

Isolation and Characterization of a Membrane Protein from Normal Human Erythrocytes That Inhibits Reactive Lysis of the Erythrocytes of Paroxysmal Nocturnal Hemoglobinuria

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Abstract

The observation that type III erythrocytes of paroxysmal nocturnal hemoglobinuria (PNH) are susceptible to hemolysis initiated by activated cobra venom factor complexes (CoFBb), whereas normal erythrocytes are resistant, implies that the PNH III cells are deficient in a membrane constituent that regulates this process. To isolate the inhibitory factor from normal erythrocytes, membrane proteins were first extracted with butanol and then subjected to sequential anion exchange, hydroxylapatite, and hydrophobic chromatography. Analysis by SDS-PAGE and silver stain of the inhibitory fractions showed a single band corresponding to a protein with an apparent M_r of 18 kD. PNH erythrocytes were incubated with incremental concentrations of the radiolabeled protein and then washed. In a dose-dependent fashion, the protein incorporated into the cell membrane and inhibited CoFBb-initiated lysis. This protein inhibitor functioned by restricting the assembly of the membrane attack complex at the level of C7 and C8 incorporation. By using a monospecific antibody to block the function of the inhibitor, it was shown that normal erythrocytes are rendered susceptible to CoFBb-initiated hemolysis. Analysis by Western blot of membrane proteins revealed that PNH III erythrocytes are deficient in the 18-kD protein. By virtue of its molecular weight and inhibitory activity, the 18-kD protein appears to be discrete from other previously described erythrocyte membrane proteins that regulate complement. These studies also indicate that the susceptibility of PNH III erythrocytes to reactive lysis is causally related to a deficiency of the 18-kD membrane inhibitor.

Introduction

Cobra venom factor (CoF)¹ is a functional analogue of human C3b, but structurally resembles human C3c (1). The phenomenon whereby CoF induces cytolysis is called either reactive or

bystander lysis. In this instance, complement is not activated directly on the cell surface; rather, activated complement complexes that are generated in the fluid-phase bind to a nearby cell and thereby initiate formation of the cytolytic membrane attack complex (MAC) on the innocent bystander cell. After incubation with normal human serum and activated CoF complexes (CoFBb, an alternative pathway C3/C5 convertase), the most complement-sensitive type of paroxysmal nocturnal hemoglobinuria (PNH) erythrocytes (PNH III) are hemolyzed, but PNH I (cells that are nearly normal in their susceptibility to complement), PNH II (cells that are of intermediate sensitivity), and normal erythrocytes are not (2, 3). This observation implies that normal (and PNH I and PNH II) erythrocytes have a membrane constituent that inhibits CoFBb-initiated hemolysis and that this factor is missing from PNH III erythrocytes (4).

PNH III erythrocytes have been shown to be deficient in decay-accelerating factor (DAF) (5, 6). DAF is a single-chain glycoprotein with an M_r of ~ 70 kD that both inhibits formation and induces destabilization of the C3 convertase of the classical and alternative pathways (7). In some cases, PNH II erythrocytes also appear to be missing DAF entirely (8), although, in other instances, the deficiency may be partial (5) or qualitative (9). Even the PNH I cells have, in most instances, an amount of DAF that is less than that of normal erythrocytes (8). The greater sensitivity of PNH cells to complement appears, at least in part, to be causally related to the deficiency of DAF because reconstitution of the abnormal cells with DAF ameliorates their greater susceptibility to lysis (10). In addition, when normal erythrocytes are treated with antibodies against DAF, their complement susceptibility is enhanced (8). DAF, however, has no inhibitory activity in the process of CoF-initiated hemolysis (8).

Homologous species restriction of complement-mediated lysis is a term used to describe the observation that C5b-9 (the MAC) is much less efficient in inducing hemolysis of homologous erythrocytes than in lysing heterologous cells (11). Two groups have isolated a membrane constituent from human erythrocytes that has a molecular mass of ~ 65 kD and that inhibits the functional activity of the MAC (12, 13). This glycoprotein, called homologous restriction factor (HRF) (13) or C8-binding protein (C8 bp) (12), appears to interact with both C8 and C9 and to inhibit binding and polymerization of C9. HRF/C8 bp was not detected in PNH III erythrocytes (14, 15), and this deficiency appears to contribute to the greater sensitivity of these cells to reactive lysis.

Inasmuch as CoF-initiated hemolysis is a form of reactive lysis, it would seem logical to assume that the greater susceptibility of PNH III erythrocytes to lysis induced by CoFBb is due to a deficiency of HRF/C8 bp. Previous studies, however, have demonstrated that after incubation with serum and CoFBb,

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1. Abbreviations used in this paper: CoF, cobra venom factor; DAF, decay-accelerating factor; GVB, veronal-buffered saline containing 0.1% gelatin; MAC, membrane attack complex; MIRL, membrane inhibitor of reactive lysis; PNH, paroxysmal nocturnal hemoglobinuria; VBS, veronal-buffered saline.

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PNH III E bind relatively large amounts of C5b67, whereas normal and PNH II E bind virtually none (2). Because HRF/C8 bp has been reported to have no effect on C7 binding (13), we postulated that a membrane protein other than HRF/C8 bp must be responsible for regulation of CoF-initiated hemolysis. The details of the isolation and characterization of this protein that has been termed membrane inhibitor of reactive lysis (MIRL) are described herein.

Methods

Erythrocytes. Whole blood from volunteer donors and the patient with PNH were obtained by venopuncture and stored in Alsever's solution at 4°C. The patient with PNH had ~ 88% type III and 12% type I cells (2). For isolation of MIRL, packed normal human erythrocytes were purchased from Latter Day Saints Hospital, Intermountain Hospital Corporation blood services (Salt Lake City, UT). Whole blood from guinea pigs was obtained by cardiac puncture and stored in Alsever's solution at 4°C.

Buffers and reagents. The following buffers were used: veronal-buffered saline, pH 7.5 (VBS), containing 5 mM sodium barbital (Sigma Chemical Co., St. Louis, MO) and 150 mM NaCl; VBS containing 0.1% gelatin (GVB); GVB containing 15 mM of the sodium salt of EDTA (GVB-EDTA) and 5 mM sodium phosphate, pH 8.0, containing 1 mM PMSF (Sigma Chemical Co.) and 1 mM EDTA (ghosting buffer). Serum-EDTA was prepared by making 10 mM serum with EDTA by using 0.1 M EDTA, pH 7.4 and incubating the mixture for 5 min at 37°C. (The sample was kept at 4°C before use.)

Complement components. C7 (2), C8 (2) CoF from *Naja naja naja* (Miami Serpenterium, Salt Lake City, UT) (16), factor B (17), and factor D (17) were isolated using previously described methods. Activated CoF complexes (CoFBb) were prepared by incubating 100 μ l of CoF (200 μ g/ml) with 100 μ l of factor B (1 mg/ml), 50 μ l of factor D, and 50 μ l of GVB containing 5 mM MgCl₂ at 37°C. After 30 min, excess Mg²⁺ was chelated by adding 40 μ l of 0.1 M EDTA, pH 7.4, and continuing the incubation for 5 min (18). (The complexes were either used immediately or stored at -70°C.)

Complement-deficient sera. Human serum was immunochemically depleted of C7 as previously described (2). C8-depleted serum was purchased from Cytotech, Inc. (San Diego, CA).

Anti-MIRL antiserum. An antibody to MIRL was generated by injecting a New Zealand White rabbit subcutaneously with CFA containing 5 μ g of purified MIRL. The procedure was repeated twice at biweekly intervals but Freund's incomplete adjuvant was used to prepare the emulsion. 2 wk after the third injection, whole blood was obtained by venopuncture and the serum was isolated subsequently. Nonimmune serum was obtained from New Zealand White rabbits before immunization.

Determination of protein concentration. Protein concentrations for CoF (16), factor B (16), C7 (2), and C8 (2) were determined spectrophotometrically. Other protein concentrations were determined with the bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL) using BSA as the standard.

