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### Research Article

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# Relationship between the Membrane Inhibitor of Reactive Lysis and the Erythrocyte Phenotypes of Paroxysmal Nocturnal Hemoglobinuria

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## Abstract

Susceptibility to hemolysis initiated by activated cobra venom factor (CoF) complexes is a characteristic that distinguishes the most complement-sensitive type III erythrocytes of paroxysmal nocturnal hemoglobinuria (PNH) from the intermediately sensitive type II and the normally sensitive type I cells. Recently we isolated a membrane constituent from normal erythrocytes that inhibits CoFBb-initiated hemolysis, and this protein was designated membrane inhibitor of reactive lysis (MIRL). To investigate the molecular basis of the variability in complement sensitivity among PNH erythrocytes, the surface expression of MIRL and decay accelerating factor (DAF) on the three phenotypes of PNH was quantified immunochemically. Both complement regulatory proteins were markedly deficient on the erythrocytes from a patient with predominately type III cells. The erythrocytes from patients with a majority of either type II or I cells were also significantly deficient in both MIRL and DAF. While cytofluorometric analysis confirmed the quantitative deficiencies, segregation of erythrocytes into discrete subpopulations that expressed either no MIRL or normal amounts of MIRL was not observed. The results of immunoprecipitation studies were consistent with quantitative, but not qualitative abnormalities of MIRL and DAF. Selective removal of the sensitive erythrocytes indicated that ~20% of the normal amount of MIRL is sufficient to protect cells from CoF-initiated lysis. These studies suggest that relatively subtle quantitative differences in membrane complement regulatory proteins underlie the variability in complement sensitivity of PNH erythrocytes.

## Introduction

Paroxysmal nocturnal hemoglobinuria (PNH)<sup>1</sup> is an acquired hemolytic anemia in which the erythrocytes are abnormally

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1. *Abbreviations used in this paper:* AET, 2-(aminoethyl)isothiuronium bromide; CB, cell blank; CBC, cell blank controlled for spontaneous hemolysis in the presence of serum; CLS, complement lysis sensitivity; CoF, cobra venom factor; CoFBb, activated CoF complexes in which activated factor B (Bb) is bound to CoF forming a stable C3/C5 convertase; C8bp, C8 binding protein; DAF, decay accelerating factor; GVB, Veronal-buffered saline containing 0.1% gelatin; HRF, homologous restriction factor; MAC, membrane attack complex; MIRL, membrane inhibitor of reactive lysis; NHS, normal

susceptible to complement-mediated hemolysis (1). The syndrome appears to arise as a result of a somatic mutation involving the pluripotential hematopoietic stem cell, since the leukocytes and platelets of PNH are also abnormal. A precise *in vitro* assay designed to quantify the complement sensitivity of the erythrocytes showed that the cells can be segregated into discrete subpopulations that differ in susceptibility to hemolysis mediated by the classical pathway. The following phenotypic classification of PNH erythrocytes is based on this assay: PNH I, normal or nearly normal in sensitivity; PNH II, intermediately sensitive; and PNH III, markedly sensitive.

Compelling evidence indicates that the greater hemolytic susceptibility of PNH erythrocytes is due to deficiencies of membrane constituents that regulate the functional activity of complement. Decay accelerating factor (DAF) is a 70-kD glycoprotein that limits the formation and stability of the C3 convertases of the classical and alternative pathways (2, 3). PNH III erythrocytes are completely deficient in DAF (4–6). In some cases PNH II erythrocytes appear to be missing DAF entirely, while in others the deficiency may be either partial (4) or qualitative (7). Quantitatively abnormal DAF expression has also been reported for PNH I erythrocytes (6). PNH III erythrocytes are also deficient in C8 binding protein (C8bp) homologous restriction factor (HRF; 8, 9), a 65-kD protein that inhibits the cytolytic activity of the membrane attack complex (MAC; 10, 11). Analysis of expression of HRF/C8bp by PNH I and PNH II erythrocytes has not been reported.

Susceptibility to complement-mediated hemolysis initiated by cobra venom factor (CoF) also distinguishes PNH III erythrocytes from PNH I and II cells (12). In this process (sometimes called reactive or bystander lysis), complement is activated by an alternative pathway C3/C5 convertase (CoFBb) that is formed when factor B that has bound to CoF (a functional analogue of human C3b; 13) is enzymatically activated by factor D. PNH III erythrocytes are readily lysed when incubated with CoFBb and serum (the complement source) because the cells fail to restrict formation of the cytolytic MAC of complement (12). In contrast, PNH I, PNH II, and normal erythrocytes are resistant to this process because assembly of the MAC is inhibited. We have recently isolated an 18-kD protein from normal erythrocytes (designated membrane inhibitor of reactive lysis [MIRL]) that protects PNH III erythrocytes from CoFBb-initiated hemolysis (14). By blocking the function of MIRL with a monospecific antibody, normal erythrocytes were made susceptible to reactive lysis, and immunoblotting experiments showed that PNH III erythrocytes were deficient in MIRL. Those studies indicated that the susceptibility of PNH III erythrocytes to CoFBb-initiated hemo-

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human serum; PNH, paroxysmal nocturnal hemoglobinuria; RIP, radioimmunoprecipitation; TBS, Tris-buffered saline; VBS, Veronal-buffered saline.

lysis is causally related to a deficiency of MIRL. Based on their resistance to reactive lysis, we hypothesized that MIRL is present on PNH I and II erythrocytes. Accordingly, studies aimed at defining the relationship between MIRL and the erythrocyte phenotypes of PNH have been undertaken.

## Methods

**Erythrocytes.** Whole blood from volunteer donors and patients with PNH was obtained by venopuncture and stored in Alsever's solution at 4°C. Normal erythrocytes were treated with 2-(aminoethyl)isothiuronium bromide (AET; 15) or trypsin (16) using previously described methods.

