Molecular Basis of the Enhanced Susceptibility of the Erythrocytes of Paroxysmal Nocturnal Hemoglobinuria to Hemolysis in Acidified Serum

By Linda A. Wilcox, John L. Ezzell, Nicole J. Bernshaw, and Charles J. Parker

When incubated in acidified serum, the erythrocytes of paroxysmal nocturnal hemoglobinuria (PNH) are hemolyzed through activation of the alternative pathway of complement (APC), but normal erythrocytes are resistant to this process. PNH cells are deficient in decay-accelerating factor (DAF), a complement regulatory protein that inhibits the activity of both the classical and the alternative pathways. However, deficiency of DAF alone does not account entirely for the aberrant effects of acidified serum on PNH cells. Recently, we have shown that PNH erythrocytes are also deficient in another complement control protein called membrane inhibitor of reactive lysis (MIRL) that restricts complement-mediated lysis by blocking formation of the membrane attack complex (MAC). To determine the effects of the DAF and MIRL on susceptibility to acidified serum lysis, PNH cells were replated with the purified proteins. DAF partially inhibited acidified serum lysis by blocking the activity of the amplification C3 convertase. MIRL inhibited acidified serum lysis both by blocking the activity of the MAC and by inhibiting the activity the C3 convertase. When DAF function was blocked with antibody, normal erythrocytes became partially susceptible to acidified serum lysis. By blocking MIRL, cells were made completely susceptible to lysis, and control of C3 convertase activity was partially lost. When both DAF and MIRL were blocked, the capacity of normal erythrocytes to control the activity of the APC and the MAC was destroyed, and the cells hemolyzed even in unacidified serum. These studies demonstrate that DAF and MIRL act in concert to control susceptibility to acidified serum lysis; of the two proteins, MIRL is the more important. In addition to its regulatory effects on the MAC, MIRL also influences the activity of the C3 convertase of the APC. Further, in the absence of DAF and MIRL, the plasma regulators (factor H and factor I) lack the capacity to control membrane-associated activation of the APC.

© 1991 by The American Society of Hematology.

THE SEMINAL observations that connected the mechanism of hemolysis of paroxysmal nocturnal hemoglobinuria (PNH) erythrocytes to complement were reported by Ham and by Ham and Dingle. In those studies, it was demonstrated that PNH erythrocytes are hemolyzed when incubated in acidified serum and that the hemolysis is complement dependent.

Acidified serum lysis is mediated through activation of the alternative pathway of complement (APC). The mechanism whereby acidification of serum favors activation of the APC appears to involve the binding of factor B to C3b. At acid pH, the binding affinity of factor B for C3b is enhanced, and as a consequence, formation of the amplification C3 convertase (C3bBb) of the APC is favored. Logue et al demonstrated that following incubation in acidified serum, PNH erythrocytes have bound much greater amounts of activated C3 fragments than normal cells, suggesting that PNH cells are deficient in membrane constituents that regulate the activity of the amplification C3 convertase.

An explanation for the aberrant effects of acidified serum on PNH cells seemed apparent when it was discovered that the erythrocytes of PNH are deficient in decay-accelerating factor (DAF). DAF (CD 55) is a 70-Kd glycoprotein that regulates the activity of the amplification C3 convertase. When DAF function was blocked with antibody, normal erythrocytes became partially susceptible to acidified serum lysis. By blocking MIRL, cells were made completely susceptible to lysis, and control of C3 convertase activity was partially lost. When both DAF and MIRL were blocked, the capacity of normal erythrocytes to control the activity of the APC and the MAC was destroyed, and the cells hemolyzed even in unacidified serum. These studies demonstrate that DAF and MIRL act in concert to control susceptibility to acidified serum lysis; of the two proteins, MIRL is the more important. In addition to its regulatory effects on the MAC, MIRL also influences the activity of the C3 convertase of the APC. Further, in the absence of DAF and MIRL, the plasma regulators (factor H and factor I) lack the capacity to control membrane-associated activation of the APC.

