min, the cells were spun and the supernate was recovered, made 20 mM with cold EDTA, and placed at 0°C. The cells were washed three times in 5P(8)BS-PE in preparation for isolation of the membrane proteins as described below. For controls in these experiments, PNH and normal erythrocytes that did not bear the alternative pathway convertase were exposed to  $^{125}\text{I}$ -C3 under the same conditions as described above (PNH and normal EC3b\*).

**Preparation of radiolabeled PNH and normal erythrocytes bearing C3b (PNH and normal E\*C3b).** Radiolabeled PNH and normal erythrocytes bearing the stabilized alternative pathway convertase were prepared exactly as were their unlabeled counterparts. Subsequently, 100  $\mu\text{l}$  of unlabeled C3 (10 mg/ml) were added to these radiolabeled convertase-bearing cells, and the mixture was incubated for 30 min at 37°C. After washing twice in GVB-EDTA and twice in 5P(8)BS-PE, the membrane proteins were isolated as described below. For controls in these experiments, radiolabeled PNH and normal erythrocytes that did not bear the alternative pathway convertase were exposed to fluid-phase C3 under the same conditions as described above (PNH and normal E\*C3).

**Trypsin treatment of PNH and normal erythrocytes.** These cells were prepared using a modification of the method of Winzler et al. (24). 1 vol of packed erythrocytes which had been washed three times in PBS was incubated with an equal volume of PBS containing 250  $\mu\text{g}/\text{ml}$  of L-(tosylamide 2-phenyl) ethyl chloromethyl ketone (TPCK)-trypsin (Worthington Biochemical Corp., Freehold, NJ) for 30 min at 37°C. The cells were washed twice with PBS containing 500  $\mu\text{g}/\text{ml}$  of trypsin inhibitor (Sigma Chemical Co.) and twice with PBS. After the trypsinization was completed, the cells were treated exactly as their untrypsinized counterpart with regard to preparation of cells bearing radiolabeled C3b, isolation of membrane proteins, and PAGE.

**Isolation of membrane proteins.** This procedure was carried out by using a modification of the method of Fairbanks et al. (25). All buffers were prechilled to 4°C, and all steps were performed at 4°C. After washing the cells in 5P(8)BS-PE, membrane ghosts were prepared by hypotonic lysis with 5P(8)-PE. The suspensions were centrifuged at 40,000 g for 10 min and the supernatant fluid was aspirated. After two additional washes under the same conditions, the ghosts were recovered and the protein concentration determined.

**Treatment of PNH and normal EC3b\* with hydroxylamine.** Nascent C3b can form ester (hydroxylamine-sensitive) or imidoester (hydroxylamine-resistant) bonds with a nearby reactive surface (10–12). To determine if there was a difference in the type of binding of C3b to PNH erythrocytes compared with normals, PNH and normal EC3b\* were treated with  $\text{NH}_2\text{OH}$  according to the method of Law et al. (10, 11, 26). Briefly described, 2 M  $\text{NH}_2\text{OH}$ , pH 10.5, was prepared by titrating the pH with 8 M NaOH. PNH and normal EC3b\* ghosts were

dissolved in 0.2% SDS and an equal volume of the 2 M  $\text{NH}_2\text{OH}$ , pH 10.5, was added to the solubilized ghosts. The reaction mixture was incubated for 30 min at 37°C, and excess  $\text{NH}_2\text{OH}$  was removed by dialysis overnight at 4°C against 0.1% SDS in deionized water. Controls for these experiments were PNH and normal EC3b\* ghosts treated in the same fashion except an equal volume of deionized water was substituted for the 2 M  $\text{NH}_2\text{OH}$ .

**PAGE in SDS.** Electrophoresis under reducing conditions was performed according to Laemmli and Favre (27) using a 6–12% gradient slab gel with a 3% stacking gel. The gels were stained with a solution of 0.125% Coomassie Brilliant Blue R250 (Bio-Rad), 10% methanol, and 10% acetic acid for 2 h at 37°C. After destaining was accomplished using a solution of 10% methanol and 10% acetic acid, the gels were photographed. Autoradiograms were prepared by exposing the dried gels to Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY) and storing them at -90°C until developed. Molecular weight protein standards (Bio-Rad) were myosin (200,000),  $\beta$ -galactosidase (116,250), phosphorylase B (92,500), bovine serum albumin (66,200), and oval albumin (45,000). Using these standards and the established molecular weights for the major erythrocyte membrane proteins [band 1 (240,000), band 2 (215,000), band 4.1 (78,000), band 4.2 (72,000), band 5 (43,000), and band 6 (35,000) (16)], the apparent molecular weights of polypeptides were determined according to Weber and Osborn (28).

For electrophoresis in the second dimension, tracks containing the proteins of interest, which had been electrophoresed in the first dimension, stained, and destained, were isolated. The track was first placed in 50 ml of deionized water for 15 min at 22°C, rinsed with deionized water, and placed in 50 ml of 1 M  $\text{NH}_2\text{OH}$ , pH 9.0 (to release ester-bound C3b from its membrane constituent) according to the method of Law et al. (10, 26). The tracks were next rinsed with deionized water and then submerged in 50 ml of deionized water for 15 min at 4°C. After submersion in 50 ml of solubilizing buffer (0.125 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 5% 2-mercaptoethanol) for 15 min at 22°C, the track was placed on top of the stacking gel and electrophoresed in the second dimension. Controls for these experiments were tracks which were treated in the same fashion as described above but were not exposed to  $\text{NH}_2\text{OH}$ .

**Determination of C3b bound to enzyme-treated normal and PNH erythrocyte bearing the stabilized alternative pathway convertase.** Trypsin-treated cells were prepared as described above. Neuraminidase treatment was performed using a modification of the method of Gahmberg et al. (29). After preparation of the erythrocytes as described above, 500  $\mu\text{l}$  of packed erythrocytes were washed three times in PBS containing 1 mM  $\text{CaCl}_2$  and resuspended to 1 ml in the same buffer. 25 U of *Vibrio cholerae* neuraminidase (Calbiochem, La Jolla, CA) were added and the suspension was incubated for 30 min at 37°C. The cells were then washed three times in PBS containing 15 mM EDTA.

For experiments involving trypsin-treated or neuraminidase-treated cells, untreated controls were prepared in parallel by incubating and washing cells under identical conditions as the treated cells, but omitting the appropriate enzyme.

Trypsin-treated and neuraminidase-treated PNH and normal erythrocytes and the appropriate incubated controls bearing EC3bBbC3NeF were prepared as described above. Three 100- $\mu\text{l}$  aliquots ( $1 \times 10^8$  cells) of each cell type were then incubated at 37°C with 100  $\mu\text{l}$  of C3 (10 mg/ml). At timed intervals, the cells were pelleted and a portion of the supernate was aspirated and kept at 0°C until frozen at -90°C. The cells were then washed three times in GVB-EDTA and the amount of cell-bound C3b was determined using monoclonal  $^{125}\text{I}$ -anti-C3 as previously described (22).

**SDS-PAGE of trypsinized and nontrypsinized,  $^{125}\text{I}$ -labeled PNH and normal erythrocytes.** PNH and normal erythrocytes were radiolabeled with  $^{125}\text{I}$  as described above.  $1 \times 10^9$  cells in a final volume of 250  $\mu\text{l}$  of PBS were then incubated with 250  $\mu\text{l}$  of PBS containing 250  $\mu\text{g}/\text{ml}$  of TPCK-trypsin for 30 min at 37°C. Control cells were incubated under similar conditions, but trypsin was omitted. The cells were washed twice in 10 ml of ice-cold PBS containing 500  $\mu\text{g}/\text{ml}$  of trypsin inhibitor and twice in 5P(8)BS-PE, and ghosts were prepared as described above. After determination of the specific activity, the membrane proteins were solubilized and electrophoresed as described above. The gels were then stained with Coomassie Blue, photographed, dried, and autoradiograms were prepared.