Radiolabeling. C7 and C8 were radiolabeled with ¹²⁵I as Nal (Amersham Corp., Arlington Heights, IL) by using Enzymobeads (Bio-Rad Laboratories, Richmond, CA) (2). MIRL was radiolabeled with ¹²⁵I by using IODO-GEN (Pierce Chemical Co., Rockford, IL) (19). No loss of functional activity of the proteins was observed after radiolabeling.

SDS-PAGE, autoradiography, and Western blotting. The methods for analysis of proteins by SDS-PAGE under reducing conditions have been published (20). In the experiments reported herein, stacking gels of 3% and resolving gradient gels of from 6 to 15% were used. To analyze samples under nonreducing conditions, the 2-mercaptoethanol was omitted from the sample buffer (20). Molecular weights were estimated (21) by reference to standards purchased from Bio-Rad Laboratories. Gels were fixed with methanol/acetic acid or ethanol/acetic

acid and stained with Coomassie blue (21) or Gelcode silver stain (Pierce Chemical Co.), respectively. Gels for autoradiography were dried and exposed to X-OMAT AR film (Eastman Kodak Co., Rochester NY) at -70°C. Analysis by Western blot was performed by using previously described methods (22), but the secondary antibody was conjugated with alkaline phosphatase (23) instead of horseradish peroxidase.

Preparation of butanol extracts derived from normal and PNH erythrocytes. To extract membrane proteins, normal and PNH III erythrocytes were washed three times in PBS. After each wash, ~ 10% of the cell pellet was aspirated to remove the buffy coat. Hemoglobin-free erythrocyte ghosts were prepared by resuspending 1 ml of packed cells in 40 ml of ghosting buffer. After vigorous agitation with a Pasteur pipette, the samples were centrifuged at 48,000 g for 10 min at 4°C. The supernate was aspirated, the cells were resuspended in ghosting buffer, and the suspension was centrifuged using the same conditions as described above. The preceding step was repeated until the ghosts showed no visual evidence of residual hemoglobin (three washes were usually required). After the last wash, the ghosts were resuspended to 2.5 ml in ghosting buffer. Next, 3 M NaCl was added to the samples so that the final concentration was 150 mM (24, 25). An aliquot of the samples was reserved for determination of total protein, and *n*-butanol (20% vol/vol) was added to the remainder of the two suspensions (24, 25). The samples were stirred for 15 min at room temperature and then centrifuged at 4°C for 10 min at 48,000 g. The butanol saturated aqueous phase was recovered and dialyzed extensively against VBS. The protein concentration of the butanol extracts from the PNH and normal cells was determined, and the sample with the highest concentration was diluted with VBS so that the concentration of the two samples was equivalent.

In preliminary experiments, the amount of normal human serum (the complement source) that was required to mediate partial lysis of guinea pig erythrocytes was determined. Guinea pig cells were washed three times in GVB-EDTA and resuspended to 5 \times 10⁹/ml. Incremental dilutions of normal human serum-EDTA were prepared using GVB-EDTA as the diluent. A 50- μ l aliquot of guinea pig erythrocytes was incubated at 37°C with a 50- μ l aliquot of one of the serum dilution, 50 μ l of CoFBb complexes (that had been diluted 1:25 with GVB-EDTA), and 50 μ l of GVB-EDTA. The cell blank (CB) that controlled for spontaneous hemolysis contained 50 μ l of erythrocytes and 150 μ l of GVB-EDTA. The reagent blank that controlled for spontaneous hemolysis in the presence of serum (CBC) contained erythrocytes and the appropriate serum dilution, but GVB-EDTA was substituted for the CoFBb complexes. Hemolysis of 100% of the cells was achieved by substituting deionized water for the other reagents. After 30 min, 4 ml of cold GVB-EDTA were added, and the reaction mixtures were centrifuged at 1,000 g for 3 min. Hemolysis was subsequently quantified by measuring free hemoglobin (aliquots of 200 μ l of the supernate were pipetted into 96-well microtitre plates and the A₄₁₀ of the samples was measured with microplate reader (model MR 700; Dynatech Laboratories, Inc., Alexandria, VA). The following formula was used to calculate the percent hemolysis: (A₄₁₀ of sample - A₄₁₀ of CBC)/(A₄₁₀ of 100% - A₄₁₀ of CB) \times 100. The amount of serum that mediated hemolysis of ~ 75% of the cells (usually in the range of 1:125 to 1:250) was used in the subsequent experiments.

Determination of the inhibitory activity of the butanol extracts by two assays. In the first assay, seven twofold dilutions of the extracts were prepared using GVB-EDTA as the diluent. A 250- μ l aliquot of the dilution was added to 50 μ l of guinea pig erythrocytes, and the reaction mixtures were incubated at 37°C. For controls in these experiments, GVB-EDTA was substituted for the dilutions of the extracts. After 30 min, the samples were washed once in GVB-EDTA and resuspended to 100 μ l. Next, 50 μ l of CoFBb and 50 μ l of the appropriate serum dilution were added. Reagent blanks that controlled for spontaneous lysis and the 100% lysis samples were prepared as described above. After an incubation period of 30 min at 37°C, 4 ml of GVB-EDTA was added, the suspensions were centrifuged, and the A₄₁₀ of the supernate was measured. The maximum lysis was defined as hemolysis that

occurred when GVB-EDTA was substituted for the dilutions of the butanol extracts.

The percent inhibition of hemolysis was calculated based on the following formula: $1 - (A_{410} \text{ of sample} - A_{410} \text{ of CBC}) / (A_{410} \text{ maximum lysis} - A_{410} \text{ of CBC}) \times 100$.

In the second assay, a 100- μ l aliquot of the dilution of the butanol extract was incubated at 37°C with 50 μ l of guinea pig erythrocytes, 50 μ l CoFBb, and 50 μ l of the appropriate serum dilution. After 30 min, 4 ml of GVB-EDTA was added, the samples were centrifuged, and the A_{410} of the supernate was measured. Inhibition of hemolysis was calculated as described above.

Isolation of MIRL. 4 U of packed human erythrocytes was extensively washed in PBS and resuspended to 1,000 ml. Next, 40 liters of ghosting buffer was added and the suspension was stirred overnight at 4°C. Using an ultrafiltration device (Pellicon; Millipore Corp., Bedford, MA) equipped with PTHK00001 membranes, the sample volume was reduced to 4 liters, and the erythrocyte membranes were washed free of hemoglobin (using ghosting buffer as the wash buffer). Next, the ghosts were concentrated by centrifugation at 48,000 g for 30 min at 4°C in a centrifuge (model J2-21) equipped with a rotor (model JA-20) (Beckman Instruments Inc., Fullerton, CA). The supernate was aspirated, and the ghosts were resuspended to 225 ml (5.9 mg/ml of protein).

A 20% suspension of ghost was prepared by adding 900 ml of 5 mM phosphate, pH 7.5. The conductivity of the solution was adjusted to 13.25 mS at RT by the addition of 3 M NaCl (24, 25). Next, *n*-butanol was added (20% vol/vol) and the mixture was stirred at room temperature for 20 min (24, 25). After centrifugation at 10,000 g for 25 min, the butanol saturated aqueous phase (1,280 ml containing 106 μ g/ml of protein) was recovered and dialyzed extensively against 20 mM sodium phosphate, 20 mM NaCl, pH 7.5. The sample was applied at a flow rate of 2 ml/min to a Mono Q HR 16/10 column (Pharmacia Fine Chemicals, Piscataway, NJ) that had been equilibrated with 20 mM sodium phosphate, pH 7.5, containing 20 mM NaCl and 0.1% NP-40 (26). The column was washed with 120 ml of the starting buffer and eluted with a linear salt gradient up to 500 mM NaCl (26).