Subsequent studies have shown that PNH cells are deficient in a number of proteins and that all of the deficient proteins share the common biochemical feature of being anchored to the membrane surface through a glycosyl phosphatidylinositol (GPI) moiety. Among the deficient GPI-linked proteins are two (other than DAF) that are concerned with regulation of complement. Homologous restriction factor (HRF) or C8 binding protein (C8bp) is a 65-Kd protein that has been reported to regulate the functional activity of the membrane attack complex (MAC) by inhibiting binding of C8 or C9 or both. However, studies aimed at determining the effects of HRF/C8bp on the process of acidified serum lysis have not been reported. Recently, we have isolated an 18-Kd protein from normal erythrocytes that inhibits complement-mediated lysis of PNH cells by blocking assembly of the MAC. Further experiments have shown that the extent of the deficiency of this GPI-linked protein, called membrane inhibitor of reactive lysis (MIRL), is a critical determinant of the sensitivity of PNH erythrocytes to complement-mediated hemolysis. Working independently, others have also iden-
tified the MIRL protein (CD 59) and elucidated some of its structural and functional characteristics.25,35

The studies reported here were designed to investigate the complement regulatory activity of DAF and MIRL both independently and in combination. The results have shown that while DAF deficiency contributes, the aberrant effects of acidified serum on PNH erythrocytes are due primarily to a deficiency of MIRL.

MATERIALS AND METHODS

Human erythrocytes. Erythrocytes from normal donors and from patients with PNH were obtained by venipuncture and stored in Alsever's solution at 4°C. A relatively homogeneous population of PNH III erythrocytes (a gift from Dr. Wendell F. Ross, Duke University Medical Center, Durham, NC) was obtained by affinity chromatography according to the method of Chow et al. This technique takes advantage of the fact that the most complement sensitive cells (type III) are deficient in acetylcholinesterase. Erythrocytes from a patient with a mixture of type I (cells with normal or nearly normal sensitivity to complement) and type III cells are chromatographed on a column of immobilized monoclonal anti-acetylcholinesterase. The type I cells that express cell-surface acetylcholinesterase are retarded by the immobilized antibody while the type III cells that are deficient in acetylcholinesterase pass through the column. The uniformity of the unretarded population is assessed by using the complement lysis sensitivity assay. Unfractionated erythrocytes from a patient (RO) with greater than 95% PNH III were also used. MIRL and DAF expression on these cells has been previously reported.29

Buffers. The following buffers were used: veronal-buffered saline (VBS) containing 10 mM sodium barbital (Sigma Chemical Co, St. Louis, MO), pH 7.5, and 145 mM NaCl; VBS containing 0.1% gelatin (GBV); GBV containing 1 mM MgCl₂ (GBV⁺); GBV⁺, pH 6.4, was prepared by titrating GBV⁺ with 1 mM HCl; GBV containing 15 mM EDTA (GBV-EDTA); tris-buffered saline containing 0.1% gelatin, 1% NP-40 (Sigma Chemical Co), and 1 mM phenylmethylsulfonyl fluoride (PMSF) (radioimmunoprecipitation [RIP] buffer).

Serum. Acidified serum was prepared by mixing 125 µL 0.2 mol/L HCl with 875 µL of normal human serum (NHS). The final pH of the serum was 6.4. In all of the experiments reported herein, acidified serum contained 2.5 mM MgCl₂ and 8 mM EGTA. This reagent was prepared by mixing 1 vol of a stock solution of 25 mM MgCl₂ and 80 mM EDTA (Sigma Chemical Co), pH 6.4, with 9 vol of acidified serum and incubating the mixture for 5 minutes at 37°C. NHS-EDTA was prepared by mixing 1 vol of 100 mM EDTA, pH 6.4, with 9 vol of NHS, pH 6.4, and incubating the mixture for 5 minutes at 37°C. NHS-MgEGTA was prepared by mixing 1 vol of a stock solution of 25 mM MgCl₂ and 80 mM EDTA (Sigma Chemical Co), pH 7.4, with 9 vol of NHS and incubating the mixture for 5 minutes at 37°C. To insure that Mg/EGTA serum did not support activation of the classical pathway of complement, sheep erythrocytes bearing antibody (EA) were prepared. Next, the EA were incubated at 37°C with incremental concentrations of either NHS, NHS-MgEGTA, or NHS-EDTA. After 30 minutes, the mixtures were centrifuged and hemolysis was quantitated by measuring free hemoglobin. Less than 1 µL of NHS supported lysis of 60% of the EA, whereas no lysis was observed using 50 µL of either NHS-MgEGTA or NHS-EDTA.