**Buffers.** The following buffers were used: Veronal-buffered saline (VBS) containing 5 mM sodium barbital (Sigma Chemical Co., St. Louis, MO), pH 7.5, and 145 mM NaCl (VBS); VBS containing 0.1% gelatin (GVB); GVB containing 15 mM sodium salt of EDTA (GVB-EDTA); 5 mM sodium phosphate, pH 8.0, containing 1 mM PMSF (Sigma Chemical Co.) and 1 mM EDTA (5,8 P-PE); 10 mM sodium phosphate, pH 7.4, containing 145 mM NaCl (PBS); 10 mM Tris-HCl (Sigma Chemical Co.), pH 7.4, containing 145 mM NaCl, (TBS); TBS containing 0.1% gelatin, 1.0% NP-40 (Sigma Chemical Co.), and 1 mM PMSF (radioimmunoprecipitation [RIP] buffer). Normal human serum (NHS) chelated with EDTA was prepared by mixing EDTA (10 mM final concentration) and incubating the mixture for 5 min at 37°C. The NHS-EDTA sample was kept at 4°C before use.

**Activated CoF complexes.** CoF from *Naja naja naja* (Miami Serpenterium, Salt Lake City, UT; 17), factor B (18), and factor D (18) were isolated according to previously described methods. Activated CoF complexes (CoFBb) were prepared by incubating 100  $\mu$ l of CoF (200  $\mu$ g/ml) with 100  $\mu$ l of factor B (1 mg/ml), 50  $\mu$ l of factor D, and 50  $\mu$ l of GVB containing 5 mM MgCl<sub>2</sub> at 37°C. After 30 min excess Mg<sup>2+</sup> was chelated by adding 40  $\mu$ l of 0.1 M EDTA, pH 7.4, and continuing the incubation for 5 min. The complexes were stored at -70°C and diluted 1:5 with GVB-EDTA before use.

**Antibodies.** Antiserum to MIRL was prepared by injecting a New Zealand white rabbit subcutaneously with Freund's complete adjuvant containing 5  $\mu$ g of purified MIRL. Two subsequent injections with 5  $\mu$ g of MIRL in Freund's incomplete adjuvant were given at 2-wk intervals. 2 wk after the last injection whole blood was obtained by venopuncture and the serum was isolated. Nonimmune serum was obtained from New Zealand white rabbits before immunization. Rabbit anti-DAF antiserum was prepared as previously described (19). The F(ab')<sub>2</sub> fragment of affinity-purified donkey anti-rabbit IgG that had been labeled with <sup>125</sup>I was purchased from Amersham Corp. (Arlington Heights, IL). Affinity-purified goat anti-rabbit IgG conjugated with FITC was purchased from Sigma Chemical Co.

**Determination of protein concentration.** The concentrations of CoF and factor B were determined spectrophotometrically (17). All other protein concentrations were determined with the BCA protein assay (Pierce Chemical Co., Rockford, IL) using BSA as the standard.

**Radiolabeling.** Normal and PNH erythrocytes were surface-labeled with <sup>125</sup>I as NaI (Amersham Corp.) using IODO-GEN (Pierce Chemical Co.; 16).

**SDS-PAGE and autoradiography.** Analysis of proteins by SDS-PAGE (16) was performed using linear gradient gels of 6–15%. Molecular weights were estimated (20) by reference to standards purchased from Bio-Rad Laboratories (Richmond, CA). Autoradiographs (16) were prepared by exposing dried gels to X-Omat AR film (Eastman Kodak Co., Rochester, NY) at -70°C.

**Complement lysis sensitivity (CLS) assay.** PNH erythrocytes were characterized according to their sensitivity to antibody-initiated complement-mediated hemolysis as described by Rosse and Dacie (21, 22). The effects of inhibition of MIRL function on the complement sensitivity of normal erythrocytes was assessed by treating cells with anti-MIRL antiserum. Erythrocytes were washed in GVB-EDTA and resuspended to 5  $\times$  10<sup>8</sup>/ml. Aliquots of the cells were incubated at 37°C

with GVB-EDTA containing either a saturating concentration of anti-MIRL antiserum or an equal volume of nonimmune rabbit serum. After 30 min the cells were washed and used in the CLS assay.

**Susceptibility of normal and PNH erythrocytes to complement-mediated hemolysis initiated by CoFBb.** Normal and PNH erythrocytes were washed three times in GVB-EDTA and resuspended to 5  $\times$  10<sup>8</sup>/ml. Seven twofold serial dilutions of NHS-EDTA were prepared using GVB-EDTA as the diluent. 100- $\mu$ l aliquots of the serum dilutions were incubated at 37°C with 50  $\mu$ l of cells and 50  $\mu$ l of CoFBb. To control for spontaneous hemolysis, a cell blank (CB) was prepared by combining 50  $\mu$ l of cells and 150  $\mu$ 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 0.04% ammonium hydroxide for the other reagents. After 30 min 3 ml of GVB-EDTA was added and the samples were centrifuged at 1,000 *g* for 3 min. Hemolysis was quantified by measuring free hemoglobin in the supernates (aliquots of 200  $\mu$ l were pipetted into the wells of microtiter plates and the A<sub>410</sub> of the samples was measured with a microplate reader ([MR 700; Dynatech Laboratories, Inc., Alexandria VA]). The following formula was used to calculate the percent hemolysis:

$$\frac{A_{410} \text{ of sample} - A_{410} \text{ of CBC}}{A_{410} \text{ of 100\%} - A_{410} \text{ of CB}} \times 100.$$

**Comparison of the sensitivity of PNH III and anti-MIRL-treated normal erythrocytes to CoFBb-initiated hemolysis.** Normal and PNH erythrocytes (from patient R.O.) were washed in GVB-EDTA and resuspended to 5  $\times$  10<sup>8</sup>/ml. A 1-ml aliquot of normal erythrocytes was incubated at 37°C with 1 ml of GVB-EDTA containing a saturating concentration of anti-MIRL antiserum. After 30 min the cells were washed in GVB-EDTA and resuspended to 1 ml. Next, 50- $\mu$ l aliquots of PNH or anti-MIRL-treated cells were incubated at 37°C with 50  $\mu$ l of CoFBb and 100  $\mu$ l of GVB-EDTA containing incremental concentrations of NHS-EDTA. Controls (the CB, CBC, and 100% lysis) were prepared as described above. After 30 min 3 ml of GVB-EDTA was added to each sample and hemolysis quantified as described above.