**SDS-PAGE of trypsinized and nontrypsinized PNH and normal erythrocytes labeled with  $\text{NaIO}_4/\text{NaB}^3\text{H}_4$ .** Trypsinized PNH and normal erythrocytes (500  $\mu\text{l}$  of packed cells) and their respective incubated controls were prepared as described above. The cells were washed three times in PBS and resuspended to 1.5 ml in PBS. Cell surface sialic acid residues were then labeled using a modification of the method of Gahmberg et al. (29, 30). The cell suspensions were made 2 mM with sodium meta-periodate ( $\text{NaIO}_4$ ) (Sigma Chemical Co.) and incubated for 10 min at room temperature in the dark. The cells were washed three times in 5P(8)BS-PE and ghosts were prepared as described above. The ghosts were resuspended to 500  $\mu\text{l}$  in 5P(8)-PE and an equal volume of 100 mM sodium phosphate, pH 8.5, containing 500  $\mu\text{Ci}$  of tritiated sodium borohydride ( $\text{NaB}^3\text{H}_4$ ) (Amersham Corp., Arlington Heights, IL) was added, and the suspension was incubated for 30 min at room temperature. The ghosts were washed three times in 30 ml of cold 50 mM sodium phosphate, pH 8.5. The ghosts were suspended in 300  $\mu\text{l}$  of 50 mM  $\text{PO}_4$ , pH 8.5, protein concentration was determined, and specific activity was then calculated by counting duplicate 10  $\mu\text{l}$  samples of the suspension, which had been dissolved in 10 ml of Aquasol-2 (New England Nuclear, Boston, MA), in a liquid scintillation counter. The radiolabeled ghosts were subsequently solubilized in SDS and electrophoresed as described above. After staining with Coomassie Blue, the gels were photographed and fluorographs were then prepared according to the method of Bonner and Laskey (31).

**SDS-PAGE of trypsinized and nontrypsinized PNH and normal erythrocytes labeled with galactose oxidase/ $\text{NaB}^3\text{H}_4$  after treatment with neuraminidase.** Three 500- $\mu\text{l}$  aliquots of packed erythrocytes (PNH and normal) were incubated with 500  $\mu\text{l}$  of PBS containing either no trypsin, 25  $\mu\text{g}/\text{ml}$  of TPCK-trypsin, or 250  $\mu\text{g}/\text{ml}$  of TPCK-trypsin for 30 min at 37°C. The cells were washed twice with cold PBS containing 500  $\mu\text{g}/\text{ml}$  of trypsin inhibitor and three times with PBS containing 1 mM  $\text{CaCl}_2$ . The cells were then suspended to 1 ml in the same buffer and treated with neuraminidase as described above. Labeling of the galactose residues of the cell-surface polysaccharides was performed using modifications of previously described methods (32–34). Each aliquot of cells was suspended to a final volume of 1 ml in PBS and 10 U of galactose oxidase (U. S. Biochemical Corp., Cleveland, OH), which had been further purified according to the method of Hatton and Regoeczi (35), was added to each suspension. After incubation for 30 min at 37°C, the cells were washed three times in cold 5P(8)BS-PE. Ghosts were then prepared and labeled with  $\text{NaB}^3\text{H}_4$  as described above. The procedures for the subsequent determination of specific activity, SDS-PAGE, and fluorography have also been described above.

## Results

**Binding pattern of  $^{125}\text{I}$ -C3b to PNH and normal erythrocytes.** To determine the topography of binding of C3b to PNH and

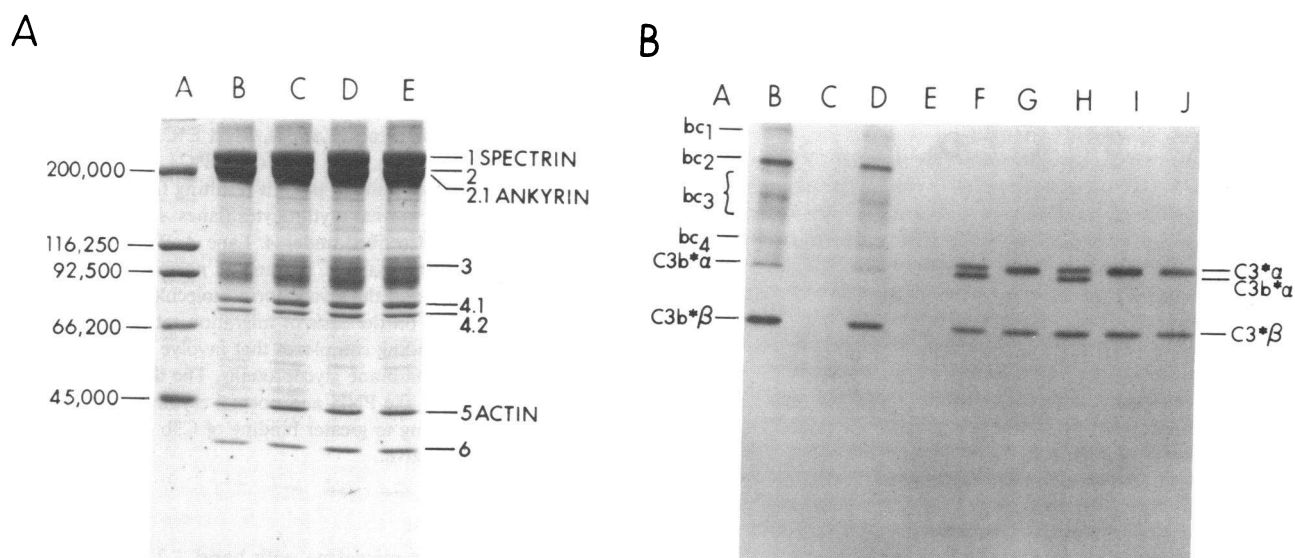
normal erythrocytes, cells bearing EC3bBbC3NeF were incubated with fluid phase  $^{125}\text{I}$ -C3. Membrane ghosts were prepared, solubilized in SDS, and electrophoresed under reducing conditions. The Coomassie-stained gels demonstrated no difference in the membrane protein staining pattern between PNH and normal erythrocytes (Fig. 1 A). The labile binding site of C3b is located within the  $\alpha$ -chain of the molecule. Therefore, when C3b forms a chemical bond with a membrane constituent, it is the  $\alpha$ -chain which becomes covalently bound and consequently displaced from its normal, uncomplexed electrophoretic position. Autoradiograms revealed the  $\alpha$ -chain of the  $^{125}\text{I}$ -C3b to be predominately part of a complex [designated binding complex 2 ( $bc_2$ ) in Fig. 1 B] whose molecular weight was estimated at 255,000 D. The film also revealed other more diffuse, less well-defined areas of  $^{125}\text{I}$ -C3b  $\alpha$ -chain binding (Fig. 1 B). The pattern of  $^{125}\text{I}$ -C3b binding to both PNH and normal erythrocytes was the same. When the gel tracks were sliced,  $\sim 60\%$  of the radioactivity above the level of the C3b  $\alpha$ -chain was found to be incorporated into the 255,000 D complex.

The nonspecific binding of radiolabeled C3 ( $^{125}\text{I}$ -C3 binding

to erythrocytes not bearing the alternative pathway convertase) was  $<1\%$  of the specific binding ( $^{125}\text{I}$ -C3b binding to EC3bBbC3NeF). There was no conversion of C3 to C3b by PNH and normal erythrocytes not bearing the alternative pathway convertase (Fig. 1 B). In these experiments, fluid-phase  $^{125}\text{I}$ -C3 was limiting. Therefore,  $^{125}\text{I}$ -C3 conversion by PNH and normal EC3bBbC3NeF was equivalent ( $\sim 70\%$  of the  $^{125}\text{I}$ -C3 was converted to  $^{125}\text{I}$ -C3b). The conversion of  $<100\%$  is attributed to loss of biological activity due to storage and to radio-labeling of the native C3.

We have previously demonstrated that the efficiency of binding of nascent C3b is the same for PNH and normal erythrocytes (5), and in that the amount of  $^{125}\text{I}$ -C3 converted was the same in these experiments, the amount of  $^{125}\text{I}$ -C3b subsequently bound to each cell type was equivalent (15,010 molecules/cell for PNH erythrocytes and 12,200 molecules/cell for normal erythrocytes).