Two assays were used to determine the inhibitory activity of the fractions obtained by the anion exchange chromatography of the aqueous phase of the butanol extract. Aliquots of the chromatographic fractions were diluted 1:5–1:20 with GVB-EDTA. In the first assay (the simultaneous assay), 50 μ l of the sample dilution were incubated at 37°C with 50 μ l of guinea pig erythrocytes, 50 μ l of CoFBb, and 50 μ l of the appropriate dilution of serum (the amount of serum that mediated lysis of ~ 75% of the cells). After 30 min, 4 ml of GVB-EDTA were added and hemolysis was quantified as described above. In the second assay (the wash assay), 50 μ l of guinea pig erythrocytes were incubated at 37°C with 50 μ l of the dilutions from the chromatographic fractions. After 30 min, the cells were washed once with GVB-EDTA, and resuspended to 100 μ l. Next 50 μ l of CoFBb and 50 μ l of the serum dilution were added, and the reaction mixture was incubated at 37°C. After 30 min, hemolysis was determined as described above. For both assays, the maximum hemolysis was the value obtained when an equal volume of VBS was substituted for the chromatographic fraction. The percent inhibition of hemolysis was calculated as described above.

A portion of the fractions that showed inhibitory activity in the wash assay were pooled and dialyzed against 20 mM Tris-HCl (26). Next, the sample was applied to a 2.5 \times 10 cm column (Bio-Gel HT; Bio-Rad Laboratories, Richmond, CA) that had been equilibrated with 5 mM sodium phosphate, pH 8.5, 40 mM NaCl, 0.1% NP-40 (26). The column was washed with 100 ml of starting buffer followed by 100 ml of 20 mM sodium phosphate, pH 7.5 containing 40 mM NaCl, 0.1% NP-40, pH 7.5 (26). 5-ml fractions were collected at a flow rate of 1 ml/min. When the column fractions were screened using the wash assay, inhibitory activity appeared in both the wash and the elution buffer eluates.

The active fractions were pooled and dialyzed against 40 mM sodium phosphate, pH 7.4 containing 300 mM NaCl (7). The sample was

applied at a flow rate of 1 ml/min to a phenyl Superose HR 5/5 column (Pharmacia Fine Chemicals) that had been equilibrated with 40 mM sodium phosphate, pH 7.4 containing 300 mM NaCl and 0.1% NP-40 (7). The column was washed with 10 ml of starting buffer and a linear gradient, formed with 10 ml of starting buffer and 10 ml of 40 mM phosphate, pH 9.5 containing 50 mM NaCl and 1.0% NP-40 (7), was applied. The fractions containing the inhibitory activity were pooled and dialyzed against VBS.

Inhibition of CoFBb-initiated hemolysis of PNH erythrocytes by isolated MIRL. Normal and PNH erythrocytes were washed three times in GVB-EDTA and resuspended to 5×10^8 /ml in GVB-EDTA. A 650- μ l aliquot of PNH erythrocytes was incubated at 37°C with either 650 μ l of VBS or 650 μ l of VBS containing 5 μ g/ml of MIRL. At the same time, 650 μ l of normal erythrocytes were incubated with 650 μ l of VBS. After 30 min, the cells were washed once with GVB-EDTA and resuspended to 650 μ l. Next, seven twofold falling dilutions of serum-EDTA were prepared using GVB-EDTA as the diluent. A 50- μ l aliquot of each of the serum dilutions was incubated at 37°C with 50 μ l of cells and 50 μ l of CoFBb (diluted 1:25). After 30 min, 3 ml of GVB-EDTA were added to each tube, the cells were centrifuged at 1,000 g for 2 min, and hemolysis was calculated based on the A_{410} of the supernates.

Inhibition of CoFBb-initiated hemolysis of PNH erythrocytes by radiolabeled MIRL. PNH cells were washed three times with GVB-EDTA and resuspended to 5×10^8 /ml. Seven twofold falling dilutions of 125 I-MIRL (the most concentrated sample contained 5 μ g/ml of protein and the specific activity was 1.59×10^7 cpm/ μ g) were prepared by using GVB-EDTA as the diluent. An 100- μ l aliquot of each dilution was incubated at 37°C with 100 μ l of PNH erythrocytes. After 30 min, the cells were washed once with GVB-EDTA and resuspended to 100 μ l. Next, 50 μ l of cells were layered onto 200 μ l of a combination of phthalate esters (19) in 400- μ l polyethylene microfuge tubes (Analytical Laboratories, Rockville Centre, NY). The bound and unbound 125 I-MIRL were separated by centrifuging the tubes at 11,500 g for 2 min in a microfuge (model 12; Beckman Instruments, Inc.). The tips of the tubes were cut off, and the radioactivity of the cell pellet was quantified in a gamma counter. The number of molecules of MIRL bound/cell was calculated based on the specific activity of the protein and an M_r of 18 kD.

Next, 50 μ l of CoFBb (1:25 dilution) and 50 μ l of an appropriate dilution of serum-EDTA (the amount that had been determined in preliminary experiments to mediate hemolysis of ~ 90% of the PNH III erythrocytes) were incubated at 37°C with the remainder of the cells that had been previously incubated with radiolabeled MIRL. After 30 min, 3 ml of GVB-EDTA was added, the suspensions were centrifuged, and the A_{410} of the supernates was measured. The percent inhibition of lysis was calculated using the formula described above.

Incorporation of radiolabeled MIRL into PNH erythrocytes. PNH erythrocytes were washed three times in GVB and resuspended to 5×10^8 /ml in GVB. A 2-ml aliquot was incubated with 1.5 μ g of 125 I-MIRL at 37°C. After 30 min, the cells were washed three times in GVB, hemoglobin-free ghosts were prepared as described above, and the radioactivity of the ghosts was quantified subsequently. Next, the membrane bound 125 I-MIRL was analyzed under both reducing and nonreducing conditions by SDS-PAGE and autoradiography.

Effect of varying cell concentrations on binding of radiolabeled C7 to PNH and normal erythrocytes after incubation with CoFBb and serum. PNH and normal erythrocytes were washed three times in GVB-EDTA, and aliquots were resuspended to either 2.5×10^9 /ml, 1×10^9 /ml, or 5×10^8 /ml. A 50- μ l aliquot of cells was incubated at 37°C with 50 μ l of CoFBb and 50 μ l of C7-deficient human serum that had been repleted with 50 μ g/ml 125 I-C7 (4.9×10^5 cpm/ μ g). To controls for nonspecific binding of radiolabeled C7, 50 μ l of GVB-EDTA was substituted for the CoFBb. After 30 min, 850 μ l of ghosting buffer were added, and the cells were centrifuged in the microfuge at 12,000 rpm for 5 min at room temperature (2, 4). The supernate was aspirated and the membranes were resuspended to 100 μ l in ghosting buffer. Next, a 90- μ l aliquot was removed from each tube and layered over 200 μ l of

