Induction of the Paroxysmal Nocturnal Hemoglobinuria Phenotype in Normal Human Erythrocytes: Effects of 2-Aminoethylisothiouronium Bromide on Membrane Proteins That Regulate Complement

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To investigate the mechanism by which treatment of normal human erythrocytes with the sulfhydryl reagent 2-aminoethylisothiouronium bromide (AET) induces susceptibility to complement-mediated lysis, the effects of AET on the structural and functional integrity of decay accelerating factor (DAF), membrane inhibitor of reactive lysis (MIRL), and complement receptor type 1 (CR1) were examined. Following treatment with AET, erythrocyte MIRL and CR1 were no longer recognized in situ by antibodies, and antibody binding to DAF was diminished by approximately 50%. These studies indicated that the structural integrity of the three complement regulatory proteins was either partially (DAF) or completely (MIRL and CR1) disrupted by AET. Subsequent experiments showed that functional inactivation paralleled the structural disruption. Treatment of normal erythrocytes with AET induced susceptibility to cobra venom factor-initiated hemolysis, indicating that the functional activity of MIRL had been destroyed. The capacity of erythrocyte CR1 to serve as a cofactor for factor I-mediated cleavage of iC3b to C3c and C3dg was lost following treatment with AET. C3 convertase activity increased markedly following treatment of erythrocytes with AET, but convertase activity on AET cells was approximately 50% less than that observed when DAF function on normal cells was completely inhibited by antibody. Susceptibility of AET cells to acidified serum lysis was shown to be due primarily to inactivation of MIRL. Unexpectedly, in acidified serum the activity of the amplification C3 convertase of the APC was found to be controlled by MIRL as well as by DAF. These studies show that AET induces susceptibility to complement-mediated lysis by disrupting the structural and functional integrity of membrane constituents that regulate the activity of both the C3 convertases and the membrane attack complex of complement.

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proteins, the deficiencies were less profound and PNH I cells were less severely affected than PNH II cells.

In 1965, Sirchia et al reported that normal human erythrocytes treated with the sulfhydryl reagent 2-amino-ethylisothiouronium bromide (AET) manifested a sensitivity to complement similar to that observed for PNH erythrocytes. Subsequently, AET cells have been used in studies aimed at determining the nature of the aberrant interactions of complement with PNH cells. Phenotypically, AET cells resemble the most complement-sensitive PNH III cells in that they have a similar hemolytic profile in the complement lysis sensitivity assay, they are hemolyzed in acidified serum, and they are susceptible to hemolysis initiated by cobra venom factor (CoF). Further, like PNH III erythrocytes, AET cells bind relatively large amounts of C5b-9 during CoF-initiated hemolysis. However, unlike PNH III erythrocytes, AET cells bind only slightly more C3b than normal erythrocytes following activation of the classical pathway of complement.

The mechanism whereby AET induces the transformation of normal erythrocytes into the PNH-like phenotype has been controversial. However, recent observations on the effects of reducing agents on MIRL appear to have provided new insights into the basis of the AET-induced transformation. We have observed that in Western blotting experiments, MIRL was not recognized by antibody when the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed under reducing conditions. Further, following treatment with AET, erythrocytes no longer bind anti-MIRL antibody. Together, these results suggest that AET destroys the structural and functional integrity of MIRL by disrupting intrachain disulfide bonds. To define more precisely the basis for the enhanced susceptibility of AET cells to complement-mediated hemolysis, the effects of AET on the structural and functional integrity of MIRL, DAF, and CR1 were investigated.

**MATERIALS AND METHODS**

**Buffers.** The following buffers were used: veronal-buffered saline (VBS) containing 10 mmol/L sodium barbital (Sigma Chemical Co, St Louis, MO), pH 7.5 and 145 mmol/L NaCl; VBS containing 0.1% gelatin (GVB); GVB containing 1 mmol/L MgCl₂ (GVB'); GVB', pH 6.4 was prepared by titrating GVB' with 1 mol/L HCl; GVB containing 5 mol/L EDTA (GVB-EDTA); GVB containing 0.15 mmol/L NiCl₂ (GVB-Ni); phosphate-buffered saline (PBS) containing 150 mmol/L sodium chloride and 10 mmol/L sodium phosphate, pH 7.4.