*Treatment of PNH and normal EC3b\* with hydroxylamine.* To determine if the  $^{125}\text{I}$ -C3b was bound to the membrane surface via an ester (hydroxylamine-sensitive) or imidoester (hydrox-

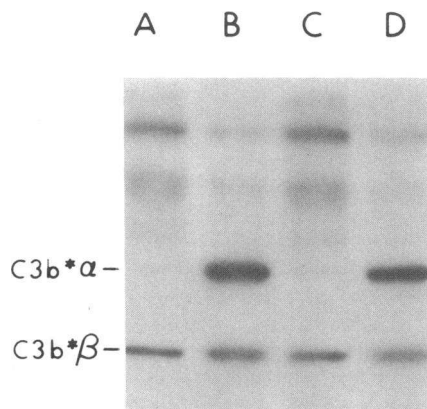


**Figure 1.** (A) SDS-PAGE of PNH and normal erythrocytes bearing the alternative pathway convertase incubated with fluid-phase  $^{125}\text{I}$ -C3 (PNH and normal EC3b\*). To control for nonspecific binding of  $^{125}\text{I}$ -C3, PNH and normal erythrocytes that did not bear the alternative pathway convertase were incubated with fluid-phase  $^{125}\text{I}$ -C3 in the same fashion (PNH and normal EC3\*). The cells were then pelleted and an aliquot of the supernate was recovered. The erythrocyte membranes from each category were isolated and, along with their respective supernates, analyzed by SDS-PAGE and stained with Coomassie Blue. The tracks containing the supernates were loaded with 10,000 cpm to approximate the number of counts loaded onto the tracks containing the ghost proteins from PNH and normal EC3b\*. Since the specific activity of the supernate was 100,000 cpm/ $\mu\text{g}$  of protein, only 100 ng of protein were loaded. This was insufficient protein to allow visualization of the bands. For this reason,

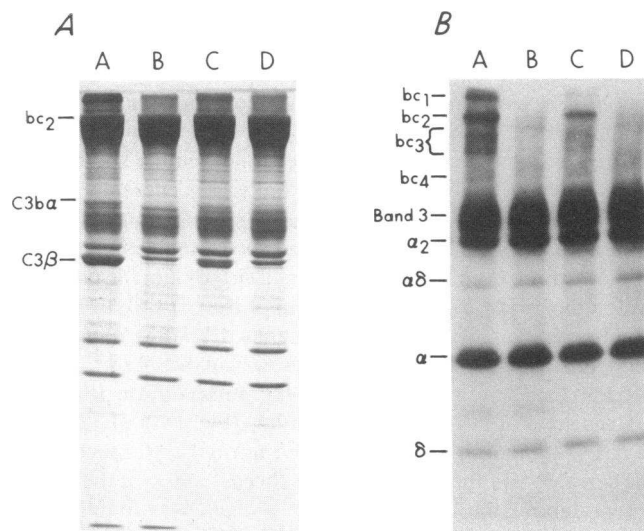
the lanes containing the supernates are not shown. However, the autoradiograms depicted in (B) includes these tracks. Approximately 25  $\mu\text{g}$  of ghost protein were applied to lanes B-E. Lane A, molecular weight standards; lane B, PNH EC3b\*; lane C, PNH EC3\*; lane D, normal EC3b\*; lane E, normal EC3\*. There was no obvious difference in the Coomassie staining pattern among the four cell types. (B) Autoradiogram of gel described under (A). Lanes B, D, and F-J had  $\sim 10,000$  cpm/track. Lane A, molecular weight standards; lane B, PNH EC3b\*; lane C, PNH EC3\*; lane D, normal EC3b\*; lane E, normal EC3\*; lane F, supernate from PNH EC3b\*; lane G, supernate from PNH EC3\*; lane H, supernate from normal EC3b\*; lane I, supernate from normal EC3\*; lane J,  $^{125}\text{I}$ -C3 not exposed to erythrocytes.  $bc_{1-4}$  are the binding complexes that involve C3b covalently linked to erythrocyte membrane glycoproteins. The pattern of binding of  $^{125}\text{I}$ -C3b is the same for PNH and normal EC3b (lanes B and D).

ylamine-insensitive) bond (26), PNH and normal EC3b\* ghosts were treated with hydroxylamine. A control set of PNH and normal EC3b\* was treated in exactly the same fashion except hydroxylamine was omitted. For both PNH and normal EC3b\*, most of the  $^{125}\text{I}$ -C3b  $\alpha$ -chain was released from the high molecular weight complexes (Fig. 2), suggesting that the  $\alpha$ -chain of C3b had been covalently bound to free hydroxyl groups on the PNH and normal erythrocytes surface via an ester bond. The modest amount of residual, unreleased radiolabeled C3b  $\alpha$ -chain suggests that some of the binding may be mediated by hydroxylamine-resistant imidoester bonds.

**Binding pattern of C3b to radiolabeled PNH and normal erythrocytes.** To determine the membrane constituents to which C3b binds, intact PNH and normal erythrocytes were labeled with  $^{125}\text{I}$  using Iodo-Gen. Radiolabeled PNH and normal erythrocytes bearing the alternative pathway convertase were then prepared and exposed to unlabeled fluid phase C3. The membranes from these E\*C3b were subsequently isolated, solubilized, and electrophoresed using SDS-PAGE. Coomassie-stained gels revealed differences in the protein staining pattern resulting from the binding of C3b to the erythrocyte surface (Fig. 3 A). For both PNH and normal E\*C3b, a new band whose molecular weight was consistent with the  $\beta$ -chain of C3b ( $M_r = 75,000$ )



**Figure 2.** Autoradiograph of SDS-PAGE of PNH and normal EC3b\* treated with hydroxylamine to release ester bound  $^{125}\text{I}$ -C3b. PNH and normal EC3b\* were prepared and the membranes were isolated. Half of each preparation was treated with hydroxylamine. The other half, which served as a control, was treated in exactly the same fashion except deionized water was substituted for hydroxylamine. The membrane proteins were then analyzed by SDS-PAGE. Lane A, PNH EC3b\* not treated with hydroxylamine; lane B, PNH EC3b\* treated with hydroxylamine; lane C, normal EC3b\*, not treated with hydroxylamine; lane D, normal EC3b\* treated with hydroxylamine. For both PNH and normal EC3b, the majority of the  $^{125}\text{I}$ -C3b $\alpha$ -chain was released by treatment with hydroxylamine, indicating that most of the C3b had been found to free hydroxyl groups via ester bonds. The modest amount of residual, unreleased  $^{125}\text{I}$ -C3b $\alpha$ -chain suggests that some of the binding may be mediated by hydroxylamine-resistant imidoester bonds.



**Figure 3.** (A) SDS-PAGE of radiolabeled PNH and normal erythrocytes bearing the alternative pathway convertase incubated with unlabeled fluid-phase C3 (PNH and normal E\*C3b). To control for non-specific binding of C3, PNH and normal erythrocytes not bearing the convertase were incubated with C3 in the same fashion (PNH and normal E\*C3). Membrane preparations were analyzed by SDS-PAGE and stained with Coomassie Blue. Approximately 25  $\mu\text{g}$  of ghost protein were loaded onto each track. Lane A, PNH E\*C3b; lane B, PNH E\*C3; lane C, normal E\*C3b; lane D, normal E\*C3. There are differences in the Coomassie staining pattern resulting from the binding of C3b to the PNH and normal erythrocytes (lanes A and C). (B) Autoradiographs of gel described under A. Lane A, PNH E\*C3b; lane B, PNH E\*C3; lane C, normal E\*C3b; lane D, normal E\*C3. The identification of band 3 and the glycoprotein molecules (denoted by Greek letters) was made on the basis of migration rate and labeling pattern.  $bc_{1-4}$  are the binding complexes that involve C3b covalently linked to erythrocyte membrane glycoproteins. The difference in the labeling pattern between the PNH and normal erythrocytes bearing C3b is quantitative (owing to greater binding of C3b to the PNH cells) rather than qualitative.

was seen, essentially comigrating with band 4.2 ( $M_r = 72,000$ ). Another new band was seen just above the trailing edge of band 3 ( $M_r = 116,000$ ). The molecular weight of this band was consistent with that of the uncomplexed  $\alpha$ -chain of C3b ( $M_r = 117,000$ ).