Antibodies. Rabbit antisera against MIRL and DAF were prepared using previously described methods.27 Rabbit antisera against glycophorin (Research Plus, Inc, Bayonne, NJ) and against whole human erythrocyte stroma (United States Biochemical Corporation, Cleveland, OH) were purchased from commercial vendors. Monoclonal anti-C3d was purchased from Quidel (San Diego, CA). The antibody recognizes an epitope that is located in the C3d region of C3 and that is expressed by C3b, IC3b, C3dg, and C3d. Monoclonal antihuman IgG was purchased from Sigma Chemical Co in the form of ascites, and the IgG was isolated by using caprylic acid.27

Radioimmunoassay21. Monoclonal anti-C3d, monoclonal anti-rabbit IgG, erythrocyte membrane proteins, purified MIRL, and purified DAF were labeled with 125I as NaI (Amersham Corp, Arlington Heights, IL) by using IODO-GEN (Pierce Chemical Co, Rockford, IL).36

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

RIP. Radiolabeled erythrocyte membrane proteins were immunoprecipitated with anti-MIRL and anti-DAF using methods that have been previously described.21

Quantitation of MIRL and DAF on erythrocytes. Quantitation of MIRL and DAF by radioimmunobinding assay was performed using the following modification of the method of Holguin et al. Monoclonal antihuman IgG was substituted for radiolabeled affinity-purified polyclonal antirabbit IgG. Equilibrium binding studies were performed to determine the amount of the antibody required to produce saturation binding.

Susceptibility of normal and PNH erythrocytes to acidified serum lysis. Normal and affinity-isolated PNH III erythrocytes were washed three times in GVB and resuspended to 5 x 10⁸/mL. Next, the cells were washed once in GVB⁺, pH 6.4, and resuspended to 5 x 10⁸/mL. Serial dilutions of acidified serum were prepared using GVB⁺, pH 6.4, as the diluent. Aliquots of 50 µL of cells were incubated at 37°C with 100 µL of each serum dilution. After 30 minutes, 3 µL of cold GVB-EDTA was added and the samples were centrifuged for 3 minutes at 1,000g. Free hemoglobin was determined by measuring the A₄₅₀ of the supernate, and hemolysis (percent) was calculated by using a previously described formula.21

Quantitation of C3 deposition on erythrocyte membranes. Following incubation in acidified serum, 800 µL of GVB-EDTA was added, and the reaction mixtures were transferred to 1.5-mL microfuge tubes. For controls in these experiments, acidified NHS-EDTA was substituted for acidified serum. The samples were centrifuged (Beckman Microfuge 12; Beckman Instruments, Inc, Palo Alto, CA) for 5 minutes at 11,500g. The supernate was recovered and used to determine hemoglobin release. The cells were resuspended to 100 µL in GVB-EDTA. In duplicate, 40 µL of cells was incubated at 37°C with 50 µL of radiolabeled anti-C3d (20 µg/mL). Preliminary experiments had shown that this amount of antibody was saturating. After 30 minutes, aliquots of 75 µL of each reaction mixture were pipetted into a 400-µL polystyrene microfuge tube containing 250 µL of 20% sucrose. After centrifugation in the microfuge for 15 minutes at 11,500g, the tips containing the cells were cut off, and the radioactivity of the pellet was quantified in a gamma counter. The radioactivity of the control samples was subtracted to determine specific binding. Binding of 125I-anti-C3d was calculated based on specific activity and an Mr of 180 Kd.

Incorporation of radiolabeled MIRL and DAF into erythrocytes. MIRL2 and DAF were purified using previously described methods. Guinea pig erythrocytes were washed three times with VBS and resuspended to 1 x 10⁸/mL. Aliquots of 1 mL of guinea pig erythrocytes were incubated at 37°C with VBS containing either...
radiolabeled MIRL, radiolabeled DAF, or radiolabeled DAF and MIRL. After 60 minutes, the cells were washed with VBS and hemoglobin-free ghosts were prepared. The radioactivity associated with the ghosts was quantitated, and the membrane proteins were analyzed by SDS-PAGE and autoradiography.

Effects of purified MIRL and DAF on susceptibility of PNH erythrocytes to acidified serum lysis. Affinity-isolated PNH type III erythrocytes were washed three times in GVB and resuspended to 5 × 10⁷/mL. Aliquots of 50 μL of cells were incubated at 37°C with 500 μL of VBS or with 500 μL of VBS containing 1.2 μg of DAF, 1.2 μg of MIRL, or 600 ng of MIRL and 600 ng of DAF. After 30 minutes, the cells were washed once with GVB+, pH 6.4, and resuspended to 100 μL. Next, 100 μL of acidified serum was added, and the samples were incubated for 30 minutes at 37°C. Subsequently, hemolysis and C3 deposition were quantified as described above.