**Quantification of the amount of anti-MIRL required to induce sensitivity of erythrocytes to CoFBb-initiated hemolysis.** Normal and PNH erythrocytes were washed in GVB-EDTA and resuspended to 5  $\times$  10<sup>8</sup>/ml. 200- $\mu$ l aliquots of cells were incubated at 37°C with 200  $\mu$ l of GVB-EDTA containing incremental concentrations of anti-MIRL antiserum. As controls, cells were also incubated with GVB-EDTA alone and with GVB-EDTA containing nonimmune rabbit serum. After 30 min the cells were washed with GVB-EDTA and resuspended to 200  $\mu$ l. 50- $\mu$ l aliquots of cells were incubated at 37°C with 100  $\mu$ l of NHS-EDTA and 50  $\mu$ l of CoFBb. After 30 min hemolysis was quantified as described above.

**Binding of anti-MIRL and anti-DAF to normal and PNH erythrocytes.** Normal and PNH erythrocytes were washed three times in GVB-EDTA and resuspended to 5  $\times$  10<sup>8</sup>/ml. 50- $\mu$ l aliquots of cells were incubated at 37°C with 50  $\mu$ l of GVB-EDTA that contained a saturating concentration of anti-MIRL or anti-DAF antiserum. Saturating concentrations were determined in preliminary experiments by generating binding isotherms for the two antibodies. To control for nonspecific binding, an equal amount of nonimmune rabbit serum was substituted for the specific antisera. After 30 min the samples were washed with GVB-EDTA and resuspended to 100  $\mu$ l. An aliquot of 100  $\mu$ l of GVB-EDTA that contained a saturating amount of <sup>125</sup>I-donkey anti-rabbit IgG was added to each sample, and the reaction mixtures were incubated for 30 min at 37°C. Next, two 75- $\mu$ l aliquots from each sample were pipetted onto a 200- $\mu$ l layer of a combination of phthalate esters in 400- $\mu$ l polyethylene microfuge tubes. After centrifugation at 8,000 *g* in a microfuge (model 12; Beckman Instruments Inc., Fullerton, CA), the tips of the microfuge tubes containing the cells were cut off, and the radioactivity of the pellet was quantified in a gamma counter.

Quantitation of binding of anti-MIRL to AET- and trypsin-treated erythrocytes was performed using the same methods.

**Binding of anti-DAF and anti-MIRL to PNH erythrocytes after CoFbB-initiated hemolysis.** Normal and PNH erythrocytes were washed in GVB-EDTA and resuspended to  $5 \times 10^8$ /ml. A 1-ml aliquot of the PNH cells was incubated at 37°C with 1 ml of CoFbB, 500  $\mu$ l of NHS-EDTA, and 1.5 ml of GVB-EDTA. After 30 min the unlysed cells were washed in GVB-EDTA and resuspended to  $6 \times 10^7$ /ml. The control cells (normal and PNH erythrocytes that had not been exposed to CoFbB and serum) were also diluted to  $6 \times 10^7$ /ml. Next, binding of anti-MIRL and anti-DAF was quantified as described above.

**RIP of normal and PNH erythrocyte membrane proteins with anti-MIRL and anti-DAF.** Normal and PNH erythrocytes that had been surface-labeled with  $^{125}$ I were washed and resuspended to  $5 \times 10^8$ /ml in PBS. 2-ml aliquots were centrifuged at 1,000 g for 3 min and the supernate was aspirated. Hemoglobin-free erythrocyte ghosts were prepared by washing the cells in hypotonic buffer (5,8 P-PE; 16). The radiolabeled ghosts were resuspended to 1 ml in 5,8 P-PE, and a 250- $\mu$ l aliquot was pipetted into a separate microfuge tube and kept at 4°C before analysis by SDS-PAGE and autoradiography. A 750- $\mu$ l aliquot of RIP buffer was added to the remaining ghosts, the suspension was incubated for 10 min at 37°C and 20 min on ice, and then centrifuged for 5 min at 12,000 g. Next, the supernate was recovered and incubated overnight at 4°C with 500  $\mu$ l RIP buffer containing 10% (vol/vol) protein A-Sepharose CL-4B (Sigma Chemical Co.). The suspensions were centrifuged at 12,000 g for 2 min, the supernate was recovered, and the volume was adjusted to 2 ml with RIP buffer. Next, 500- $\mu$ l aliquots were pipetted into 1.5-ml microfuge tubes and 10  $\mu$ l of anti-MIRL, anti-DAF, or nonimmune rabbit serum were added. After incubation for 30 min at 37°C, 100  $\mu$ l of RIP buffer containing 10% protein A-Sepharose was added, and the samples were incubated at room temperature. After 30 min the samples were centrifuged at 12,000 g for 2 min and the supernate was aspirated. The protein A-Sepharose beads were washed three times with RIP buffer. After the final wash the supernate was aspirated as completely as possible without disturbing the pellet, 50  $\mu$ l of reduced sample buffer (16) was added, and the tubes were placed in a boiling water bath for 5 min. Next, the samples were centrifuged for 5 min at 12,000 g and the supernates were recovered and subjected to analysis by SDS-PAGE and autoradiography.

**Cytofluorographic analysis of MIRL and DAF on normal and PNH erythrocytes.** Normal and PNH erythrocytes were washed three times in GVB-EDTA and resuspended to  $2 \times 10^7$ /ml. 50- $\mu$ l aliquots of cells were incubated at 37°C with 50  $\mu$ l of GVB-EDTA containing a saturating concentration of anti-MIRL, anti-DAF, or an equivalent amount of nonimmune rabbit serum. After 30 min the cells were washed in GVB-EDTA and resuspended to 50  $\mu$ l. Next, 50  $\mu$ l of a 1:40 dilution of FITC-conjugated goat anti-rabbit IgG was added and the reaction mixtures were incubated at 37°C. After 30 min the cells were washed in GVB-EDTA and resuspended to 1 ml. The samples were analyzed with a cytofluorograph II (Ortho Diagnostic Systems, Inc., Raritan, NJ). A minimum of 5,000 cells were counted.