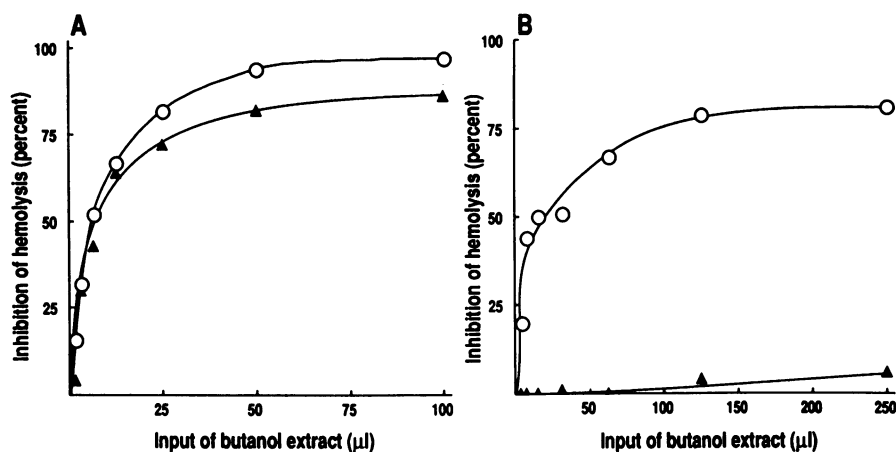


Figure 1. Inhibition of CoFBb-initiated hemolysis of guinea pig erythrocytes by butanol extracts derived from normal and PNH erythrocytes. (A) Guinea pig erythrocytes were incubated simultaneously with the following reagents: (a) CoFBb; (b) an aliquot of human serum that would mediate hemolysis of ~ 75% of the cells; (c) incremental dilutions of butanol extracts derived from normal (○) and PNH (▲) erythrocytes. The protein concentration of the butanol extracts was 86 µg/ml. Inhibition of hemolysis was calculated based on the formula described in Methods. The results are representative of two experiments. The capacity of the two extracts to inhibit reactive lysis of guinea pig erythrocytes was

similar. (B) The same reagents were used, but the guinea pig erythrocytes were first incubated with the dilutions of the butanol extracts from normal (○) or PNH (▲) erythrocytes and then washed. Next, the CoFBb complexes and the serum dilution were added and hemolysis was quantified. The butanol extract from normal, but not PNH erythrocytes, contains a factor that binds to guinea pig cells and inhibits CoFBb-initiated lysis.

20% sucrose in 400-µl microfuge tubes. To separate bound from unbound radiolabeled C7, the tubes were centrifuged for 15 min at 12,000 rpm (2, 4). The tips of the microfuge tubes were cut off just above the membranes pellet and counted in a gamma counter. The number of molecules of ^{125}I -C7 bound per cell was calculated based on an M_r of 92,400 (2). Nonspecific binding was subtracted to define specific binding.

Effect of MIRL on binding of radiolabeled C7 and C8 to PNH erythrocytes after incubation with CoFBb and serum. Normal and PNH erythrocytes were washed three times in GVB-EDTA and aliquots were resuspended to either $2.5 \times 10^9/\text{ml}$ or $1 \times 10^9/\text{ml}$ in GVB-EDTA. Next, 650 µl of each cell suspension were incubated at 37°C with either 650 µl of VBS or 650 µl of VBS containing 5 µg/ml of MIRL. After 30 min, the cells were washed with GVB-EDTA and resuspended to 650 µl. An aliquot of 50 µl from each reaction mixture was incubated at 37°C with 50 µl of CoFBb and 50 µl of C7 or C8 depleted serum that had been replenished with 50 µl/ml of ^{125}I -C7 (1.58×10^6 cpm/µg) or ^{125}I -C8 (1.99×10^6 cpm/µg) respectively. Controls for nonspecific binding of the radiolabeled proteins were prepared as described above. After 30 min, 850 µl of ghosting buffer were added and the tubes were centrifuged in the microfuge at 12,000 rpm for 5 min at RT. The remainder of the experiment was performed as described above for binding of ^{125}I -C7. Calculation of binding of C8 was based on an M_r of 150 kD (2).

CoFBb-initiated hemolysis of normal erythrocytes treated with anti-MIRL. Normal erythrocytes were washed three times in GVB-EDTA and resuspended to $5 \times 10^8/\text{ml}$. An aliquot of 50 µl of cells was incubated at 37°C with 50 µl of GVB-EDTA containing incremental concentrations of anti-MIRL antiserum or nonimmune rabbit serum. After 30 min, the cells were washed with GVB-EDTA and resuspended to 50 µl. Next, 50 µl of CoFBb (diluted 1:5 with GVB-EDTA) and 100 µl of EDTA-serum were added to the cell suspensions. Controls were prepared as described above and the reaction mixtures were incubated at 37°C. After 30 min, 3 ml of GVB-EDTA was added, the samples were centrifuged and hemolysis was calculated after measuring the A_{410} of the supernates.

Results

Inhibition of CoFBb-initiated hemolysis by butanol extracts derived from normal and PNH erythrocytes. To determine if normal (but not PNH) erythrocytes have a constituent that inhibits complement-mediated hemolysis initiated by CoFBb, membrane proteins were first extracted with butanol. After

recovery of the butanol-saturated aqueous phase, the samples were dialyzed, and the protein concentration was determined subsequently. A 1-ml aliquot of packed PNH erythrocytes yielded 3.52 mg of total membrane protein, whereas 3.63 mg of protein was obtained from the same volume of normal erythrocytes. The butanol-saturated aqueous phase from the PNH ghosts contained 245 µg of protein (7.4% of the initial total membrane protein), and 259 µg of protein (6.7% of the initial membrane protein) was present in the corresponding extract from normal erythrocytes.

After the protein concentration of the PNH extract was adjusted so that it was equal to that of the extract from the normal erythrocytes, the capacity of the two samples to inhibit CoFBb-initiated hemolysis of guinea pig erythrocytes was compared. When the single-step simultaneous assay was used, the inhibitory activity of the two samples was similar (Fig. 1 A). When the two-step wash assay was used, however, a marked difference in inhibitory activity was observed (Fig. 1 B). These results led to two hypotheses. The first possibility was that a single inhibitor of CoFBb-initiated lysis existed, and this inhibitor was associated with both PNH and normal erythrocytes. According to this hypothesis, the inhibitor would function in the fluid-phase, but would also have the capability of binding to cells and inhibiting reactive lysis. The inhibitor associated with PNH cells would be qualitatively abnormal because either it could not bind to cells, or else if it could bind, its functional activity was not expressed. The second possibility was that two inhibitors existed. According to this hypothesis, one inhibitor (that is present in the butanol extract derived from both PNH and normal cells) either does not bind to the membrane or else if it does bind, its functional activity is not expressed. The other inhibitor (that is associated only with normal cells) binds to the membrane and remains functional.

Isolation of MIRL. To test the two hypotheses stated above, the aqueous phase derived from butanol extraction of normal erythrocyte membrane proteins was subjected to anion exchange chromatography. When the one-step assay (simultaneous incubation of the reagents) was used to test the column fractions, two major areas of inhibitory activity were observed (Fig. 2 A). The first peak of inhibition eluted along with a protein peak shortly after the wash buffer was applied. The

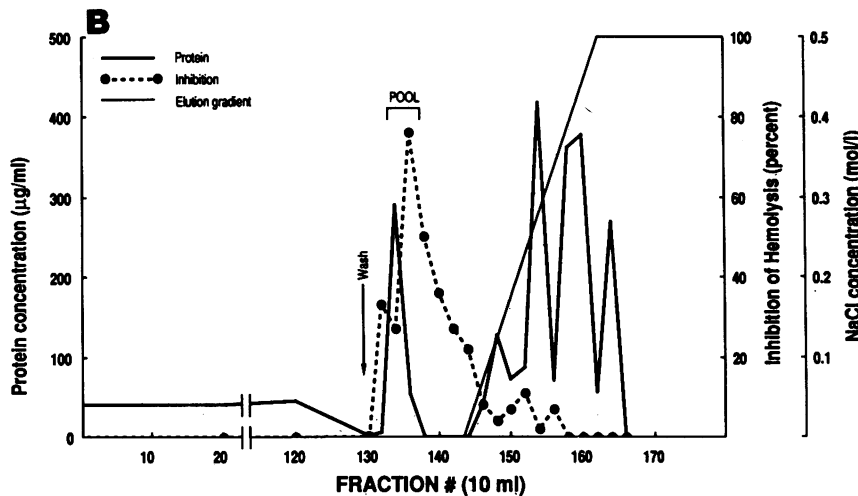
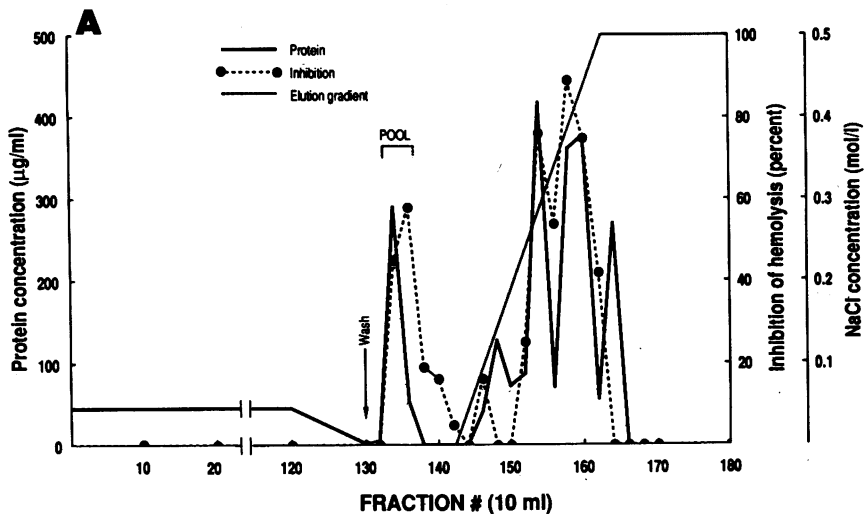