**Preparation of AET erythrocytes.** Erythrocytes from normal donors were obtained by phlebotomy and stored in Alsever's solution at 4°C. AET was purchased from Sigma Chemical Co. Normal erythrocytes were washed three times in PBS, and after each wash the top 10% of the cell pellet was aspirated to remove theuffy coat. An aliquot of 1 mL of packed cells was added to 4 mL of an 8% (wt/vol) solution of AET that had been titrated to pH 8.0 with HCl. The mixture was incubated at 37°C with constant agitation. After exactly 9 minutes, the cells were washed three times with GVB.

**Antibodies and complement components.** Polyclonal antiserum against MIRL and DAF was prepared using previously described methods. Monoclonal antibody (MoAb) against CR1 was a gift from Dr Eric Brown (Washington University School of Medicine, St Louis, MO). The IgG was isolated from ascites by using caprylic acid. 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. Affinity-purified antimouse IgG and monoclonal antirabbit IgG were purchased from Sigma Chemical Co. The monoclonal antirabbit IgG was in the form of ascites, and the IgG was isolated by using caprylic acid. CoF, C3, factor B, factor D, and C3 nephritic factor were isolated using methods that have been previously described. Protein concentrations were determined either spectrophotometrically or by using the bichinchoninic acid (BCA) protein assay (Pierce Chemical Co, Rockford, IL) using bovine serum albumin (BSA) as the standard. Activated C3 complexes (CoFBb) were prepared according to reference 9.

**Radiolabeling.** Monoclonal anti-C3d, monoclonal antirabbit IgG, affinity-purified antimouse IgG, and human C3 were labeled with ¹¹¹I as NaI (Amersham Corp, Arlington Heights, IL) by using IODO-GEN (Pierce Chemical Co).

**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 ¹²⁵I-labeled standards purchased from Amersham Corp. Autoradiographs were prepared by exposing dried gels to X-Omat AR film (Eastman Kodak Co, Rochester, NY) at -70°C.

**Quantitation of MIRL, DAF, and CR1 on normal and AET erythrocytes.** Quantitation of MIRL, DAF, and CR1 by using an indirect radioimmunoassay was performed using a minor modification of the method of Holguin et al. In that radioiodinated monoclonal antirabbit IgG (for MIRL and DAF quantitation) or affinity-purified antimouse IgG (for CR1 quantitation) were substituted for radiolabeled affinity-purified polyclonal antirabbit IgG. In each case, equilibrium binding studies were performed to determine the amount of the primary and secondary antibodies that were required to produce saturation binding.

**Susceptibility of AET erythrocytes to lysis by CoFBb complexes.** AET erythrocytes were incubated with CoFBb and incremental concentrations of normal serum (the complement source) that had been eluted with EDTA. The methods for these experiments are described in detail elsewhere. Briefly, normal and AET cells bearing the nephritic factor-stabilized APC C3 convertase were incubated with radiolabeled C3. After washing, each set of cells was divided into equal aliquots, and one aliquot was incubated at 37°C with heat-inactivated serum as a source of factor H and factor I according to the method of Ross et al. The other aliquot was incubated with GVB. After 30 minutes, the cells were washed with GVB-EDTA. Next, hemoglobin-free ghosts were prepared, and membrane proteins were solubilized in SDS. Ester-bound activation and degradation products of C3 were dissociated by incubating the membrane proteins in 1 mol/L NH₄OH at alkaline pH. After dialysis, C5 fragments were analyzed by SDS-PAGE and autoradiography.