## Results

**Characterization of the complement sensitivity of PNH erythrocytes.** Two methods were used to test the susceptibility of the erythrocytes from the three patients with PNH to complement-mediated hemolysis (Table I). The standard method for classifying PNH erythrocytes is the CLS. In this assay erythrocytes are sensitized with an optimal amount of anti-IgM (isolated from a patient with chronic cold agglutinin disease), and dilutions of NHS serve as the complement source. The relative sensitivity of erythrocytes is defined by the amount of serum required to induce hemolysis of 50% of a particular population of cells. Accordingly, the erythrocytes of PNH have been clas-

Table I. Characterization of the Complement Sensitivity of the PNH Erythrocytes

Patient	PNH I*	PNH II*	PNH III*	Maximum hemolysis induced by CoFbB <sup>†</sup>
				%
R.O.	0	0	>95	84
B.A.	29	64	7	9
O.B.	79	0	21	21

\* Determined in the CLS assay.

<sup>†</sup> Derived from the results illustrated in Fig. 2.

sified into the following three groups: PNH I cells are equivalent or (nearly equivalent) in sensitivity to normal erythrocytes; PNH II cells require 25–33% as much serum and are therefore considered to be intermediate in sensitivity; and PNH III cells are markedly sensitive, requiring 4–7% as much serum for an equal degree of lysis compared with normal erythrocytes. The results of the CLS assay for the erythrocytes used in this study are shown in Table I.

To determine the effects of inhibition of MIRL function on susceptibility to hemolysis mediated by the classical pathway of complement, normal erythrocytes were incubated with either anti-MIRL antiserum or nonimmune rabbit serum. After washing, the cells were used in the CLS assay (Fig. 1). For comparison, the CLS curve for the erythrocytes of patient R.O. is shown. The anti-MIRL treatment increased the sensitivity of normal erythrocytes to that of PNH III cells. These results

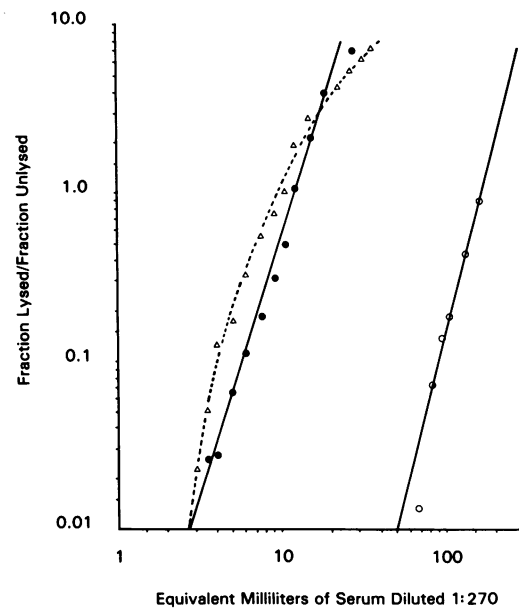


Figure 1. Comparison of the susceptibility of normal, anti-MIRL-treated normal, and PNH III erythrocytes to hemolysis in the CLS assay. Normal erythrocytes were incubated with buffer containing either saturating amounts of anti-MIRL antiserum (●) or an equivalent amount of nonimmune rabbit serum (○). After washing, the cells were used in the CLS assay along with the erythrocytes from patient R.O. (△). By blocking MIRL function, normal erythrocytes were made as susceptible to antibody-initiated complement-mediated hemolysis as PNH III cells.

indicate that MIRL is an important inhibitor of antibody-initiated hemolysis as well as of reactive lysis. In the CLS assay the IgM antibody is used in excess with the complement source being the limiting factor. Thus, direct activation of the classical pathway by anti-MIRL would not influence the result of the assay. We also observed that by allowing purified MIRL to incorporate into the cells (14), PNH III erythrocytes isolated by cell-affinity chromatography (6) can be made to manifest a CLS profile similar to that of PNH I cells (data not shown).

In the second assay used to assess the complement sensitivity of the PNH erythrocytes, CoFBb were used to initiate hemolysis (Fig. 2). The maximum susceptibility of the PNH erythrocytes to CoFBb-initiated hemolysis was similar to the percentage of cells that were classified as type III in the CLS assay (Table I).

Previous studies have shown that MIRL protects PNH erythrocytes from CoFBb-initiated hemolysis and that PNH III erythrocytes are deficient in MIRL (14). In addition, PNH III erythrocytes have been shown to be deficient in HRF/C8bp (8, 9). Based on its reported function, HRF/C8bp would be expected to offer some protection against CoFBb-initiated hemolysis. To test this possibility, MIRL function was blocked by treating normal erythrocytes with optimal amounts of anti-MIRL antiserum. After washing, the susceptibility of the anti-MIRL-treated cells to CoFBb-initiated hemolysis was compared with that of the PNH III cells. As illustrated in Fig. 3, the susceptibility of the anti-MIRL-treated cells was equivalent to that of the PNH III cells. Identical results were observed when purified anti-MIRL IgG was used instead of anti-MIRL antiserum, but isolated IgG from a nonimmune rabbit had no effect on the susceptibility of normal cells to CoFBb-initiated hemolysis (not shown). These experiments demonstrate that the susceptibility of PNH III cells to CoFBb-initiated hemolysis is due primarily to a deficiency of MIRL.