In a second set of experiments, the unfractionated erythrocytes from a patient (RO) with greater than 95% PNH type III cells were used to assess the effects of incorporation of MIRL on deposition following incubation of cells in acidified serum. Aliquots of 300 μL of PNH erythrocytes (1 × 10⁹/mL in VBS) were incubated at 37°C with 300 μL of either VBS or VBS containing purified MIRL (29 μg/mL). After 30 minutes, the PNH cells were washed with GVB+, pH 6.4, and resuspended to 300 μL. Normal erythrocytes (1 × 10⁹/mL in GVB+, pH 6.4) served as an additional control. Aliquots of 50 μL of cells were incubated at 37°C with incremental concentrations of acidified serum (diluted in GVB+, pH 6.4). After 30 minutes, C3 deposition was quantified as described above.

Comparison of the susceptibility of anti-DAF- and anti-MIRL-treated erythrocytes to acidified serum lysis. Normal erythrocytes were washed three times in VBS and resuspended to 5 × 10⁸/mL. The calcium and magnesium in rabbit serum (either nonimmune or anti-DAF or anti-MIRL antisera) was chelated by adding EDTA to a final concentration of 10 mmol/L and incubating the sample for 5 minutes at 37°C. Serial dilutions of the rabbit serum were prepared using GVB as the diluent. Aliquots of 50 μL of cells were incubated at 37°C with dilutions of the rabbit serum. After 30 minutes, the cells were washed once with 4 mL of GVB+, pH 6.4, and resuspended to 100 μL. Next, 100 μL of acidified serum was added, and the samples were incubated for 30 minutes at 37°C. Subsequently, hemolysis and C3 deposition were quantified as described above.

In a second set of experiments, 50 μL of normal erythrocytes (5 × 10⁹/mL in GVB+) was incubated at 37°C with incremental concentrations of either anti-MIRL or nonimmune rabbit serum (NIRS) that had been treated with EDTA as described above. After 30 minutes, the cells were washed once with 4 mL of GVB+, pH 6.4, and resuspended to 100 μL. Next, 100 μL of acidified serum was added, and the samples were incubated for 30 minutes at 37°C. Subsequently, C3 deposition was quantified as described above.

Comparison of the susceptibility of anti-DAF- and anti-MIRL-treated normal erythrocytes to hemolysis in NHS-Mg/EGTA. These experiments were performed exactly as those described above except that NHS-Mg/EGTA was substituted for acidified serum.

Statistical analysis. Unpaired data was analyzed by using the two-tailed Student's t-test. P < .05 was considered to be statistically significant.

RESULTS

Quantitation of DAF and MIRL on PNH erythrocytes. Quantitation by radioimmunobinding assay showed that the affinity-isolated PNH type III erythrocytes expressed 9.33% ± 0.58% (n = 3) of the normal amount of DAF and 11.67% ± 1.15% (n = 3) of the normal amount of MIRL. Expression of MIRL and DAF on the unfractionated cells from patient RO have been previously reported.

Susceptibility of normal and PNH cells to acidified serum lysis. Normal human erythrocytes are resistant to hemolysis in acidified serum, whereas PNH cells are hemolyzed by acidified serum in a dose-dependent fashion (Fig 1A). In this and all subsequent experiments, the acidified serum contained Mg/EGTA, thereby inhibiting classical pathway activation while allowing alternative pathway activity to proceed. Following exposure to acidified serum, PNH but not normal erythrocytes had bound large amounts of

![Fig 1](image-url)

**Fig 1.** Susceptibility of normal and PNH erythrocytes to acidified serum lysis. (A) Normal (○) and affinity-isolated PNH type III cells (▲) were incubated in acidified serum and hemolysis was subsequently quantified. The data points represent the mean of duplicate determinations. PNH erythrocytes showed a dose-dependent susceptibility to acidified serum, whereas normal cells were resistant to this process. (B) Following incubation in acidified serum, the amount of activated C3 products bound to the cells was quantitated by using a radiolabeled monoclonal antibody (MoAb). The bars depict the mean ± SEM (n = 6). PNH erythrocytes had bound large amounts of C3, indicating that the APC had been activated on the cell surface.
activated C3 products (Fig 1B). Those results show that acidification of serum induces activation of the APC and that PNH erythrocytes are deficient in the membrane constituents that control the activity of the amplification C3 convertase on normal cells.