**Determination of the activity of the amplification C3 convertase of the APC on erythrocytes.** The procedure has been described in detail elsewhere. Briefly, erythrocytes were washed three times with GVB and resuspended to 1 x 10⁷/mL. To serve subsequently as a control for nonspecific binding of radiolabeled anti-C3d, an aliquot of 100 μL was diluted to 500 μL in GVB and stored on ice. To form the nidus for formation of the amplification C3 convertase, cells were incubated with C3 and activated CoF complexes. After washing, an aliquot of these cells called EC3b, was resuspended to 2 x 10⁷/mL and kept on ice for subsequent determination of the amount of C3b bound. The remainder of the EC3b, was washed in...
GVB-Ni, and the nickel-stabilized C3 convertase (C3bBb) was generated by incubating the cells at 37°C with factor B and factor D. After 3 minutes, the cells were washed once in GVB-Ni, 100 μL of C3 (2.5 mg/mL) were added, and the incubation was continued. After 20 minutes, the cells (called EC3b) were washed three times in GVB and resuspended to 2 × 10^9/mL. The amount of C3b on EC3b, and EC3b, was determined by using radiolabeled anti-C3d.46 In triplicate, 50-μL aliquots of the samples were incubated with 50 μL of monoclonal radiolabeled anti-C3d (20 μg/mL) at 37°C. After 30 minutes, 75 μL of the sample was pipetted onto 300 μL of a mixture of phthalate esters contained in 400-μL microfuge tubes. Following centrifugation (Beckman Microfuge 12, Beckman Instruments, Inc, Palo Alto, CA) for 3 minutes at 8,000g, 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 M₀ of 160 Kd. 

Effects of blocking DAF, MIRL, and CR1 on the activity of the amplification C3 convertase of the APC. Anti-DAF, anti-MIRL, and nonimmune rabbit serum were chelated by adding EDTA to a final concentration of 10 mmol/L and incubating the mixture for 5 minutes at 37°C. Normal and AET erythrocytes were incubated with saturating amounts of anti-DAF, anti-MIRL, or anti-CR1 either alone or in combination. For controls, nonimmune rabbit serum or GVB was substituted for the antisera or for the anti-CR1 antibody.

Serum reagents. Acidified serum was prepared by mixing 125 μL of 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 here, acidified serum contained 2.5 mmol/L MgCl₂ and 8 mmol/L EGTA. This reagent was prepared by mixing 1 volume of a stock solution of 25 mmol/L MgCl₂ and 80 mmol/L EGTA (Sigma Chemical Co), pH 6.4, with 9 volumes of acidified serum and incubating the mixture for 5 minutes at 37°C.

Acidified NHS containing 10 mmol/L EDTA (acidified NHS-EDTA) was prepared by mixing 1 volume of 100 mmol/L EDTA, pH 6.4 with 9 volumes of NHS, pH 6.4 and incubating the mixture for 5 minutes at 37°C. Acidified NHS containing 10 mmol/L EDTA (acidified NHS-EDTA) was prepared by mixing 1 volume of 100 mmol/L EDTA, pH 6.4 with 9 volumes of NHS, pH 6.4 and incubating the mixture for 5 minutes at 37°C. This reagent was used as a negative control in the acidified serum lysis experiments.

To ensure 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-Mg/EGTA (pH 7.4), or NHS-EDTA. After 30 minutes, the mixtures were centrifuged and hemolysis was measured 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-Mg/EGTA or NHS-EDTA.

Susceptibility of erythrocytes to acidified serum lysis. Erythrocytes were washed three times in GVB, once in GVB', pH 6.4 and resuspended to 5 × 10⁹/mL. 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 the appropriate dilution of acidified serum. At the same time, cells were incubated with reagents that served to control for spontaneous lysis in the presence of buffer or acidified NHS-EDTA. Lysis of 100% of the cells was achieved by using 0.04% NH₄OH. After 30 minutes, 850 μL of GVB-EDTA were added, the samples were transferred to 1.5-mL microfuge tubes and centrifuged for 5 minutes at 11,500g. An aliquot of 100 μL of supernate was recovered and free hemoglobin was determined by measuring the A₄₁₀. The values were used to calculate hemolysis.