Figure 2. Anion exchange chromatography of the butanol-saturated aqueous phase of normal erythrocyte ghosts on a Mono Q column. Aliquots of the fractions were assayed for their capacity to mediate inhibition of CoFBb-initiated lysis of guinea pig erythrocytes. Two assays were used. (A) In the first assay, aliquots of the column fractions were incubated simultaneously with guinea pig erythrocytes, CoFBb and a dilution of normal human serum (the complement source). Two major peaks of inhibitory activity were observed. (B) In the second assay, guinea pig erythrocytes were incubated with aliquots of the column fractions and then washed. Next, CoFBb and the serum dilution were added and the incubation was continued. One major peak of inhibitory activity was observed when the two-step wash assay was used.

maximum inhibitory activity, however, was not coincident with the highest protein concentration. A second major area of inhibitory activity was detected after the gradient was applied in association with the first two of three major protein peaks eluted by the increasing salt concentration.

When the two-step wash assay was used to test the column fractions, inhibitory activity was present within a single major peak (Fig. 2 B). These results are consistent with the second of the two hypothesis stated above, that normal erythrocytes have two constituents that can inhibit CoFBb-initiated hemolysis. Inasmuch as PNH III erythrocytes appear to be deficient in the inhibitory factor that is detected by the two-step wash assay (Fig. 1), column fractions 133–137 were pooled in preparation for further isolation of the membrane inhibitor of reactive lysis.

Approximately 10% (136 mg) of the starting membrane protein was present in the butanol-saturated aqueous phase that was applied to the Mono Q column. The pool consisting of fractions 133–137 contained 3.35 mg of protein and was further chromatographed on hydroxylapatite (Fig. 3). The active fractions (21–51) from hydroxylapatite, containing 2.4 mg of protein, were dialyzed and subjected to hydrophobic chromatography. A single peak of inhibitory activity was eluted by the gradient (Fig. 4). When fractions 70–72 eluted from the phenyl superose column were pooled and analyzed by SDS-

PAGE followed by silver staining, a band corresponding to a protein with an apparent M_r of 18 kD was observed (Fig. 5). After dialysis against VBS, the inhibitory pool contained 166

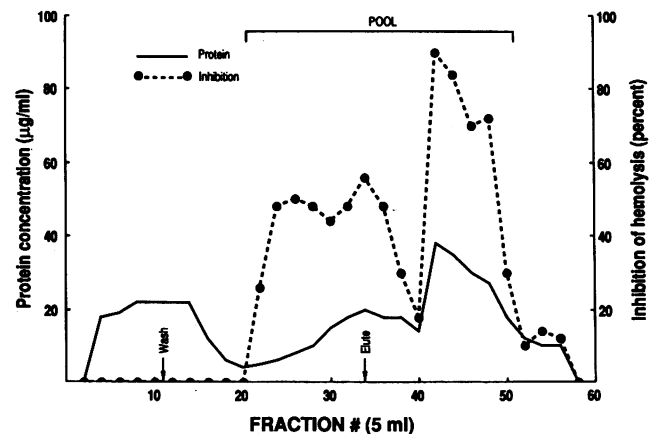


Figure 3. Hydroxylapatite chromatography of fractions 133–137 from Mono Q. After dialysis, the sample was applied, and the column was exposed sequentially to equilibration (wash) and elution buffers. The two-step wash assay was used to screen the column fractions. A peak of inhibitory activity was observed after the wash buffer was applied and again after the elution buffer was applied.

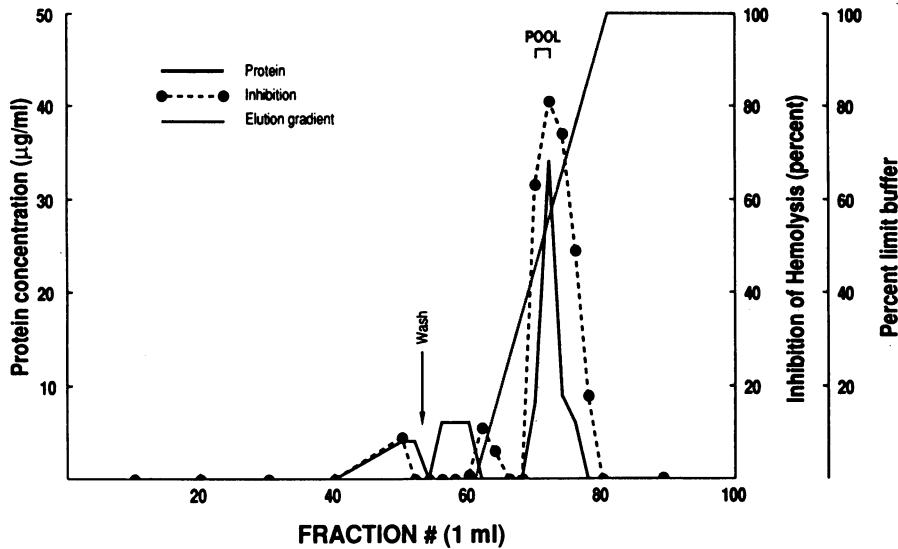


Figure 4. Chromatography on phenyl Superose of fractions 21-51 from hydroxylapatite. The two-step wash assay was used to screen the column fractions. The inhibitory activity eluted in the middle portion of the gradient along with the majority of the protein.

µg of protein. The results of the isolation procedure described above are representative of two MIRL purifications.

Effects of MIRL on CoFBb-initiated hemolysis of PNH III erythrocytes. PNH erythrocytes were incubated with buffer or with buffer containing a fixed amount of MIRL. At the same time, normal erythrocytes that had been incubated with buffer were also evaluated for susceptibility to reactive lysis. After washing, the cells were incubated with CoFBb and incremental concentrations of normal human serum as the complement source. Throughout the concentration range of serum used in these experiments, MIRL protected PNH III erythrocytes from hemolysis (Fig. 6).

After radiolabeling, the capacity of ^{125}I -MIRL to inhibit reactive lysis of PNH erythrocytes was compared with that of an equal concentration of the unlabeled MIRL. No difference in inhibitory activity was observed (data not shown). Next, incremental concentrations of ^{125}I -MIRL were incubated with PNH erythrocytes, and the amount of MIRL that remained bound to the cells was quantified. Under the conditions used in these experiments, MIRL binding to PNH erythrocytes did not demonstrate characteristics of saturation binding because the binding isotherm was linear throughout the dose-response range (Fig. 7 A).

The specific activity of ^{125}I -MIRL was tested by incubating PNH erythrocytes with incremental concentrations of the radiolabeled protein. After washing, a portion of the cells were incubated with CoFBb and an amount of serum that mediated lysis of ~ 90% of the affected cells (Fig. 7 B). A second aliquot of the cells was used to determine the amount of ^{125}I -MIRL that was bound (Fig. 7 C). An initial input of ~ 30 ng of MIRL resulted in binding of ~ 400 molecules/cell and produced 50% inhibition of hemolysis. Complete inhibition of hemolysis was observed when > 1,000 molecules/cell were bound and when the initial input of MIRL was > 60 ng.