Based on their known function, we hypothesized that DAF and MIRL control the susceptibility of human erythrocytes to acidified serum lysis. If this hypothesis is correct, repletion of PNH cells with DAF and MIRL should inhibit not only hemolysis but also the activity of the APC C3 convertase. Further, by blocking the function of DAF and MIRL, normal cells should lose the capacity to control the activity of the APC and the MAC and, consequently, become susceptible to acidified serum lysis. Analysis of the purity and specificity of the reagents used to test the hypothesis are shown in Fig 2. Following incubation with radiolabeled DAF and MIRL, bands representing proteins with Mrs of 68 Kd and 18 Kd were observed by autoradiography (lane 1). When cells were incubated with DAF alone (lane 2) or with MIRL alone (lane 4) bands representing proteins with Mrs of 68 Kd and 18 Kd, respectively, were seen. Immunoprecipitation studies showed that anti-DAF specifically precipitated a protein with an Mr of 68 Kd (lane 3), whereas anti-MIRL specifically precipitated a protein with an Mr of 18 Kd (lane 5).

Effects of isolated DAF and MIRL on the susceptibility of PNH erythrocytes to acidified serum lysis. Affinity-isolated PNH III cells were incubated with buffer or with buffer containing either isolated DAF and MIRL, isolated MIRL, or isolated DAF. The total amount of protein (on a weight basis) in each reaction mixture was the same (ie, the sample containing the combination of DAF and MIRL had 600 ng of each protein, whereas the samples with DAF or MIRL alone contained 1.2 μg of protein). By radioimmunobinding assay, incubation of the PNH cells with DAF and MIRL increased expression (compared with normal) by 9% and 6%, respectively. Incubation with DAF alone increased expression by 11% and incubation with MIRL alone increased expression by 8%.

The treated cells were incubated in acidified serum, and

![Image](http://example.com/image.jpg)

Fig 2. Reagents used to determine the effects of DAF and MIRL on susceptibility to acidified serum lysis. DAF and MIRL were radiolabeled and incubated either in combination (lane 1) or alone (lane 2 for DAF and lane 4 for MIRL) with guinea pig erythrocytes. After washing, hemoglobin-free ghosts were prepared and membrane proteins were analyzed by SDS-PAGE (reducing conditions) and autoradiography. DAF and MIRL incorporated into the erythrocytes. Normal human erythrocytes were radiolabeled and immunoprecipitated with anti-DAF (lane 3), anti-MIRL (lane 5), or NIRS (lane 6). Subsequently, the samples were analyzed by SDS-PAGE (reducing conditions) and autoradiography. Anti-DAF specifically precipitated a protein with an Mr of 68 Kd, whereas anti-MIRL precipitated a protein with an Mr of 18 Kd. Radiolabeled erythrocyte membrane proteins are shown in lane 7. Left, the positions of DAF and MIRL and the molecular weight standards are indicated. Right, the positions of the radiolabeled erythrocyte membrane proteins are shown. The Greek letters denote the positions of the glycophorin monomers and of the homo and heterodimers.
hemolysis and C3 deposition were subsequently quantified. The combination of MIRL and DAF inhibited lysis by 48% (P < .001), MIRL alone inhibited 56% of the lysis (P < .001), whereas DAF alone inhibited lysis by 23% (P = .002) (Fig 3A).

The combination of DAF and MIRL inhibited C3 deposition by 26% (P = .002), while DAF alone inhibited 46% of the deposition (P < .001), and MIRL alone inhibited C3 deposition by 21% (P = .008) (Fig 3B). This latter finding was unexpected because MIRL was previously thought to inhibit complement-mediated lysis only through regulation of formation of the MAC. Together, these experiments demonstrate that DAF and MIRL inhibit acidified serum lysis by restricting the activity of both the APC C3 convertase and the MAC.

The effects of incorporation of isolated MIRL into the erythrocytes of patient RO were also examined (Fig 4). At each concentration of acidified serum, the MIRL-treated PNH cells had bound approximately 50% less C3 than the untreated PNH cells. These experiments confirm that MIRL inhibits the activity of the amplification C3 convertase of the APC.

**Effects of blocking DAF and MIRL function on susceptibility of normal erythrocytes to acidified serum lysis.** The activities of DAF and MIRL were examined independently and in combination by treating normal erythrocytes with antibodies (Fig 5A). To inhibit activation of the classical but not the APC, acidified serum was treated with Mg/EGTA. By inhibiting the function of both DAF and MIRL, 100% of normal erythrocytes became susceptible to acidified serum lysis. Inhibition of MIRL function also induced complete susceptibility, although the combined antibodies were more effective at the lower concentrations. This observation suggests that when inhibition of MIRL function is incomplete, by blocking DAF function, the residual activity of MIRL becomes overwhelmed. Blockade of DAF function alone caused only partial susceptibility to acidified serum lysis. However, the fact that the dose-response curve developed a plateau phase indicated that DAF inhibition was maximal. As anticipated, nonimmune serum had no effect on susceptibility. These results indicate that both DAF and MIRL participate in regulation of susceptibility to acidified serum lysis, but that MIRL is the primary regulatory factor. Even when the functional activity of DAF is blocked, MIRL is able to inhibit lysis; however, the converse of this is not true.