In both the PNH and normal E\*C3b tracks, a group of high molecular weight bands (some of which entered the gel only slightly) were visible (Fig. 3 A, lanes A and C). Because of its overlap with the trailing edge of band 1, the  $bc_2$  band (Fig. 3 A, lanes A and C) is difficult to visualize in the black and white photograph; however, in the original Coomassie-stained gel, it is clearly the most intensely staining of the higher molecular weight series and has an  $M_r$  of 255,000. There were no qualitative differences in the staining pattern between PNH and normal E\*C3b. Because excess fluid-phase C3 was used in these ex-

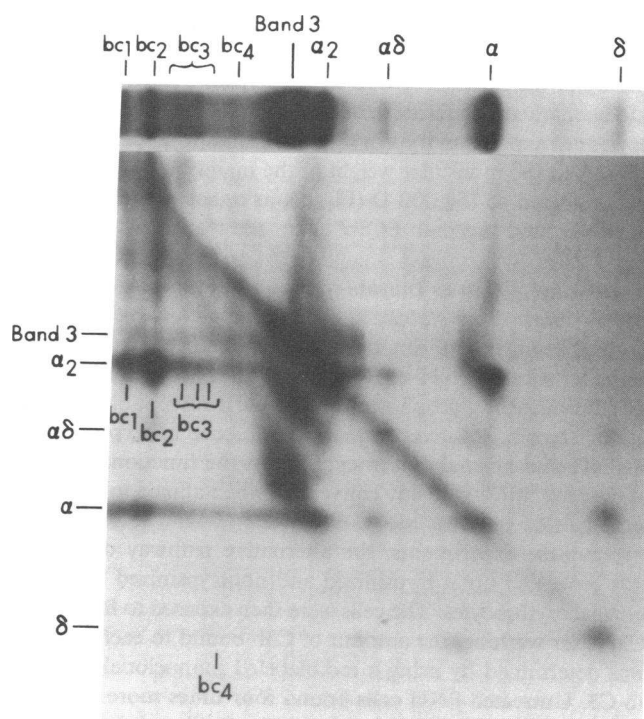
periments, the PNH E\*C3b bound more C3b than did their normal counterpart (5), accounting for the greater staining intensity of the new bands.

Autoradiograms revealed no obvious difference between the labeling pattern of the radiolabeled PNH and normal erythrocytes bearing no C3b (Fig. 3 *B*, lanes *B* and *D*). For the E\*C3b (Fig. 3 *B*, lanes *A* and *C*), high molecular weight bands were visualized, the most prominent ( $bc_2$ ) corresponding to the 255,000 D band seen on the Coomassie-stained gel. Also visualized are a less prominent higher molecular weight band ( $bc_1$ ) and two lower molecular weight species, one which is diffuse ( $bc_3$ ) and the other which is very faint ( $bc_4$ ). This binding pattern appears analogous to that seen when  $^{125}\text{I}$ -C3 is used in similar experiments (Fig. 1 *B*). All of these bands are more prominent on the PNH E\*C3b. The apparent differences in the labeling pattern between the two cell types can be accounted for by the fact that the PNH E\*C3b had more C3b bound than did their normal counterpart (Fig. 3 *A*). On prolonged exposure of the film, there was no difference in the labeling pattern between the two cell types (data not shown).

*Two dimensional electrophoresis of radiolabeled PNH and normal EC3b (E\*C3b).* To determine the electrophoretic mobility of the membrane constituent when unbound to C3b, the PNH and normal E\*C3b described above were electrophoresed in the first dimension, the tracks of interest were isolated, treated with hydroxylamine, and electrophoresed in the second dimension.

After release of C3b by hydroxylamine, the membrane component of both the major C3b binding complex ( $bc_2$ ) and the less prominent higher molecular weight complex ( $bc_1$ ) were seen to migrate in the same horizontal plane as glycophorin- $\alpha$  dimer (Fig. 4). The membrane constituents of the diffuse  $bc_3$  area were also seen to migrate in the same horizontal plane as glycophorin- $\alpha_2$  and to consist of three separate, distinct components (Fig. 4, denoted by the pointers). The membrane constituent of the  $bc_4$  area was faintly visible, appearing in the same horizontal plane as glycophorin- $\delta$  monomer. Thus for PNH and normal erythrocytes, nascent C3b binds to the glycophorin molecules, predominately to glycophorin- $\alpha$ , the major erythrocyte sialoglycoprotein.

The glycophorin- $\alpha$  monomer and homodimer interconvert in SDS-PAGE systems even in the absence of treatment with hydroxylamine and reducing agents (36–38). It seems likely that this interconversion phenomenon also involves the glycophorin- $\delta$  monomer and homodimer and the glycophorin- $\alpha\delta$  heterodimer, thereby accounting for the exposed areas which appear off-diagonal in the same vertical planes as the on-diagonal glycophorins (Fig. 4). The horizontal plane in which these off-diagonal glycoproteins appear defines their molecular composition. Similarly, it seems likely that the areas of exposure which are off-diagonal just below the on-diagonal position of band 3 represent a subpopulation of band 3 molecules whose electrophoretic mobility has been modified as a result of changes in their biophysical/biochemical properties induced during the process of two-dimensional electrophoretic analysis (Fig. 4).



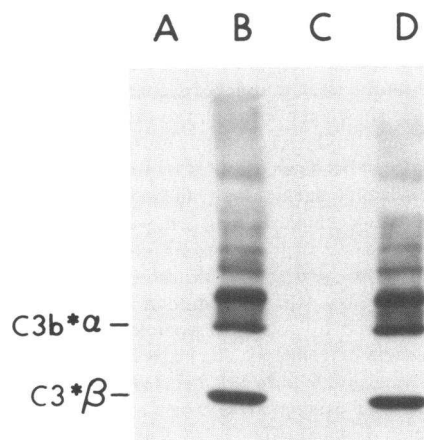
**Figure 4.** Autoradiograph of two-dimensional gel electrophoresis of PNH E\*C3b after treatment with hydroxylamine. Tracks from the gels described in Fig. 3 were isolated, treated with hydroxylamine, and run in the second dimension. After staining and destaining the gels, autoradiographs were prepared. The film of the PNH E\*C3b is shown. An autoradiograph of the track as it appears after electrophoresis in the first dimension is shown above the film to define the vertical planes of the labeled bands in the second dimension. The horizontal planes of the on-diagonal bands are denoted by the pointers to the left of the film. Hydroxylamine releases the radiolabeled membrane constituents of the binding complexes ( $bc_{1-4}$ ) allowing them to run in the same horizontal planes as their on-diagonal counterparts. The membrane constituents of  $bc_{1-3}$  are in the same horizontal plane as the glycophorin- $\alpha$  dimer. The  $bc_3$ , which is diffuse in the one-dimensional autoradiograph, appears to have three separate, distinct membrane components (denote by pointers). The  $bc_4$  is faintly visible in the same horizontal plane as glycophorin- $\delta$ . A detailed explanation of the etiology of the off-diagonal exposed areas, which are in the same vertical planes as the on-diagonal radiolabeled glycoproteins, is provided in Results.

*Binding pattern of  $^{125}\text{I}$ -C3b to PNH and normal erythrocytes treated with trypsin.* The glycophorin- $\alpha$  molecule is susceptible to cleavage by trypsin (39). It therefore seemed likely that, if nascent C3b bound predominately to this particular membrane constituent, treatment of PNH and normal erythrocytes with trypsin would alter the C3b binding pattern seen by SDS-PAGE. To test this hypothesis, PNH and normal erythrocytes were treated with trypsin, the alternative pathway convertase was established on the cells, and these trypsinized EC3bBbC3NeF were exposed to fluid-phase  $^{125}\text{I}$ -C3. The membranes were iso-

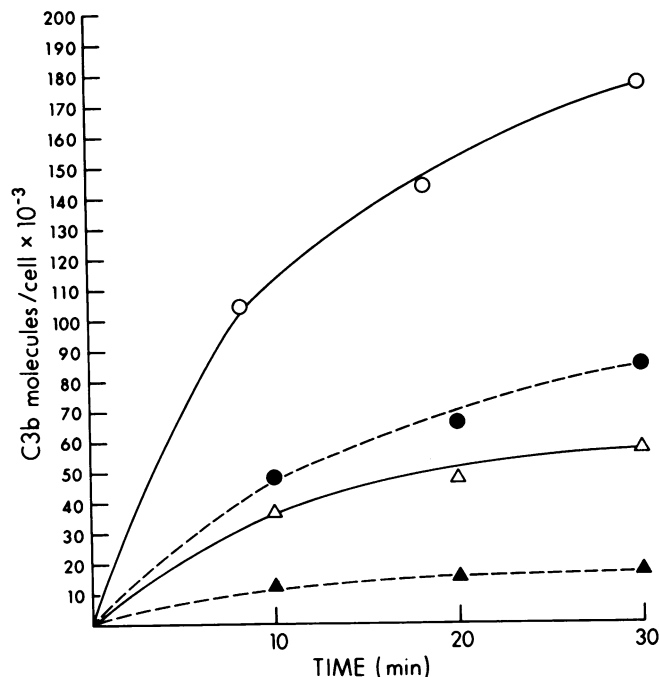
lated, solubilized under reducing conditions, and electrophoresed using SDS-PAGE. Autoradiograms revealed the pattern of binding of the  $\alpha$ -chain of C3b to the trypsin-treated cells (Fig. 5) to be markedly different from that to PNH and normal erythrocytes not exposed to trypsin (Fig. 1 B). Binding appeared more diffuse and the molecular weight of the major binding complex was estimated at 152,000 D (Fig. 5), as opposed to 255,000 D for PNH and normal EC3b\* not pretreated with trypsin (Fig. 1 B).