Analysis of radiolabeled MIRL that binds to PNH erythrocytes. After incubation of radiolabeled MIRL with PNH erythrocytes, the cells were washed and hemoglobin-free ghosts were generated. Approximately 7% of the ^{125}I -MIRL that was

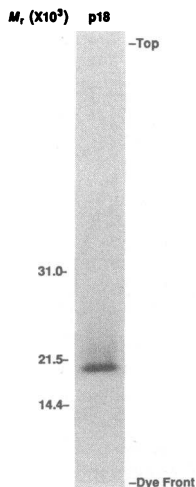


Figure 5. Analysis by SDS-PAGE (non-reducing conditions) and silver stain of isolated MIRL (500 ng). (Left) The positions of the molecular weight standards; (right) the top of the gel and the dye front. A single band with an electrophoretic mobility consistent with an M_r of 18 kD was observed. When the sample was electrophoresed under reducing conditions, the estimated molecular weight of MIRL did not change appreciably.

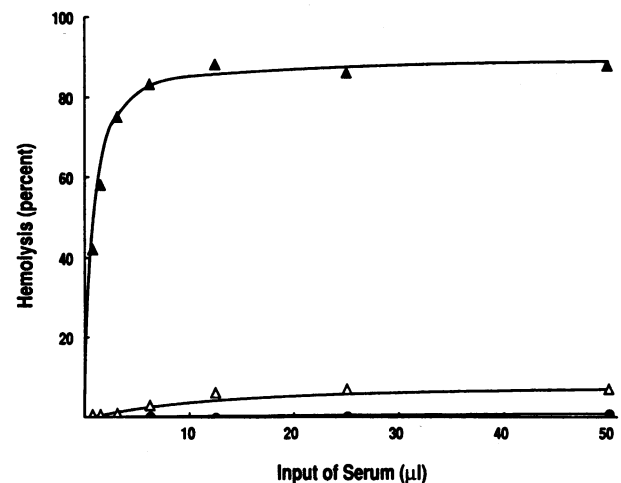


Figure 6. Effects of MIRL on CoFBb-initiated hemolysis of PNH III erythrocytes. Normal erythrocytes were incubated with buffer (○) and PNH erythrocytes were incubated with buffer (△) or with buffer containing MIRL (5 µg/ml) (▽). After washing, the cells were incubated with CoFBb and incremental concentrations of human serum. The data points represent the mean of three determinations. Pre-treatment with MIRL protected PNH III erythrocytes against reactive lysis.

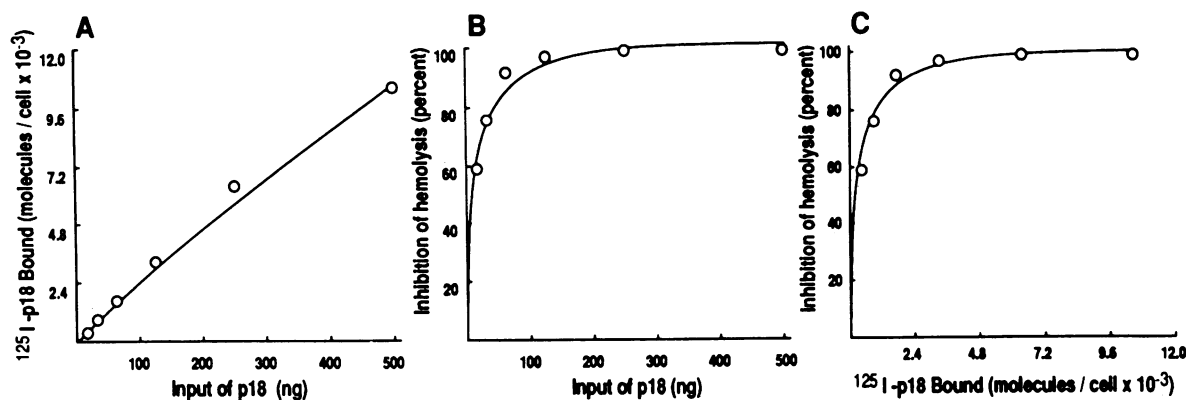


Figure 7. Treatment of PNH III erythrocytes with ¹²⁵I-MIRL. MIRL was radiolabeled without loss of functional activity. (A) PNH erythrocytes were incubated with incremental concentration of ¹²⁵I-MIRL and after washing, binding was quantified. The data points represent the mean of triplicate determinations. The binding of radiolabeled MIRL was linear throughout the concentration range used in these experiments. (B) Aliquots of PNH III erythrocytes were incubated with incremental concentrations of ¹²⁵I-MIRL and then washed with GVB-EDTA. Next, the cells were incubated with CoFBb and a dilution of human serum that mediated hemolysis of ~90% of the abnormal cells. The percent inhibition of hemolysis was calculated based on a comparison of hemolysis of MIRL-treated cells with that of buffer-treated controls. The input of MIRL (shown on the abscissa) was the amount of protein present in the reaction mixture before the wash step. The values represent the mean of triplicate determinations. MIRL inhibited hemolysis of PNH III erythrocytes in a dose-dependent fashion. (C) Inhibition of hemolysis was compared with the number of molecules of MIRL present on the cells after the wash step. The values represent the mean of triplicate determinations. Approximately 400 molecules/cell caused 50% inhibition of lysis, and ~1,000 molecules/cell completely inhibited lysis.

originally added to the cell suspension remained bound. The erythrocyte proteins were solubilized and analyzed by SDS-PAGE and autoradiography (Fig. 8). There was no apparent difference in the molecular size of MIRL under reducing (Fig.

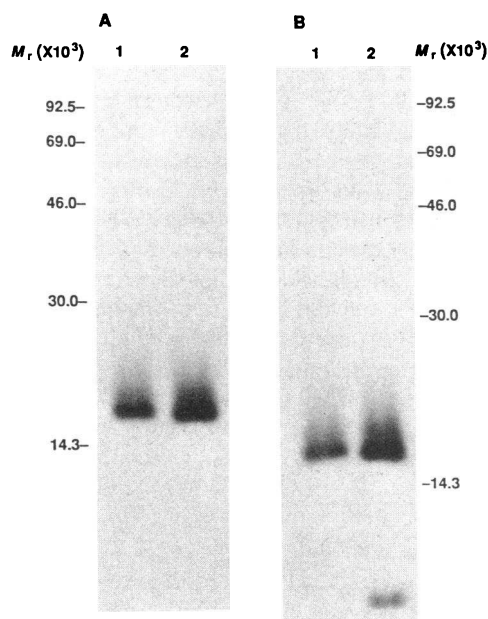


Figure 8. Analysis by SDS-PAGE and autoradiography of ¹²⁵I-MIRL that bound to PNH III erythrocytes. Radiolabeled MIRL was incubated with PNH erythrocytes. The cells were washed and erythrocyte ghosts were prepared subsequently. The membrane proteins were analyzed under reducing (A) and nonreducing (B) conditions. In A and B, lane 1 contains isolated radiolabeled MIRL, whereas lane 2 contains the membrane proteins. The positions of the molecular weight markers are indicated beside the autoradiographs. There was no apparent difference in the molecular weight of MIRL under reducing and nonreducing conditions. Under nonreducing conditions, a band was observed just above the dye front.

8 A) and nonreducing (Fig. 8 B) conditions. An additional band that migrated to a position just above the dye front was seen in the track containing the membrane proteins (but not in the track containing the isolated MIRL) when the samples were electrophoresed under reducing conditions. It is conceivable that the low-molecular weight protein is a degradation product of MIRL that is generated by an erythrocyte protease, and that the activity of the enzyme is partially inhibited under reducing conditions.