*Comparison of surface expression of MIRL and DAF on PNH erythrocytes by using a radioimmunobinding assay.* Monospecific polyclonal antibodies were used to quantitate the amount of DAF and MIRL on the PNH erythrocytes (Fig. 4). The erythrocytes from all three patients demonstrated a

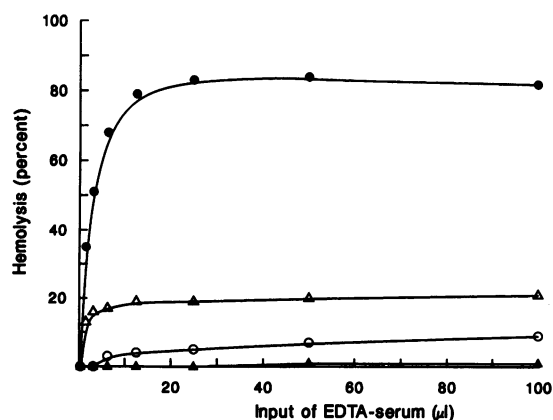


Figure 2. CoFBb-initiated hemolysis of PNH and normal erythrocytes. Normal (▲) and PNH erythrocytes from patients B.A. (○), O.B. (△), and R.O. (●) were incubated with CoFBb and incremental concentrations of EDTA-chelated serum. Hemolysis was subsequently quantified by measuring free hemoglobin. The data points represent the mean of triplicate determinations. Susceptibility to CoFBb-initiated hemolysis is a characteristic of PNH III erythrocytes.

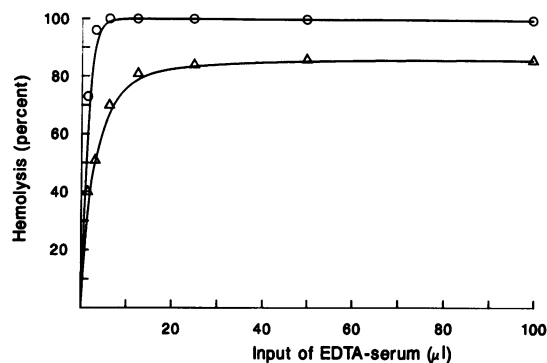


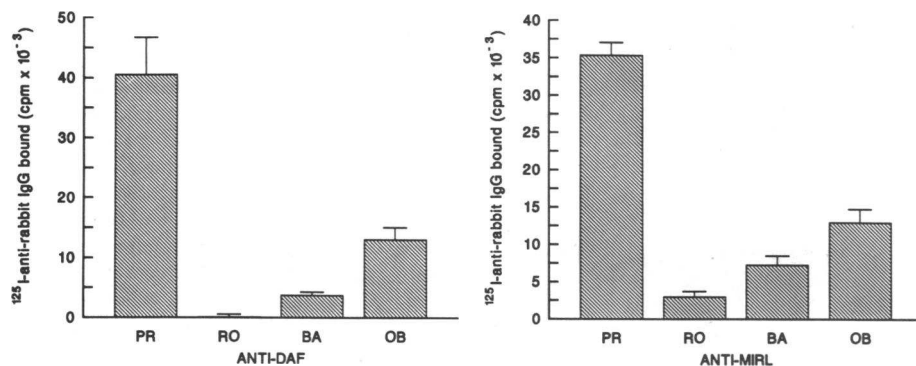
Figure 3. Comparison of PNH III and anti-MIRL-treated normal erythrocytes to CoFBb-initiated hemolysis. Normal erythrocytes were incubated with a saturating amount of anti-MIRL antiserum. After washing, the antibody-treated cells (○) and the erythrocytes from patient R.O. (△) were incubated with CoFBb and incremental concentrations of EDTA-chelated serum and hemolysis subsequently quantified. The data points represent the mean of triplicate determinations. By blocking MIRL function, normal erythrocytes are made as susceptible to CoFBb-initiated hemolysis as PNH III cells.

parallel deficiency of both regulatory proteins. Among volunteer donors, the number of molecules of DAF/erythrocyte (~ 2,000) was reported to be relatively uniform (6). Uniformity of expression also appears to be the case for MIRL, because by using the indirect binding assay the counts per minute for anti-MIRL-treated erythrocytes from 10 volunteer donors was  $29,944 \pm 3,460$  (mean  $\pm$  SD,  $n = 5$  for each subject, range of means = 25,666–36,662).

Normal erythrocytes treated with the sulfhydryl reagent, AET, are susceptible to CoFBb-initiated hemolysis (12), but trypsin-treated erythrocytes are not (Parker, C. J., unpublished observations). When AET-treated cells were assessed for surface expression of MIRL, no antibody binding was observed (Fig. 5). In contrast, trypsin treatment produced a modest reduction in binding of anti-MIRL. These results are consistent with the hypothesis that AET disrupts the structural/functional integrity of MIRL and thereby induces susceptibility to reactive lysis.

*Cytofluorographic analysis of the surface expression of MIRL on PNH erythrocytes.* To determine if MIRL expression among PNH I, II, and III erythrocytes is discrete and homogeneous, cells were analyzed using the FACS (Fig. 6). Consistent with the phenotypic profile of the patient's erythrocytes (Table I), the cells of patient R.O. demonstrated a marked deficiency of MIRL (Fig. 6 C). Analysis of the erythrocytes of patient B.A. (Fig. 6 B), however, were inconsistent with the concept that PNH II cells express a normal amount of MIRL. Rather, except for a modest quantitative difference, the pattern of MIRL expression on B.A.'s erythrocytes was similar to that of patient R.O. The FACS analysis of the erythrocytes of patient O.B. was also more consistent with a continuum of MIRL expression than with division into discrete subpopulations with either normal or no MIRL expression (Fig. 6 D).

*Analysis by using RIP of MIRL and DAF on PNH erythrocytes.* These studies were performed to determine if MIRL and DAF are qualitatively as well as quantitatively abnormal on PNH erythrocytes. The deficiencies of MIRL observed in the RIP studies (Fig. 7 B) paralleled those seen in the radioimmunobinding studies (Fig. 4), and no qualitative differences of



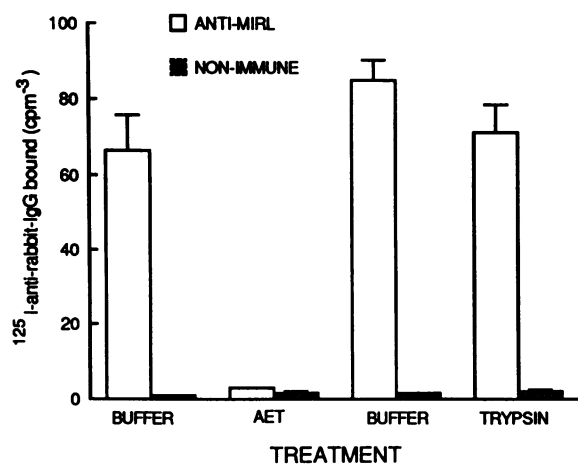
**Figure 4.** Quantitation of erythrocyte DAF and MIRL by using a radio-immunobinding assay. Normal (PR) and PNH erythrocytes from patients R.O., B.A., and O.B. were incubated with either saturating amounts of anti-DAF or anti-MIRL antiserum or with an equal amount of nonimmune rabbit serum (to control for nonspecific binding). Subsequently, binding of rabbit IgG was quantified by incubating the cells with a saturating amount of <sup>125</sup>I-anti-rabbit IgG. The values (specific binding) depicted by the bars represent the mean + SD ( $n = 5$ ). The PNH erythrocytes demonstrate a parallel deficiency of DAF and MIRL.