*Binding of C3b to enzyme-treated PNH and normal erythrocytes bearing the stabilized alternative pathway convertase.* Normal erythrocytes can be made to manifest many of the characteristics of PNH erythrocytes by treatment with trypsin (40). Because the glycoprotein- $\alpha$  molecule is susceptible to cleavage by trypsin, we investigated the influence of trypsinization of PNH and normal erythrocytes upon the functional activity of the alternative pathway convertase when affixed to such protease-treated cells.

In these experiments, the alternative pathway convertase was generated upon trypsinized and nontrypsinized PNH and normal erythrocytes. The cells were then exposed to fluid-phase C3. After washing, the amount of C3b bound to each cell type was determined by using a radiolabeled monoclonal antibody to C3. Untreated PNH cells bound four times more C3b than did untreated normal erythrocytes (Fig. 6). Trypsinized normal



**Figure 5.** SDS-PAGE of PNH and normal erythrocytes treated with TPCK-trypsin. Cells bearing the alternative pathway convertase were prepared and incubated with fluid-phase  $^{125}\text{I}$ -C3 (trypsinized PNH and normal EC3b\*). To control for nonspecific binding of  $^{125}\text{I}$ -C3, trypsinized PNH and normal erythrocytes not bearing the convertase were incubated with fluid-phase  $^{125}\text{I}$ -C3 in the same fashion (trypsinized PNH and normal EC3\*). Membrane preparations were analyzed by SDS-PAGE and an autoradiogram of the gel was prepared. Lane A, trypsinized normal EC3\*; lane B, trypsinized normal EC3b\*; lane C, trypsinized PNH EC3\*; lane D, trypsinized PNH EC3b\*. Trypsinization of the erythrocytes markedly alters the pattern of binding of  $^{125}\text{I}$ -C3b to both normal and PNH erythrocytes compared to that of their untrypsinized counterparts (Fig. 1 A).



**Figure 6.** Binding of C3b to trypsinized (open symbols) and nontrypsinized (closed symbols) normal (triangles) and PNH (circles) EC3bBbC3NeF as a function of time. Data points represent the mean of duplicate experiments.

erythrocytes bound three times more C3b than did their untreated counterpart, whereas trypsinized PNH cells bound 10 times more C3b than did the untreated normal erythrocytes.

The cleavage by trypsin of the major erythrocyte sialoglycoprotein (glycophorin- $\alpha$ ) results in the release of one-third to one-half of the total erythrocyte sialic acid (24). To determine if the observations described above regarding modification of alternative pathway functional activity by treatment of the cells with trypsin were the consequence of the removal of sialic acid, we performed similar experiments on PNH and normal erythrocytes treated with neuraminidase. Untreated PNH erythrocytes bearing the alternative pathway convertase bound five times more C3b than the normal EC3bBbC3NeF (Fig. 7). However, in contrast to the trypsin-treated cells, both PNH and normal EC3bBbC3NeF that had been treated with neuraminidase bound approximately one-third less C3b than did their untreated counterparts. Thus, the greater fixation of C3b to trypsinized PNH and normal cells is not a consequence of removal of sialic acid alone.

These experiments suggested that trypsin treatment of PNH and normal erythrocytes results in the modification of a cell-surface glycoprotein(s), which modulates the functional activity of the alternative pathway convertase. To further investigate this observation, we examined the effects of trypsinization upon the electrophoretic pattern of radiolabeled normal and PNH erythrocyte membrane glycoproteins.

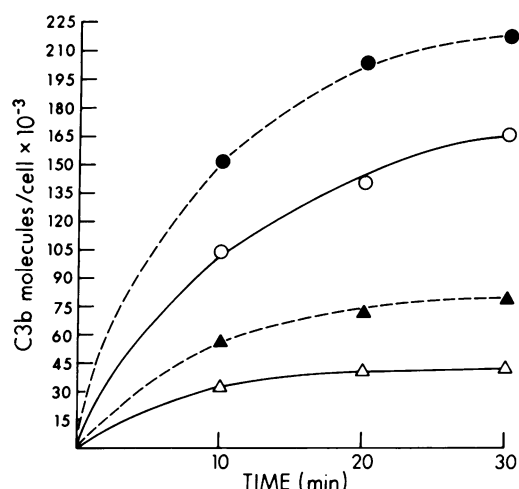


Figure 7. Binding of C3b to neuraminidase-treated (open symbols) and nontreated (closed symbols) normal (triangles) and PNH (circles) EC3bBbC3NeF as a function of time. Data points represent the mean of duplicate experiments.

**SDS-PAGE of trypsinized and nontrypsinized PNH and normal erythrocytes radiolabeled with  $^{125}\text{I}$ .** In these experiments, Iodo-Gen was used to label the tyrosine residues of the cell surface glycoproteins. There was no obvious difference in the labeling pattern between the two cell types for the untrypsinized erythrocytes (Fig. 8, lanes A and B). Lanes C and D contain the membrane proteins from the trypsin-treated normal and PNH erythrocytes and demonstrate for both cell types complete cleavage by trypsin of the glycophorin- $\alpha$  dimer. However, for the PNH erythrocytes, there was a marked decrease in the radioactivity of the band designated as the trypsin cleavage peptide (TCP). This band putatively represents a residual part of the glycophorin- $\alpha$  dimer. Lanes E and F contain the membrane proteins from another set of trypsin-treated normal and PNH erythrocytes, again depicting the decrease in radioactivity of the TCP for the PNH cells. These data are consistent with a qualitative difference in glycophorin- $\alpha$  on PNH erythrocytes.

**SDS-PAGE of trypsinized and nontrypsinized PNH and normal erythrocytes radiolabeled using  $\text{NaIO}_4/\text{NaB}^3\text{H}_4$ .** To determine if abnormalities in glycosylation contribute to the observed differences in the glycophorin- $\alpha$  molecule on the PNH cells, the terminal sialic acid residues of trypsinized and nontrypsinized PNH and normal erythrocytes were oxidized with sodium metaperiodate, then radiolabeled with tritiated sodium borohydride. After determination of the specific activity of the membrane proteins, the ghosts were solubilized and electrophoresed and fluorographs were subsequently prepared. For the two patients with PNH, the specific activity of their radiolabeled erythrocyte membrane glycoproteins was 30–35% less than that of the normal control. Thus, PNH erythrocytes have fewer periodate oxidizable constituents than normal erythrocytes. This may account for the differences in the glycophorin- $\alpha$  dimer between the untryp-

sinized PNH and normal erythrocytes, seen in the fluorographs (Fig. 9). The glycophorin- $\alpha$  dimer band is less diffuse for the PNH cells (lane B vs. lane A and lane F vs. lane E), suggesting a quantitative difference in the radioactivity of the band. Alternatively (or in addition), aberrations in glycosylation may alter the electrophoretic pattern of the dimer. Further, for the PNH erythrocytes, there appears to be a modest reduction in the radioactivity of glycophorin- $\delta_2$ , glycophorin- $\alpha$ , and possibly glycophorin- $\beta$  suggesting that abnormalities in glycosylation involve these and possibly other membrane glycoproteins.

As with the iodinated cells, these  $\text{NaIO}_4/\text{NaB}^3\text{H}_4$ -labeled cells also demonstrate a marked decrease in the radioactivity of the TCP for the PNH cells (lane D vs. lane C and lane H compared with lane G). Using techniques identical to those described above, we have also examined the membrane glycoproteins from erythrocytes of patients with other hematological abnormalities. In data not shown, we found no difference from normal controls in the cells of two patients with high reticulocyte counts (>20%) who were recovering from bleeding episodes, one patient with sickle-cell anemia, one patient who was a double heterozygote for sickle-cell anemia and  $\beta$ -thalassemia, and three patients with polycythemia vera. A modest decrease in the radioactivity of the TCP was observed in two patients with acquired sideroblastic anemia, a myelodysplastic disease as is PNH.