Inhibition of formation of the membrane attack complex by MIRL. Previous studies have shown that after incubation with CoFBb and serum, PNH erythrocytes bind much greater amounts of C7 than normal erythrocytes (2). Those results lead to the hypothesis that MIRL might protect PNH erythrocytes from CoFBb-initiated hemolysis by inhibiting the assembly of the trimolecular C5b-7 complex on the cell surface. In preparation for testing the above stated hypothesis, the binding of C7 to PNH and normal erythrocytes was compared using three different concentrations of cells (Table I). When the initial concentration of cells was 2.5×10^9 /ml, no specific binding of C7 was observed to the normal erythrocytes. Those results were consistent with previous observations (2, 4); however, when the concentration of cells was either 1.0×10^9 /ml or 0.5×10^9 /ml specific binding of ¹²⁵I-C7 to normal and PNH erythrocytes was nearly equivalent (Table I). Inasmuch as normal erythrocytes (and MIRL-treated PNH erythrocytes) are not susceptible to CoFBb-initiated hemolysis when the initial cell concentration is 0.5×10^9 /ml (Fig. 6), mechanisms other than inhibition of membrane-associated formation of the C5b-7 complex must account for the resistance.

As was the case with normal erythrocytes, when the initial concentration of cells was 2.5×10^9 /ml, no specific binding of C7 to MIRL-treated PNH erythrocytes was observed; and when the initial cell concentration was 1×10^9 /ml, MIRL-treated PNH cells bound C7 as efficiently as the buffer-treated PNH erythrocytes (Fig. 9). No specific binding of C8 was observed, however, when the initial concentration of the MIRL-

Table I. Binding of ^{125}I -C7 to Varying Concentrations of PNH and Normal Erythrocytes after Incubation with CoFBb and Serum*

Cell concentration [§]	^{125}I -C7 Bound [‡]	
	PNH	Normal
2.5	1,420	0
1.0	4,980	5,260
0.5	15,950	12,890

* C7-deficient serum was repleted with 50 $\mu\text{g}/\text{ml}$ of ^{125}I -C7.

[‡] The specific binding (molecules/cell) is presented as the mean of duplicate experiments. No specific binding of ^{125}I -C7 was observed to the normal erythrocytes when the initial concentration of cell was $2.5 \times 10^9/\text{ml}$.

[§] The initial concentration of erythrocytes ($\times 10^{-9}$).

treated cells was $1 \times 10^9/\text{ml}$. These data suggest that MIRL protects erythrocytes against CoFBb-initiated hemolysis by inhibiting formation of the membrane attack complex. Under some circumstances, MIRL can block formation of the trimolecular C5b-7 complex, but under other conditions, the inhibitor appears to prevent binding of C8 to membrane-associated C5b-7.

For the buffer-treated PNH erythrocytes, the amount of C7 that was bound was significantly greater than the amount of C8. These results are consistent with previous observations that the ratio of C7:C8 binding is > 1 (2), and suggest that even on PNH III erythrocytes, not all of the C5b-7 complexes are equally available for C8 binding.

Inhibition of MIRL function by anti-MIRL. To determine if MIRL protects normal erythrocytes from reactive lysis initiated by CoFBb, cells were treated with anti-MIRL antiserum or with nonimmune rabbit serum. After washing, CoFBb and EDTA-serum was added and hemolysis was subsequently quantified. In a dose-dependent fashion, anti-MIRL induced susceptibility to reactive lysis, whereas nonimmune serum had

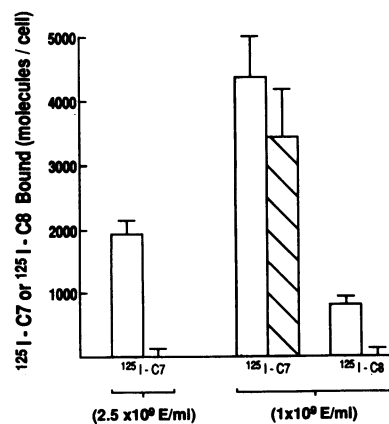


Figure 9. Effects of MIRL on binding of C7 and C8 to PNH erythrocytes after incubation with CoFBb and serum. PNH erythrocytes were washed and resuspended to either $2.5 \times 10^9/\text{ml}$ or $1 \times 10^9/\text{ml}$. Next, aliquots of cells were incubated with buffer alone (open bars) or with buffer containing MIRL (hatched bars). After washing, the cells were incubated with CoFBb

and C7- or C8-depleted serum that had been repleted with radiolabeled C7 or C8, respectively. To control for nonspecific binding of the radiolabeled proteins, GVB-EDTA was substituted for CoFBb. The values depicted by the bars represent the mean plus SD ($n = 5$). Depending upon the concentration of cells used, MIRL inhibits binding of both C7 and C8 to PNH erythrocytes.

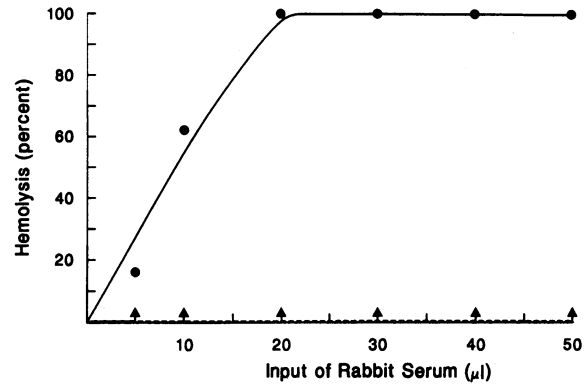


Figure 10. Normal erythrocytes are rendered susceptible to CoFBb-initiated hemolysis after treatment with anti-MIRL antiserum. Normal erythrocytes were incubated with incremental concentrations of anti-MIRL antiserum (\bullet) or nonimmune serum (\blacktriangle). After washing, the cells were incubated with CoFBb complexes and EDTA-serum and hemolysis was quantified subsequently. The data points represent the mean of triplicate determinations. Inhibition of MIRL function induces susceptibility to CoFBb-initiated hemolysis.

no effect (Fig. 10). Inasmuch as all buffers contained EDTA, hemolysis could not be mediated by antibody activation of the classical pathway. These results demonstrate that MIRL is the cellular constituent that controls susceptibility to CoFBb-initiated hemolysis.

PNH erythrocytes are deficient in MIRL. Previous observations (4) and the results of experiments described above were consistent with the concept that PNH III erythrocytes are deficient in MIRL. This hypothesis was tested by analyzing membrane proteins from normal and PNH erythrocytes by Western blot using anti-MIRL antiserum as the source of the primary antibody (Fig. 11). A band representing a protein with an apparent M_r of 18 kD was observed in the lanes containing the normal erythrocyte membrane proteins but not in the lane containing the PNH proteins. When the proteins were electrophoresed under reducing conditions, however, no staining of MIRL was observed in any of the lanes (not shown). Further, when isolated MIRL was analyzed by the same methods, antibody binding was observed under nonreducing conditions but not under reducing conditions (not shown). These results suggests that the epitope recognized by the antibody is lost upon treatment with the reducing agent. The functional and the structural integrity of MIRL may be dependent on intrachain

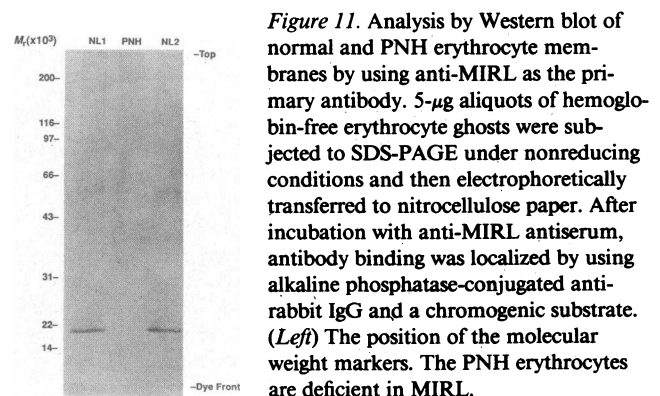


Figure 11. Analysis by Western blot of normal and PNH erythrocyte membranes by using anti-MIRL as the primary antibody. 5- μg aliquots of hemoglobin-free erythrocyte ghosts were subjected to SDS-PAGE under nonreducing conditions and then electrophoretically transferred to nitrocellulose paper. After incubation with anti-MIRL antiserum, antibody binding was localized by using alkaline phosphatase-conjugated anti-rabbit IgG and a chromogenic substrate. (Left) The position of the molecular weight markers. The PNH erythrocytes are deficient in MIRL.

disulfide bonds because normal erythrocytes that are treated with the sulfhydryl reagent 2-aminoethylisothiuronium bromide manifest a susceptibility to CoFBb-initiated lysis that is identical to that of PNH erythrocytes (2).