MIRL were apparent. Similar results were obtained with anti-DAF except that a diffuse band was observed in the molecular weight range  $\leq 14$  kD in the lane containing the immunoprecipitate from patient R.O. (Fig. 7 B, lane 3). The nature of this band is unknown, but it was not identified by anti-DAF in Western blot analysis (not shown).

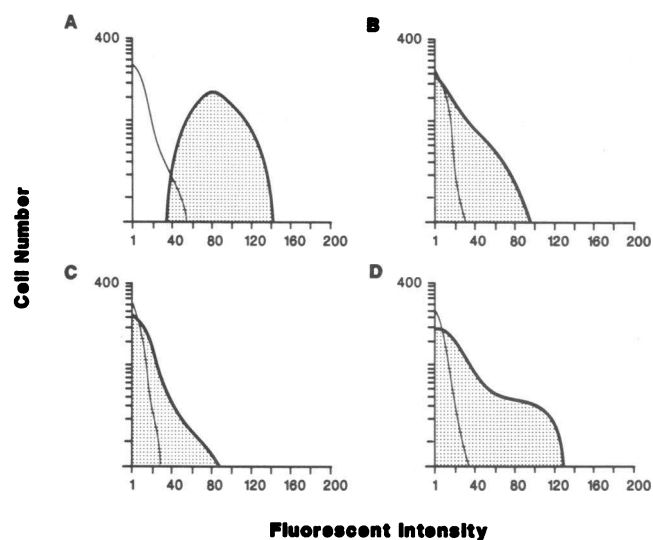
**Effects of anti-MIRL on the susceptibility of PNH erythrocytes to CoFBb-initiated hemolysis.** The preceding studies have suggested that the difference in complement sensitivity among PNH I, II, and III cells is not due to the existence of discrete subpopulations of cells, one of which is completely deficient in a complement regulatory protein, while the other expresses a normal amount. This conclusion was directly challenged by pretreating the erythrocytes from a volunteer donor and from patients B.A. and O.B. with incremental concentrations of anti-MIRL. After washing, the cells were analyzed for susceptibility to CoFBb-initiated hemolysis. The experimental conditions were designed so that the dose-response curve for the anti-MIRL-treated normal erythrocytes manifested a sigmoid appearance (Fig. 8). If a subpopulation of erythrocytes bearing a normal amount of MIRL existed

among the PNH erythrocytes, that subpopulation should produce a dose-response curve that would parallel that of the anti-MIRL-treated normal erythrocytes. This, however, was not the case. These results suggest that the entire population of erythrocytes from patients B.A. and O.B. are deficient in MIRL, and that the erythrocytes that are hemolyzed in the absence of anti-MIRL treatment are simply more profoundly deficient.

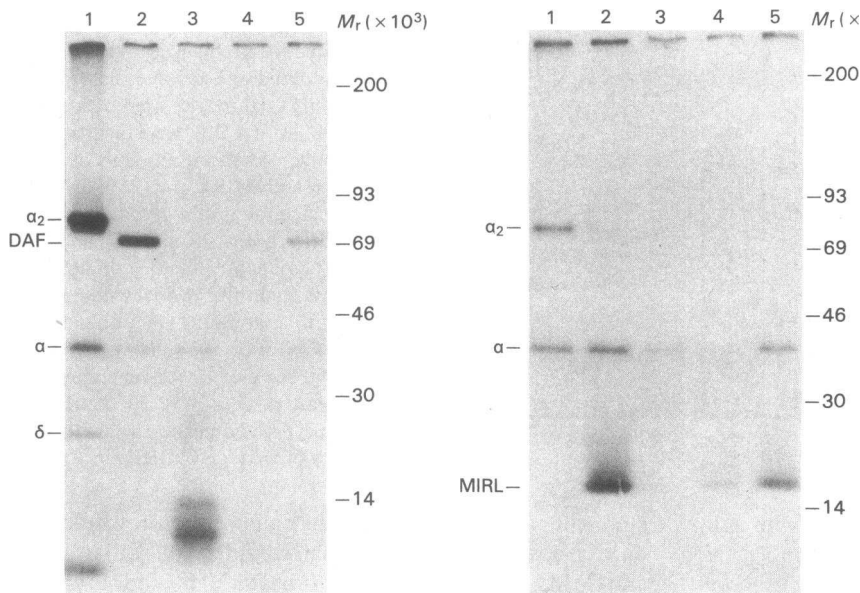
**Quantitation of MIRL on PNH erythrocytes after CoFBb-initiated hemolysis.** To examine further the question of whether the erythrocytes that are insensitive to hemolysis induced by CoFBb possess normal amounts of MIRL, DAF, or both, PNH cells were incubated with CoFBb and NHS-EDTA. Next, the unlysed cells were recovered and the amount of MIRL and DAF on these insensitive cells was quantified using the radioimmunobinding assay. At the same time, the



**Figure 5.** Binding of anti-MIRL to AET and trypsin-treated erythrocytes. The binding of anti-MIRL to normal erythrocytes treated with AET, trypsin, or buffer was assessed using the same methods as described in the legend to Fig. 4. The values depicted by the bars represent the mean + SD ( $n = 5$ ). Treatment of cells with AET completely abolished the binding of anti-MIRL, whereas trypsin treatment had little effect.