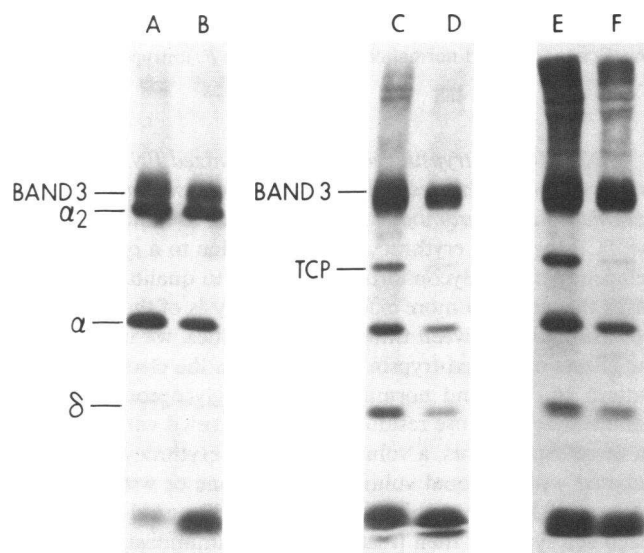
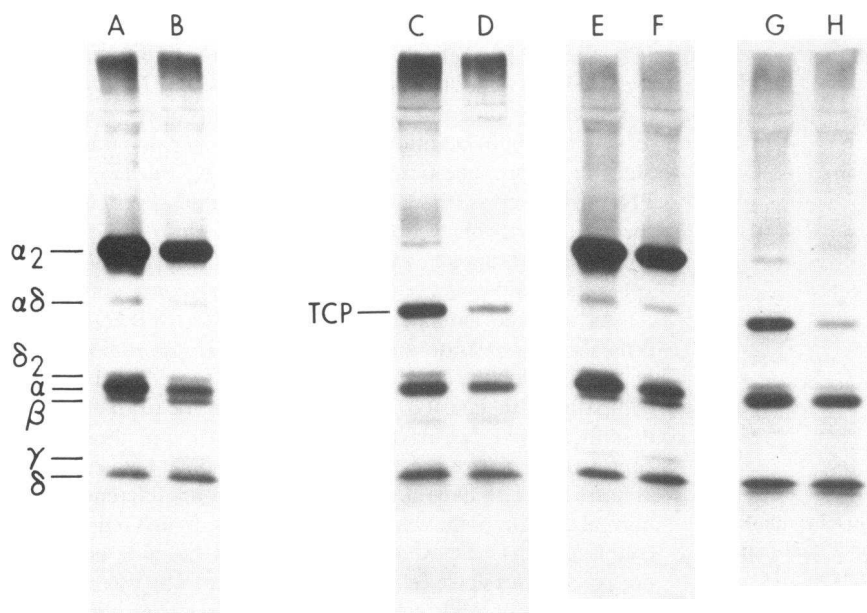


Figure 8. Autoradiograph of  $^{125}\text{I}$ -labeled normal and PNH erythrocytes. After radiolabeling the cells, half were treated with TPCK-trypsin, and  $1 \times 10^5$  cpm were loaded onto each track. Lane A, untreated normal erythrocytes; lane B, untreated PNH erythrocytes; lane C, trypsinized normal erythrocytes; lane D, trypsinized PNH erythrocytes; lane E, trypsinized normal erythrocytes; lane F, trypsinized erythrocytes from a second patient with PNH. The Greek letters denote glycophorin monomers and homo- and heterodimers. The radioactivity of the TCP is markedly diminished on the PNH cells (lanes D and F).



**Figure 9.** Fluorograph of normal and PNH erythrocytes labeled by treatment with  $\text{NaIO}_4/\text{NaB}^3\text{H}_4$ . Trypsinized and nontrypsinized PNH and normal erythrocytes were radiolabeled, and 25  $\mu\text{g}$  of solubilized protein was loaded onto each track. Lane A, untrypsinized normal erythrocytes; lane B, untrypsinized PNH erythrocytes; lane C, trypsinized normal erythrocytes; lane D, trypsinized PNH erythrocytes; lane E, untrypsinized normal erythrocytes; lane F, untrypsinized

erythrocytes from a second patient with PNH; lane G, trypsinized normal erythrocytes; lane H, trypsinized erythrocytes from the second patient with PNH. The glycophorin monomers, and homo- and heterodimers are denoted by the Greek letters. For the PNH erythrocytes, there is less radioactivity in the area of the glycophorin- $\alpha$  dimer and the TCP (lanes B and D and F and H).

*SDS-PAGE of trypsinized and untrypsinized PNH and normal erythrocytes labeled with galactose oxidase/ $\text{NaB}^3\text{H}_4$  after treatment with neuraminidase.* The decrease in radioactivity of the TCP for PNH erythrocytes could be due to a quantitative deficiency of the glycophorin- $\alpha$  dimer, or to qualitative differences that result in more extensive proteolysis of the molecule. To distinguish between these two possibilities, we investigated the effects of limited trypsin cleavage upon the electrophoretic pattern of PNH and normal cell-surface glycoproteins whose penultimate galactose residues had been labeled with  $\text{NaB}^3\text{H}_4$ . In these experiments, a volume of packed erythrocytes was incubated with an equal volume of PBS alone or with an equal volume of PBS containing either 25 or 250  $\mu\text{g}/\text{ml}$  of trypsin. Each cell type was then treated with neuraminidase to remove the terminal sialic acid residues. After oxidation with galactose oxidase, ghosts were prepared and exposed to  $\text{NaB}^3\text{H}_4$ . The ghosts were then solubilized, and the specific activity was determined. After electrophoresis, fluorographs were prepared.

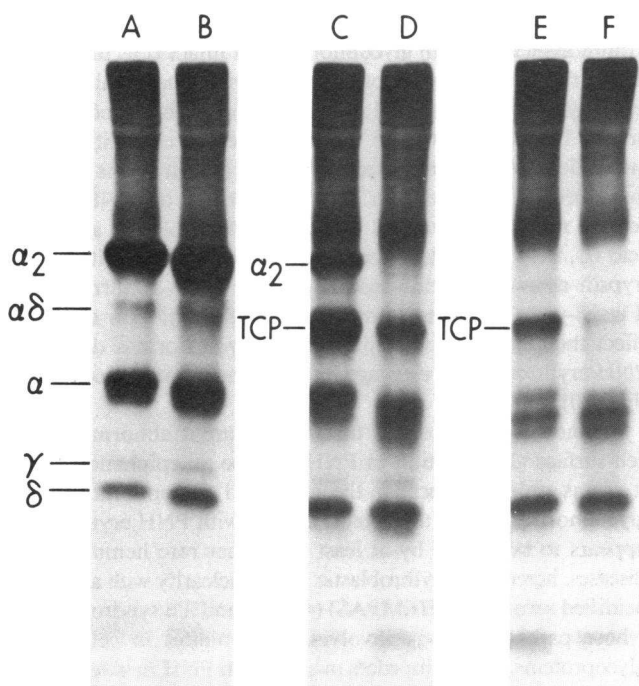
The fluorograph (Fig. 10) demonstrates a similar labeling pattern for the untrypsinized normal and PNH cells with a moderate increase in the radioactivity of the glycophorin molecules for the PNH erythrocytes (lane B vs. lane A). In concordance with this observation, the nontrypsin-treated PNH membrane proteins had a higher specific activity than did their

normal counterpart. However, for the PNH cells treated with trypsin, there was a marked decrease in radioactivity of the glycophorin- $\alpha$  dimer and of the TCP.

Lanes C and D contain, respectively, the normal and PNH cells treated with 12.5  $\mu\text{g}/\text{ml}$  of trypsin and demonstrate that while the glycophorin- $\alpha$  dimer of the normal cells is only partially cleaved at this concentration of trypsin, there is complete cleavage of this peptide on the PNH erythrocytes. Further, despite more extensive cleavage of the glycophorin- $\alpha$  dimer, there is less radioactivity in the area of TCP for the PNH erythrocytes. In addition, for the PNH cells, the diffuse band just below the TCP has a faster electrophoretic mobility than its normal counterpart.