As anticipated, inhibition of DAF function caused large amounts of activated C3 to be deposited on cells following incubation in acidified serum (Fig 5B). Together with the results of the hemolytic assay (Fig 5A), these findings indicate that MIRL can protect cells from hemolysis even when control of convertase activity is partially lost. Surprisingly, the anti-MIRL-treated cells had bound as much C3 as the anti-DAF-treated cells (P = .744). These results indicate that MIRL has a regulatory effect not only on the activity of the MAC but also on the activity of the C3
ACIDIFIED SERUM LYSIS

Fig 5. Effects of anti-DAF and anti-MIRL on susceptibility of normal human erythrocytes to acidified serum lysis. (A) Normal erythrocytes were incubated with a combination of anti-DAF and anti-MIRL (△), anti-MIRL and NIRS (○), anti-DAF and NIRS (●), or NIRS (□). The amounts shown on the abscissa indicate the total volumes of rabbit serum in the samples divided equally among the additives (eg, the 100 μL samples contained 50 μL of anti-DAF and 50 μL of anti-MIRL, or 50 μL anti-MIRL and 50 μL of NIRS, or 50 μL anti-DAF and 50 μL of NIRS, or 100 μL of NIRS). After washing, the cells were incubated in acidified serum and hemolysis was subsequently quantified. The results are representative of two experiments. Inhibition of DAF and MIRL function alone or in combination induced susceptibility to acidified serum lysis, but MIRL is the more important of the two regulators.

(B) Following incubation in acidified serum, the amount of activated C3 products bound to the cells was quantitated by using a radiolabeled MoAb. The bars depict the mean ± SEM (n = 6). Inhibition of DAF and MIRL function alone or in combination caused cells to lose the capacity to regulate the activity of the C3 convertase. Further, these experiments show that in the acidified serum lysis assay, DAF is unable to control C3 convertase activity completely if MIRL function is inhibited. The cells that were incubated with a combination of antibodies had bound approximately 40% more C3 than the cells treated separately with either anti-DAF (P < .001) or anti-MIRL (P < .001). This finding suggests that the regulatory effects of DAF and MIRL on convertase activity are additive and independent.

The effects of incremental concentrations of anti-MIRL on C3 deposition onto erythrocytes following incubation in acidified serum were also examined (Fig 6). Anti-MIRL caused a dose-dependent increase in C3 deposition with a maximum effect observed when approximately 6 μL of antiserum was used.

To confirm the specificity of the above observations, the effects of rabbit antiserum against glycoporphin and rabbit antiserum against whole human erythrocytes stroma on susceptibility of normal cells to acidified serum were examined. In preliminary experiments, the concentration of each antibody that resulted in binding of an amount of rabbit IgG equivalent to that which bound using saturating amounts of anti-DAF and anti-MIRL was determined by radioimmunobinding assay. This amount of antibody was used in subsequent experiments. Neither anti-glycoporphin nor antihuman red blood cell antiserum, either alone or in combination, induced C3 deposition or susceptibility to acidified serum lysis (data not shown). Further, these antibodies enhanced neither hemolysis nor C3 deposition when used in combination with anti-DAF or anti-MIRL in the acidified serum lysis assays (data not shown).

Effects of blocking DAF and MIRL function on susceptibility of normal erythrocytes to hemolysis in unacidified serum. To inhibit activation of the classical but not the APC, serum was treated with Mg/EGTA (pH 7.4). Inhibition of DAF function did not induce susceptibility to hemolysis in serum that was not acidified (Fig 7A). In contrast, by blocking MIRL function, a portion of the erythrocytes underwent spontaneous lysis. When both DAF and MIRL were inactivated, approximately 80% of the cells hemolyzed in unacidified serum.