**Figure 6.** FACS analysis of anti-MIRL binding to normal and PNH erythrocytes. Normal (A) and PNH erythrocytes from patients B.A. (B), R.O. (C), and O.B. (D) were incubated with either a saturating amount of anti-MIRL antiserum (shaded area) or an equal amount of nonimmune rabbit serum (area under the curve inscribed by the thinner of the two lines). After washing, the cells were incubated with a saturating amount of FITC-conjugated anti-rabbit IgG. A minimum of 5,000 cells was analyzed for each subject. A bimodal cytofluorometric pattern was not observed for the erythrocytes of patients B.A. and O.B.

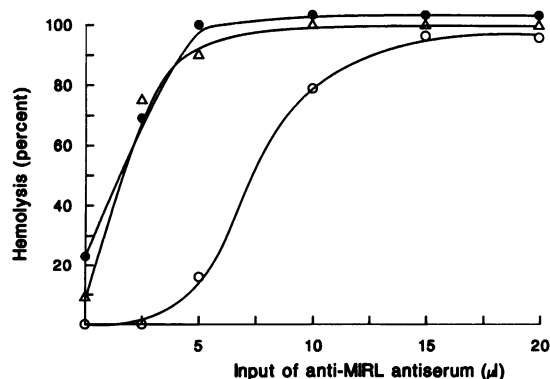


**Figure 7.** Analysis of DAF and MIRL on normal and PNH erythrocytes by using RIP. Hemoglobin-free ghosts of normal and PNH erythrocytes were prepared from cells that had been surface-labeled with  $^{125}\text{I}$ . Membrane proteins were solubilized and immunoprecipitated by using anti-DAF (A) or anti-MIRL (B). Lane 1, radio-labeled ghosts from normal erythrocytes. The other lanes contain RIP from the following sources: lane 2, normal volunteer; lane 3, patient R.O.; lane 4, patient B.A.; lane 5, patient O.B. The Greek letters denote the positions of the glycoprotein- $\alpha$  monomer and dimer and of the  $\delta$  monomer. The positions of the molecular weight standards and of DAF and MIRL are also indicated. Parallel quantitative abnormalities of DAF and MIRL are observed for the patients with PNH.

amount of MIRL and DAF present on the erythrocytes before selective lysis of the sensitive cells was also determined. Among the PNH patients, only the erythrocytes from R.O. expressed a significantly greater amount of MIRL after selective removal of the CoFBb-sensitive cells (Fig. 9). This amount of MIRL, however, remained markedly less than normal. In agreement with the other studies reported herein, these results suggest that the degree of complement sensitivity of erythrocytes from patients with PNH is due to relatively subtle quantitative variations in the amount of regulatory proteins present on the erythrocyte membrane surface.

## Discussion

The studies reported here suggest that PNH II erythrocytes are deficient in MIRL as well as DAF. Previously, either of two hypotheses seemed plausible as explanations for the molecular basis of the differences between the PNH II and III pheno-



**Figure 8.** CoFBb-initiated hemolysis of normal and PNH erythrocytes treated with anti-MIRL. Normal (○) and PNH erythrocytes from patients B.A. (△) and O.B. (●) were incubated with incremental amounts of anti-MIRL. After washing, the cells were incubated with an optimal amount of CoFBb and EDTA-chelated serum, and hemolysis was subsequently quantified. The data points depict the mean of triplicate determinations. Greater amounts of anti-MIRL are required to block MIRL function on normal erythrocytes than on PNH erythrocytes.

types. According to the first hypothesis, the two phenotypes have the same basic membrane defect, with the PNH III cells being more severely affected. In the second hypothesis, PNH III erythrocytes have two independent defects. One deficiency accounts for the intermediate complement sensitivity and the greater activation and binding of C3b, and this defect is shared by PNH II cells. The absence of a second complement regulatory protein is responsible for the greater susceptibility of PNH III cells to CoFBb-initiated hemolysis, and this other factor is normal on PNH II erythrocytes. The combined deficiency of the two regulatory proteins accounts for the marked sensitivity of PNH III erythrocytes in the CLS assay.

Inasmuch as both DAF and MIRL are markedly deficient on the erythrocytes of patient B.A. (Fig. 4), only the first of the hypotheses stated above appears tenable. This patient has only 7% type III cells (Table I and Fig. 2), yet the erythrocytes expressed  $\sim 20\%$  of the normal amount of MIRL. If the second hypothesis were correct, erythrocytes from a patient with 93% CoFBb-insensitive cells (64% type II and 29% type I) would have essentially normal amounts of MIRL.

The results of the experiments shown in Fig. 9 suggest that  $\sim 20\%$  of the normal amount of MIRL inhibits CoFBb-initiated hemolysis because this amount was present on the insensitive cells of patient R.O. The erythrocytes that are classified as PNH II appear to have an amount of MIRL that is just above the threshold that provides protection against reactive lysis (Figs. 4 and 9). Combined with a parallel DAF deficiency, however, this same degree of MIRL deficiency apparently causes the cells to display an intermediate sensitivity in the CLS assay (the PNH II phenotype).

The fact that PNH I erythrocytes are nearly normal in susceptibility to hemolysis mediated by the classical pathway has suggested that they may be the progeny of the residuum of the normal clone, and that among patients the normal and abnormal cells coexist in various proportions (1). Analysis of MIRL and DAF associated with the erythrocytes of patient O.B., however, demonstrate that PNH I erythrocytes are abnormal. This patient has 80% type I cells, and yet quantitative binding studies show that the erythrocytes express 37 and 32% of the normal amount of MIRL and DAF, respectively (Fig. 4).