These data suggest that the TCP undergoes further trypsin digestion, and that on PNH erythrocytes both the glycophorin- $\alpha$  dimer and TCP are more susceptible to trypsin-mediated proteolysis. The tracks containing the membrane proteins from the normal (lane E) and the PNH (lane F) cells treated with 125  $\mu\text{g}/\text{ml}$  of trypsin confirm this hypothesis. At this concentration of trypsin, there is complete cleavage of the glycophorin- $\alpha$  dimer for both cell types. However, the TCP has undergone more extensive proteolysis on the PNH erythrocytes as evidenced by decreased radioactivity in the area of TCP.

Thus, the glycophorin- $\alpha$  dimer appears to be qualitatively



**Figure 10.** Fluorograph of normal and PNH erythrocytes labeled using galactose oxidase/ $\text{NaB}^3\text{H}_4$  after treatment with neuraminidase. Trypsinized and nontrypsinized PNH and normal erythrocytes were treated with neuraminidase and then exposed to galactose oxidase and the oxidized galactosyl residues were labeled by reduction with  $\text{NaB}^3\text{H}_4$ . 25  $\mu\text{g}$  of solubilized protein was loaded onto each track. Lane A, untreated normal erythrocytes; lane B, untreated PNH erythrocytes; lane C, normal erythrocytes treated with 12.5  $\mu\text{g}/\text{ml}$  of TPCK-trypsin; lane D, PNH erythrocytes treated with 12.5  $\mu\text{g}/\text{ml}$  of TPCK-trypsin; lane E, normal erythrocytes treated with 125  $\mu\text{g}/\text{ml}$  of TPCK-trypsin; lane F, PNH erythrocytes treated with 125  $\mu\text{g}/\text{ml}$  of TPCK-trypsin. The glycophorin monomers, and homo- and heterodimers are denoted by Greek letters. For PNH erythrocytes, the glycophorin- $\alpha$  dimer and the TCP are more susceptible to trypsin-mediated proteolysis (lanes D and F).

abnormal on PNH cells as evidenced by its greater susceptibility to cleavage by trypsin.

## Discussion

We have compared the topography of binding of C3b to PNH and normal erythrocytes in an attempt to gain insight into the biochemistry of the membrane defect that accounts for the greater fixation of C3b to PNH erythrocytes when complement is activated. Our studies show that the pattern of binding of C3b is the same for PNH and normal erythrocytes (Figs. 1 B, 2, 3 B, and 5), and that C3b binds predominantly to glycophorin- $\alpha$  (Fig. 4), the major erythrocyte sialoglycoprotein.

The investigations of Law and Levine (10) have suggested

that activated C3b binds to cells by forming an ester bond with free hydroxyl groups on the glycosyl residues of surface glycoproteins. The structure of glycophorin- $\alpha$  (14, 39, 41, 42) makes it a likely binding site for nascent C3b. The molecule has a transmembrane orientation (i.e., it spans the lipid bilayer and has external and cytoplasmic domains), and it is heavily glycosylated (60% carbohydrate by weight). 9 of the first 15 amino acids of the amino terminal end (external domain) are *O*-glycosidically linked with oligosaccharides. It is the major erythrocyte glycoprotein contributing 60% of the membrane carbohydrate, and there are an estimated  $1 \times 10^6$  copies per cell.

An understanding of the biochemistry of the glycophorin- $\alpha$  molecule aids in the interpretation of the experimental data. Glycophorin- $\alpha$  dimer and glycophorin- $\alpha$  monomer interconvert in SDS-PAGE systems (36–38) (this accounts for the presence of the exposed areas above and below the diagonal in the same horizontal planes as glycophorin- $\alpha$  monomer and dimer seen in Fig. 4). This phenomenon may partially explain the heterogeneous nature of the binding pattern between the C3b $\alpha$ -chain and the glycophorin- $\alpha$  molecule seen in the one-dimensional gels (i.e., the C3b $\alpha$ -chain-glycophorin- $\alpha$  complex may in some cases consist of a C3b $\alpha$ -chain covalently linked to the glycophorin- $\alpha$  monomer, and in other cases, the binding may be to the dimer or perhaps even higher polymers). Also, because the molecule is so heavily glycosylated, it is possible that more than one molecule of C3b may bind to a molecule of glycophorin- $\alpha$ . Further complicating the issue is the anomalous behavior of heavily glycosylated molecules in SDS-PAGE systems, making tenuous the determination of molecular weights based on relative mobility. Thus the exact subunit composition of the C3b $\alpha$ -chain-glycophorin- $\alpha$  heteropolymers cannot be determined from these experiments.

The sensitivity of the membrane-C3b binding complex to hydroxylamine is consistent with ester bond formation involving the exposed thioester of nascent C3b and free hydroxyls on the carbohydrate moieties of the glycophorin- $\alpha$  molecule (Fig. 2) (12). However, a minor portion of the C3b appears to be bound via hydroxylamine-resistant imidoester bonds (Fig. 2).

When intact erythrocytes are treated with trypsin, glycophorin- $\alpha$  is cleaved (24). As a result, approximately half of the 70 amino acids that comprise the molecule's external domain (and concurrently three-fourths of the 16 oligosaccharide residues) are released into the supernate (14). Trypsinization markedly alters the binding pattern of C3b to PNH and normal erythrocytes (Fig. 5). The more diffuse pattern suggests that removal of the bulk of the glycosyl residues from the glycophorin- $\alpha$  allows binding of C3b to other membrane glycoproteins.

We have previously demonstrated that the C3NeF-stabilized alternative pathway convertase is enzymatically more active when affixed to PNH erythrocytes than to normal erythrocytes (5). We hypothesized that this greater enzymatic activity of the alternative pathway convertase (and the subsequent greater binding of C3b) on PNH cells might be due to a difference in the steric configuration of the C3b molecule inasmuch as it acts as the nidus for the formation of the convertase. These studies

demonstrate that the pattern of binding of C3b to PNH erythrocytes is the same as to normal erythrocytes (Fig. 1 *B*). Thus C3b binding to a different membrane constituent does not appear to account for the greater enzymatic activity of the alternative pathway convertase when affixed to PNH erythrocytes.

However, it seemed possible that the glycoproteins to which C3b binds on PNH erythrocytes might be qualitatively abnormal and that structural differences in these molecules could impose alterations in the steric orientation of the cell-bound C3b, thereby modifying the enzyme-substrate interactions of the alternative pathway convertase. In this perspective, the glycophorin- $\alpha$  molecule is considered a subunit of the convertase complex and may therefore be seen as having a regulatory role in the convertase's activity.

Trypsin treatment of normal and PNH erythrocytes markedly alters the activity of the C3 nephritic factor stabilized alternative pathway convertase, implying the proteolytic modification of a cell-surface glycoprotein which modulates the functional activity of the convertase (Fig. 6). After trypsin treatment, normal EC3bBbC3NeF bind only slightly less C3b than untrypsinized PNH EC3bBbC3NeF. However, trypsin-treated PNH EC3bBbC3NeF bind approximately four times more C3b than their normal counterpart. There appear to be two plausible explanations for these observations: (*a*) The trypsin-sensitive regulatory glycoprotein is abnormal on the PNH erythrocytes accounting for the greater fixation of C3b to the untrypsinized PNH EC3bBbC3NeF. Trypsinization more extensively modifies the abnormal glycoprotein on the PNH cells resulting in greater binding of C3b to the trypsinized PNH EC3bBbC3NeF compared to their normal counterpart. (*b*) There is no difference in the trypsin-sensitive regulatory glycoprotein on the two cell types. Trypsinization modifies the glycoprotein equally on both cell types, and the greater binding of C3b to the PNH cells is the result of a membrane abnormality which is not susceptible to inactivation by trypsin. [These experiments do, however, appear to rule out membrane abnormalities involving glycoproteins whose regulatory activity is completely susceptible to trypsin inactivation (e.g., the C3b receptor). In this case, trypsin-treated normal EC3bBbC3NeF would be expected to bind the same amount of C3b as trypsin-treated PNH erythrocytes bearing the stabilized convertase.] To help distinguish between the above two possibilities, we investigated the effects of trypsinization upon the electrophoretic pattern of radiolabeled PNH and normal erythrocytes (Figs. 8–10). The results of these experiments favor the former explanation because they suggest that the glycophorin- $\alpha$  dimer (the glycoprotein to which C3b predominately binds) is qualitatively abnormal on PNH erythrocytes as manifested by its greater susceptibility to trypsin-mediated proteolysis. The cleavage of glycophorin- $\alpha$  by trypsin has been extensively investigated (14, 15, 34, 35, 43). On intact erythrocytes, proteolysis occurs primarily at amino acid residues 30 and 39. However, using purified glycophorin- $\alpha$ , an additional trypsin-sensitive peptide bond has been identified at amino acid 61, which is potentially available for cleavage on intact erythrocytes, in that it is external to the membrane portion of the molecule.