Discussion

The purpose of our studies was to isolate and characterize the membrane constituent that restricts the susceptibility of normal human erythrocytes to complement-mediated hemolysis initiated by CoFBb. The existence of this inhibitor was implied from previous data that showed that PNH III erythrocytes are hemolyzed after incubation with CoFBb and human serum, whereas normal erythrocytes are not (2, 4).

The capacity of membrane proteins extracted from normal and PNH erythrocytes to inhibit CoFBb-initiated hemolysis was similar in the assay in which reagents were incubated simultaneously (Fig. 1 A). These results are reminiscent of those reported by Chua et al. (27). In their studies (in which the reagents were also incubated simultaneously) the capacity of membrane proteins derived from normal and PNH erythrocytes to inhibit antibody-initiated hemolysis of sheep erythrocytes (using guinea pig serum as the complement source) was observed to be similar. The inhibitory effects of the extracts were not examined in a two-step wash assay, however, and the methods used by Chua and colleagues to solubilize the normal and PNH erythrocyte membranes appears to have been different from those used in the present investigations.

When the butanol extract was subjected to anion exchange chromatography, two major areas of inhibitory activity were observed by using the one-step simultaneous assay to analyze the eluted fractions (Fig. 2 A). The inhibitory activity that was eluted by the gradient was associated with two of the three major protein peaks that were also eluted by the gradient. When fractions from the three protein peaks were analyzed by SDS-PAGE followed by silver staining or by Western blot using anti-glycophorin-A as the primary antibody, the first two peaks (but not the third peak) were found to be rich in glycophorin-A (DAF eluted just before the first of the three peaks. Those three protein peaks contained no DAF activity, and the fractions that had DAF activity had no effect on CoFBb-initiated lysis). Further isolation and characterization of the second inhibitory factor has not been undertaken, although others have reported that purified glycophorin-A can inhibit reactive lysis (28). It is possible that glycophorin-A accounts for the inhibitory activity observed in the experiments whose results are depicted in Fig. 1 A. Whether glycophorin-A functions *in situ* as a regulator of the membrane attack complex remains to be determined.

Two lines of evidence indicate that MIRL and HRF/C8 bp are discrete proteins. First, MIRL has an M_r (Fig. 5) that is different from HRF/C8 bp (12, 13); and second, anti-MIRL does not recognize proteins whose molecular weights are consistent with HRF/C8 bp or its primary degradation product (13) (Fig. 11). Whether HRF/C8 bp is present in the butanol extract from which MIRL is isolated is not known because assays (functional or immunochemical) specifically designed to detect the presence of HRF/C8 bp were not performed.

Previous studies have shown that purified DAF has no effect on the susceptibility of PNH III erythrocytes to CoFBb-initiated hemolysis (8), but the effect of HRF/C8 bp on CoFBb-initiated lysis has not been reported. By blocking

MIRL function with specific antibody, normal erythrocytes are rendered susceptible to CoFBb-initiated lysis (Fig. 10). These results suggest that HRF/C8 bp exerts little, if any, control over reactive lysis initiated by CoFBb.

The process of reactive lysis initiated by CoFBb in which whole serum is used as the complement source is not entirely analogous to reactive lysis mediated by isolated components of the MAC; because in the former, but not in the latter system, PNH III erythrocytes bind much more C5b-7 than normal erythrocytes (2, 4). Preliminary experiments from our laboratory have shown that MIRL inhibits reactive lysis of PNH erythrocytes when purified components are used to generate the trimolecular C5b-7 complex on cells. Inhibition was similar whether MIRL was added simultaneously with the isolated C5b6 and C7 or sequentially, after the PNH EC5b-7 had been generated and the cells had been washed. Inhibition by MIRL, however, was found to be significantly greater in experiments in which normal human serum was used as the source of C8 and C9 as compared with experiments in which purified C8 and C9 were used to induce hemolysis. This observation is consistent with previous studies that have suggested that serum and membrane constituents act in concert to restrict the activity of the MAC (4).

Recently, Sugita et al. (29) have reported the isolation from human erythrocytes of an 18-kD membrane protein that inhibits reactive lysis of guinea pig EC5b-7. Based on its functional activity and molecular weight, that protein appears to be the same as MIRL.

We have also observed that MIRL reduces the susceptibility of PNH III erythrocytes to acidified serum (hemolysis mediated by the alternative pathway) and to hemolysis mediated by the classical pathway (using whole serum as the complement source). In studies using purified components, however, MIRL, appears to have no effect on the functional activity of the C3 convertase of the alternative pathway (Holguin, M. H., and C. J. Parker, unpublished observations). Together, these observations suggest that MIRL controls complement-mediated hemolysis by restricting formation of the MAC rather than by regulating the formation and stability of the C3/C5 convertases.

Depending upon the experimental conditions used, MIRL can inhibit the assembly of the MAC by blocking binding of C7 or C8 (Fig. 9). Experiments designed to determine if MIRL affects C9 binding, however, have not been performed. The C7 binding site on the C5b6 complex is not known, but the C5b component of the trimolecular C5b-7 complex appears to be the binding site for C8 (30). If C7 binds to C5b in close proximity to C8 then MIRL could inhibit formation of both C5b-7 and C5b-8 complexes by blocking the binding sites on C5b for C7 and C8, respectively. Further studies aimed at defining more precisely the molecular interactions of MIRL with components of the MAC will be undertaken.

Previous studies have shown that PNH III erythrocytes are missing both DAF (5, 6) and HRF/C8 bp (14, 15). These studies present evidence that PNH III erythrocytes are deficient in an additional complement regulatory protein, the MIRL (Fig. 11). Unlike PNH III erythrocytes, PNH II cells are not susceptible to CoFBb-initiated hemolysis (2, 3). This observation argues that the intermediately sensitive PNH II cells have an amount of MIRL that is sufficient to inhibit reactive lysis. Inasmuch as the number of MIRL molecules/cell required to inhibit CoFBb-initiated hemolysis is relatively low

(Fig. 7 C), it is conceivable that PNH II cells could have a partial deficiency of MIRL but still be resistant to reactive lysis. The relationship between the complement regulatory proteins (MIRL, DAF, and HRF/C8 bp) and the phenotypes of PNH (I, II, and III) needs to be defined more clearly.

Incorporation of MIRL into erythrocytes (Figs. 7 and 8) appears to be analogous to that of DAF (31) and HRF/C8 bp (15). Whether the mechanism by which MIRL associates with erythrocytes is similar to that of DAF and HRF/C8 bp (both appear to be anchored to the cell membrane by a glycosylphosphatidylinositol moiety [32–34]) remains to be determined. The fact that other membrane proteins of this class have been shown to be missing from PNH cells (35–39) would suggest that the molecular basis of the PNH defect may involve abnormalities in the biogenesis of glycosylphosphatidylinositols.

The complement system is activated in response to injury and infection. As a consequence, cellular constituents of the immune response are at risk for complement-mediated injury because activated components are generated in the fluid phase at the site of inflammation. Conceivably, MIRL has evolved as a mechanism for protecting cells against nonspecific damage by complement. Accordingly, the presence of MIRL on hematopoietic elements other than erythrocytes should be determined (40–45).

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