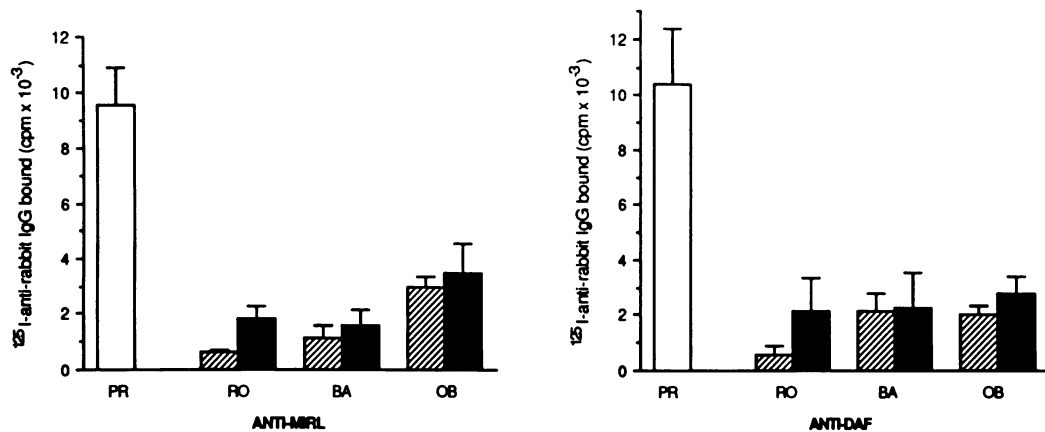


Figure 9. Quantitation of MIRL and DAF on PNH erythrocytes that are insensitive to CoFBb-initiated hemolysis. Erythrocytes from patients R.O., B.A., and O.B. were incubated with optimal amounts of CoFBb and NHS-EDTA, and the insensitive cells were subsequently recovered. Next, binding of anti-MIRL and anti-DAF to the unlysed population (closed bars) was compared with

binding to the unfractionated population (cells that had not been incubated with CoFBb and NHS-EDTA; hatched bars) and to normal erythrocytes (open bars). The results depicted by the bars represent the mean + SD ( $n = 5$ ). The insensitive erythrocytes are deficient in MIRL and DAF.

Further, the cytofluorographic analysis of MIRL expression demonstrates that only 47% of the patient's erythrocytes have a fluorescence intensity that falls within the normal range, and that the cells do not display the bimodal pattern expected if two distinct populations exist (Fig. 6 D).

Two other lines of evidence indicate that PNH I erythrocytes are abnormal. First, the amount of antibody that was required to block MIRL function on the cells that were initially insensitive to CoFBb-initiated hemolysis was much less than that required for inhibition of MIRL function on normal erythrocytes (Fig. 8). Second, when the PNH III erythrocytes of patients O.B., R.O., and B.A. were selectively hemolyzed, the residual PNH I cells were observed to be deficient in MIRL and DAF (Fig. 9). Based on studies that compared expression of DAF and acetylcholine esterase by PNH erythrocytes that were insensitive to CoFBb-initiated hemolysis, Medof and colleagues also concluded that PNH I cells are abnormal (6). Thus it appears that by the time the PNH syndrome becomes evident clinically, normal erythrocytes have been replaced almost entirely by the progeny of the abnormal clone. The mechanisms that account for the heterogeneity of expression of the various membrane proteins among patients remain speculative.

Despite the fact that they are significantly deficient in complement regulatory proteins, PNH I erythrocytes demonstrate a nearly normal susceptibility to antibody-initiated hemolysis. These observations suggest that on normal erythrocytes MIRL and DAF are present in a relative excess. Apparently the degree of deficiency of the inhibitory constituents must be profound before in vitro and in vivo evidence of disordered regulation of complement is manifested.

Normal erythrocytes treated with the sulfhydryl reagent AET have been used as models for investigating the pathophysiological basis of PNH (1). In the CLS assay the chemically modified cells display a susceptibility similar to that of PNH III erythrocytes (15). Further, like PNH III cells AET-treated erythrocytes are susceptible to CoFBb-initiated hemolysis, and after incubation with CoFBb and NHS-EDTA they bind relative large amounts of the trimolecular C5b-7 complex (12). In immunoblotting studies anti-MIRL does not bind to MIRL if the SDS-PAGE is performed under reducing condition (Parker, C. J., unpublished observations), and normal erythrocytes that have been treated with AET no longer bind

anti-MIRL (Fig. 5). Taken together, these observations suggest that AET destroys the structural and functional integrity of MIRL by disrupting intrachain disulfide bonds. While AET may affect other erythrocyte constituents (23), abolition of MIRL function appears to account for most of the disordered regulation of complement, because the behavior of normal erythrocytes in which MIRL function has been blocked by antibody is analogous to that of the chemically modified cells (Figs. 1 and 3).

Previous studies have suggested that AET does not disrupt the structural or functional integrity of DAF (23). Normal DAF function may account for the observation that AET-treated erythrocytes do not bind as much C3 as PNH II and III erythrocytes after activation of the classical pathway (12). As evidenced by the results illustrated in Fig. 1, however, control of the classical pathway C3 convertase by DAF appears to offer relatively little protection against complement-mediated hemolysis if MIRL is not functional.

The relationship between the expression of HRF/C8bp and the erythrocyte phenotypes of PNH has not been analyzed in this report. Our studies suggest, however, that HRF/C8bp has a limited capacity to control complement-mediated hemolysis initiated either by the classical pathway activation (Fig. 1) or by CoFBb (Fig. 3) because anti-MIRL-treated normal erythrocytes are as sensitive as PNH III erythrocytes (cells that are presumably missing HRF/C8bp). In the studies reported here, CoFBb and serum have been used to induce reactive lysis. Sugita et al. (24) have isolated a complement regulatory protein that appears to be identical to MIRL. In those studies, the 18-kD protein was shown to block reactive lysis induced by isolated C5b6, C7, C8, and C9, but an inhibitor with the reported properties of HRF/C8bp could not be identified. We have made similar observations.

The molecular basis of PNH remains enigmatic. To date, all of the proteins that are deficient in PNH have been shown to share the common biochemical feature of being anchored to the cell membrane by a glycosyl-phosphatidylinositol moiety (25-31). This observation has led to the hypothesis that PNH is the manifestation of an abnormality in the complex systems of either synthesis, attachment, or degradation of phosphatidylinositols. Accordingly, studies to determine if MIRL is anchored by a phosphatidylinositol-glycan are being pursued (32, 33).



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