Proteolysis at this site would remove any radioactive iodine or tritium associated with glycophorin- $\alpha$  on intact cells inasmuch as this trypsin-sensitive peptide bond is proximal to all the external tyrosine and glycosyl residues. Because the carbohydrate moiety has been shown to influence the susceptibility of the molecule to trypsin-mediated proteolysis (34), it seems possible that aberrant glycosylation of glycophorin- $\alpha$  on PNH erythrocytes results in enhanced cleavage of the molecule at amino acid 61, thus accounting for the decreased radioactivity of the trypsin cleavage peptide associated with these abnormal cells (Figs. 8–10). This same abnormality in glycosylation may also effect the greater susceptibility of the glycophorin- $\alpha$  dimer on PNH erythrocytes to cleavage by low concentrations of trypsin (Fig. 10).

Although it is possible that the apparent abnormalities in cell-surface glycosylation in PNH may be an epiphenomenon, it seems unlikely, because the enhanced susceptibility of the glycophorin- $\alpha$  dimer to proteolysis seen with PNH erythrocytes appears to be shared by at least two other rare hematological diseases, hereditary erythroblastic multinuclearity with a positive acidified serum test (HEMPAS) (44, 45) and Tn syndrome (46), whose pathophysiology involves abnormalities in cell-surface glycoproteins. These disorders overlap with PNH in several other interesting ways. HEMPAS erythrocytes have in common with PNH erythrocytes a greater susceptibility to lysis by antibody plus complement. The molecular events leading to the enhanced lysis are, however, different for the two disorders (3). As with PNH erythrocytes, HEMPAS erythrocytes are also lysed in the presence of acidified serum although the mechanism of lysis is again different. In the case of HEMPAS, the lysis is mediated by an IgM cold-reacting antibody (47) (directed against antigenic sites on the glycophorin molecules) (48) found in heterologous serum. For PNH erythrocytes, lysis in acidified serum is mediated by the alternative pathway although the molecular events leading to the convertase activation have not been defined (49). Thus, although mechanistically different, both HEMPAS and PNH erythrocytes interact aberrantly with complement.

Although the interactions of Tn cells with complement have apparently not been explored in detail, this syndrome may be compared with PNH in several other ways. Both disorders appear to involve a membrane abnormality which arises as a result of a somatic mutation that involves the pluripotent stem cell because platelets and neutrophils as well as erythrocytes are involved (50–55). Both conditions also have coexisting subpopulations of cells: one subpopulation is the abnormal clone which involves variable percentages of the total cell population; the other subpopulation, is the putative residual normal clone. Another area of overlap between PNH and Tn syndrome is the occasional development of acute leukemia in association with the disorders (54). The abnormality in glycosylation that defines the Tn syndrome has been elucidated at the molecular level: the hemotopoietic elements of Tn are deficient in 3- $\beta$ -D-galactosyltransferase, which is involved in the biosynthesis of sugar chains O-glycosidically linked to membrane glycoproteins (55). Because of the many similarities between PNH and

Tn syndrome, it is tempting to speculate that the hematopoietic elements (erythrocytes, granulocytes, and platelets) of PNH also lack a shared component essential for proper glycosylation of membrane constituents. Platelet glycoproteins are important in the maintenance of normal hemostasis (56). Aster and Enright (51) have demonstrated that, by treatment with proteases, normal platelets can be made to mimic PNH platelets with regard to increased susceptibility to lysis by complement, and preliminary experiments from this laboratory indicate that the labeling pattern of PNH platelet glycoproteins (using  $\text{NaIO}_4/\text{NaB}^3\text{H}_4$ ) is aberrant (Parker, C. J., unpublished observation). Abnormalities in membrane glycoproteins may be involved in the high incidence of thrombotic complications in PNH (57).

The membrane abnormality in PNH not only modulates the greater fixation of C3b, but also results in the cells being more efficiently lysed by the C5b-9 membrane attack complex (1-4). Abnormalities in glycosylation of glycophorin- $\alpha$  might also effect the enhanced lysis of PNH cells by a mechanism analogous to that which apparently accounts for the greater susceptibility of certain serum-sensitive strains of bacteria to complement-mediated lysis (in this case, differences in cell-surface glycosylation regulate the depth to which the membrane attack complex can penetrate, thereby determining whether or not an effective lesion will be produced) (58, 59). Of interest in this regard are the erythrocytes from En(a-) individuals. These erythrocytes have a total deficiency of glycophorin- $\alpha$ , yet there are no apparent clinical sequelae (27, 60). The lack of associated symptomatology may be accounted for by a compensatory increase in glycosylation of other membrane glycoproteins (in particular band 3) (29). In vitro, these cells are reported to show a markedly increased susceptibility to lysis by anti-I (as do HEMPAS and PNH erythrocytes) (14). It would be of great interest to define the molecular basis of the enhanced lysis of En(a-) erythrocytes.

The abnormal erythrocytes of PNH are deficient in activity of the superficial membrane glycoprotein, acetylcholinesterase; however, this deficiency apparently plays no role in the pathological manifestations of the disease (57). Whether the molecule is present but inactive or whether it is physically absent has never been clearly determined. Aberrations in glycosylation (of the enzyme itself or of microenvironmental cofactors) might effect this lack of activity by influencing the enzyme kinetics or by rendering the molecule unstable. It seems likely that other membrane surface glycoproteins may be structurally or functionally abnormal or deficient on PNH erythrocytes; however, assigning a role in the pathophysiology of the disease to minor membrane glycoproteins must be done with caution. Recently, Pangburn et al. have reported that PNH erythrocytes lack factor H-like functions (61). They postulated that a deficiency in membrane regulatory glycoproteins could account for their findings as their study suggested that PNH erythrocytes are deficient in one such regulatory glycoprotein, the C3b receptor ( $\text{CR}_1$ ) (62-65). Using a monoclonal antibody to  $\text{CR}_1$ , we have observed that PNH erythrocytes have a low, but quite variable number of  $\text{CR}_1$  sites, and that the receptor number does not vary inversely with the percentage of abnormal cells, but rather appears to

correlate with disease activity (the lowest numbers of receptor sites are seen in patients who are actively hemolyzing). Further, we have observed low numbers of  $\text{CR}_1$  sites on the erythrocytes of patients with autoimmune hemolytic anemias in which C3b is deposited on the surface (patients with chronic cold agglutinin disease who have large amounts of C3dg on their cells have extremely low numbers of  $\text{CR}_1$  sites) (Parker, C. J., C. M. Soldato, and M. J. Telen, manuscript submitted for publication). Thus the low number of  $\text{CR}_1$  sites on PNH erythrocytes may be an epiphenomenon related to the activation and fixation of C3 to the membrane surface. Further, the greater fixation of C3b to PNH erythrocytes when complement is activated is observed in whole serum systems (1-4) in which the endogenous control protein, factor H, is present in high concentrations. This raises questions concerning the pathophysiological importance of a deficiency of a minor membrane glycoprotein(s) [including decay accelerating factor (66-68)] whose role is analogous that of factor H. Although the characteristics of binding of factor H to C3b on PNH erythrocytes are aberrant, it appears unlikely that this contributes to the enhanced fixation of C3b to PNH cells when whole serum complement is activated (22). In addition, using purified complement components, we have demonstrated that, under conditions in which the alternative pathway convertase is equally stable on PNH and normal erythrocyte, PNH EC3bBbC3NeF activate and bind four to five times more C3 than their normal counterparts (5).

The biochemical composition of the surface upon which the complexes of the complement sequence are organized greatly influences the activity of the constituent components of the complexes (58, 59, 69-75). The studies reported here suggest that abnormalities in glycosylation of membrane glycoproteins may underlie the aberrant interactions of complement with the hematopoietic elements (erythrocytes, granulocytes, platelets) of PNH. Further investigations into the carbohydrate composition of these abnormal cells are needed to define the defect at the molecular level. Such studies should provide insight into the biochemical events that modulate the interactions of complement with cell membranes.

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