C3 deposition on anti-DAF–treated cells was significantly greater than on cells treated with NIRS (P < .001) (Fig 7B). Those data indicate that when DAF function is inhibited, spontaneous activation of the APC occurs. Nonetheless, MIRL was able to protect erythrocytes treated with anti-DAF from hemolysis. However, the converse was not true because erythrocytes treated with anti-MIRL were not protected by DAF. Deposition of C3 onto anti-MIRL–
treated cells was greater than onto anti-DAF–treated cells ($P = .002$), indicating that inhibition of MIRL function resulted not only in failure to regulate the MAC but also in failure to regulate the APC C3 convertase. The fact that C3 deposition was greater on cells treated with both anti-DAF and anti-MIRL compared with cells treated with either antibody alone suggests that DAF and MIRL act in concert to control the activity of the APC.

Together, these results indicate that the capacity of normal human erythrocytes to inhibit spontaneous hemolysis mediated by the APC is dependent on DAF and MIRL. Of the two regulatory proteins, MIRL appears to be the more important because it regulates the activity of both the C3 convertase and the MAC.

**DISCUSSION**

The studies reported herein have shown that the susceptibility of PNH erythrocytes to acidified serum lysis is due to a deficiency of DAF and MIRL. Under conditions of acidification, activation of the APC is favored. However, even under these conditions, the amount of DAF and MIRL on normal erythrocytes is sufficient to inhibit the activity of the APC. Accordingly, following incubation in acidified serum, normal erythrocytes bear only small amounts of C3 activation products and hemolysis is not observed (Fig 1). In contrast, the capacity of PNH erythrocytes to inhibit the activity of the APC is limited because of the deficiency of DAF and MIRL. Under conditions that favor APC activation (acidification of serum), the regulatory capacity on PNH cells is overwhelmed. As a consequence, APC activity proceeds on the cell surface (as evidenced by the presence of large amounts of bound C3 activation products) (Fig 1). PNH cells are hemolyzed in acidified serum because they are deficient in membrane constituents that regulate both the APC (DAF and MIRL) and the MAC (MIRL).

Incubation of PNH III erythrocytes with purified DAF and MIRL did not completely inhibit either hemolysis or C3 convertase activity (Fig 3). There are two plausible explanations for those results. First, proteins other than DAF and MIRL may participate in regulation of susceptibility to acidified serum lysis. Evidence against this hypothesis is provided by the observation that by blocking the functional activity of both DAF and MIRL, normal erythrocytes are rendered completely susceptible to acidified serum lysis (Fig 5). Second, the amount of DAF and MIRL that incorporated into the PNH cells was insufficient to completely inhibit the effects of acidified serum lysis. Evidence in favor of the second explanation is provided by the observation that incubation of affinity-isolated PNH type III cells with DAF and MIRL increased expression (compared with normal) by only 9% and 6%, respectively. Incubation with DAF alone increased expression by 11% and incubation with MIRL alone increased expression by 8%. Technically, it was not feasible to increase the concentration of DAF and MIRL in the reaction mixtures because of the need to have detergent in the buffers containing the proteins. The addition of greater amounts of DAF and MIRL produced concentrations of detergent that induced hemolysis of the erythrocytes.

Recently, it has been shown that antigens of the Cromer blood group complex are located on DAF, and rare cases of a null phenotype called Inab have been reported. Apparently, Inab erythrocytes are completely deficient in DAF, but, unlike PNH erythrocytes, other GPI-linked proteins that have been studied are expressed normally on Inab. Further, Inab cells are resistant to reactive lysis, suggesting that MIRL function is normal. The susceptibility of Inab cells to acidified serum lysis has been examined by two groups. Telen and Green reported that Inab cells were resistant to acidified serum lysis, whereas Merry et al. reported that approximately 5% of Inab cells were hemolyzed in acidified serum. It is unclear why normal erythrocytes treated with anti-DAF are more susceptible to acidified
serum lysis than Inab cells (Fig 5). Variations in the technical aspects of the assay may account for the differences. Alternatively, Inab cells may have quantitative or qualitative differences in other membrane constituents (eg, MIRL and CR1) that control APC activation. Regardless of the explanation, it is clear that DAF is not primarily responsible for controlling susceptibility to acidified serum lysis.

An isolated deficiency of DAF does not result in erythrocytes that manifest the PNH phenotype (individuals with Inab erythrocytes have no demonstrable hematologic abnormalities). However, our studies predict that an isolated deficiency of MIRL would produce the PNH phenotype. A recent report indicates that such a patient has been identified.

The observation that anti-MIRL–treated normal erythrocytes bind large amounts of C3 activation products following incubation in acidified serum (Figs 5B and 6) was unexpected because MIRL was previously thought to inhibit complement-mediated lysis only through regulation of formation of the MAC. Treatment of normal erythrocytes with anti-glycoporphin or anti-whole erythrocyte membrane antibodies did not induce cell-surface activation of the APC. Those results suggest that activation of the APC following incubation with anti-DAF or anti-MIRL or both is due specifically to inhibition of the functional activity of the membrane regulatory proteins rather than to antibody binding to cell surface constituents per se. While the exact site of action is not entirely clear, DAF appears to exert its effect on APC activity by direct interaction with components of the C3 convertase. Further studies are needed to establish the mechanism by which MIRL inhibits C3 convertase activity, but recent studies from our laboratory indicate that a serum cofactor is required.

Hänsch et al have suggested that acidified serum lysis of PNH erythrocytes is a type of reactive lysis. Those investigators presented evidence that acidification of serum results in the formation of a bimolecular complex consisting of C5 and C6 that has the capacity to bind C7. Transiently, the nascent trimerolecular C5–7 complex can bind to erythrocytes and initiate hemolysis by acting as the nidus for the formation of the MAC. However, for the following two reasons it seems unlikely that this mechanism contributes to hemolysis of PNH erythrocytes observed in our studies. First, following incubation in acidified serum, PNH erythrocytes had bound large amounts of activated C3, indicating that the APC had been activated on the cell surface (Fig 2). Second, as controls for our experiments, erythrocytes were incubated in acidified serum chelated with EDTA. Under these conditions, neither hemolysis nor C3 deposition was observed. Activation of the APC is magnesium-dependent but reactive lysis does not require divalent cations. Accordingly, we conclude that acidified serum lysis of PNH erythrocytes is mediated primarily through activation of the APC on the cell surface. However, the possibility that other mechanisms contribute to the hemolysis in a minor way cannot be excluded.

The effects of HRF/C8bp on susceptibility to acidified serum lysis were not investigated. However, the studies reported here suggest that if HRF/C8bp has an effect on the process of acidified serum lysis, it is extremely limited, because by blocking DAF and MIRL, normal erythrocytes are rendered completely susceptible to acidified serum lysis (Fig 5A).

When the functional activity of both DAF and MIRL are blocked, normal erythrocytes undergo hemolysis even in serum of physiologic pH (Fig 7A). The process is mediated by the APC because C3 activation products are present on the cells following the incubation (Fig 7B). Those results show that in the absence of functional DAF and MIRL, APC activation on the cell surface cannot be completely controlled by the endogenous serum regulators of the APC (factor H and factor I).

Normal erythrocytes survive in the circulation for an average of 120 days, whereas PNH III cells survive for 4 to 6 days. It seems likely that the complement-mediated destruction of PNH erythrocytes in vivo results from a profound deficiency of DAF and MIRL such that even under physiologic conditions, cell surface control of APC activation is lost. Previous studies have shown that surface expression of GPI-linked proteins diminish during cellular aging. It seems plausible to hypothesize that this process contributes to the markedly short life-span of PNH erythrocytes. According to this hypothesis, PNH erythrocytes enter the circulation with abnormally low numbers of DAF and MIRL and become more profoundly deficient as they age. When the amount of DAF and MIRL reaches a critically low level such that APC activation can no longer be controlled, the cells spontaneously hemolyze. This mechanism would account for the chronic intravascular hemolysis that is the predominant clinical feature of PNH.

ACKNOWLEDGMENT

The authors thank Dr Mark Holguin and Dr John Bohnsack for critically reviewing the manuscript. Dr Wendell F. Rosse (Duke University Medical Center, Durham, NC) kindly provided the PNH erythrocytes. The excellent technical assistance of Sharon Hall and Mary Bass (Duke University Medical Center) is gratefully acknowledged.

REFERENCES

Affered erythrocytes of patients with paroxysmal nocturnal hemoglobinuria are deficient in the complement regulatory protein decay-accelerating factor. Proc Natl Acad Sci USA 80:5066, 1983


40. Telen MJ, Green AM: The Inab phenotype: Characteriza-


44. Meri S, Morgan BP, Davis A, Daniels RH, Olavesen MG, Waldmann H, Lachmann PJ: Human protectin (CD59), an 18,000-20,000 MW complement lysis restricting factor, inhibits C5b-8 catalysed insertion of C9 into lipid bilayers. Immunology 71:1, 1990


Molecular basis of the enhanced susceptibility of the erythrocytes of paroxysmal nocturnal hemoglobinuria to hemolysis in acidified serum

LA Wilcox, JL Ezzell, NJ Bernshaw and CJ Parker