Effects of blocking DAF, MIRL, and CR1 on the susceptibility of erythrocytes to acidified serum lysis. Normal and AET erythrocytes were washed three times in GVB and resuspended to 5 × 10⁹/mL. Anti-DAF, anti-MIRL, and anti-CR1 and control reagents were prepared as described above. Erythrocytes were incubated at 37°C with optimal concentrations of the antibodies and the control reagents either alone or in combination. After 30 minutes, the cells were washed once with 4 mL of GVB', pH 6.4 and resuspended to 5 × 10⁹/mL. Aliquots of 50 μL of cells were incubated with 100 μL of the appropriate dilution of acidified serum, and hemolysis was quantified subsequently as described above.

Quantitation of C3 deposition on erythrocyte membranes following incubation in acidified serum. Following removal of an aliquot of the supernate for determination of free hemoglobin, the remainder of the supernate was aspirated and the cells were resuspended to 100 μL in GVB-EDTA. In duplicate, 40 μL of cells were incubated at 37°C with 50 μL of radiolabeled anti-C3d (20 μg/mL). Pilot experiments had shown that this concentration of antibody was saturating. After 30 minutes, aliquots of 75 μL of each reaction mixture were pipetted into a 400-μL polyethylene microfuge tube containing 300 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 M₀ of 160 Kd.

RESULTS

Effects of AET on recognition of erythrocyte DAF, MIRL, and CR1 by antibody. Following treatment of normal erythrocytes with AET, binding of anti-MIRL and anti-CR1 was abolished, suggesting that AET had disrupted the structural integrity of the two proteins (Table 1). In contrast, binding of anti-DAF to AET cells was only partially diminished (Table 1). These results are consistent with the concept that following treatment with AET a portion of crythrocyte DAF remains structurally intact.

Effects of AET on the functional integrity of DAF, MIRL, and CR1. The experiments described above suggested that the structural integrity of the three complement regulatory proteins was either partially or completely destroyed by AET. The following experiments were performed to test the effects of AET on the functional integrity of MIRL, CR1, and DAF.

Normal human erythrocytes are resistant to CoFBB-induced hemolysis because MIRL blocks assembly of the

| Table 1. Binding of Anti-DAF, Anti-MIRL, and Anti-CR1 to AET Erythrocytes |
|-----------------------------|-----------------------------|-----------------------------|
| Anti-DAF*                   | Anti-MIRL*                  | Anti-CR1†                   |
| 57 ± 5                     | 4 ± 1                       | 0                           |

The values (the mean ± SD of six determinations) are presented as a percent of normal. Specific binding of the radiolabeled second antibody to the normal controls treated with the respective primary antibodies was as follows: anti-DAF (16,377 ± 1,933); anti-MIRL (74,264 ± 5,433); anti-CR1 (14,215 ± 2,309).

* Rabbit antiserum was used as the source of the primary antibody.

† Folated IgG from ascites was used as the source of the primary antibody.
membrane attack complex on the cell surface. However, treatment with AET caused normal cells to become markedly sensitive to CoFBB-induced hemolysis (Fig 1). These results, along with those shown in Table 1, indicate that AET disrupts the functional as well as the structural integrity of MIRL.

Erythrocyte CR1 has two known functions. First, it has decay-accelerating activity for the amplification convertase of the APC. Second, it serves as a cofactor for factor I-mediated cleavage of C3b and iC3b. To test the effects of AET on CR1 cofactor activity, normal and AET erythrocytes bearing radiolabeled C3b were incubated with heat-inactivated serum as a source of factor H and factor I. Membrane-associated C3 was subsequently analyzed by SDS-PAGE and autoradiography. As evidenced by the virtual absence of the α chain of C3b and iC3b, essentially all of the C3b bound to normal erythrocytes incubated in heat-inactivated serum was degraded past the stage of iC3b (Fig 2, lane 2).

Factor H can serve as a cofactor for factor I-mediated cleavage of C3b to iC3b, but CR1 cofactor activity is required for factor I-mediated cleavage of iC3b to C3c and C3dg. Thus, the presence of a prominent α68 band in lane 3 of Fig 2 indicates that AET has destroyed the cofactor activity of CR1.

As evidenced by the presence of the α chain of C3b, when normal (Fig 2, lane 4) and AET (Fig 2, lane 5) EC3b were incubated in buffer, C3b was not degraded. Those results confirm that serum factors are required for degradation of erythrocyte-bound C3b. The C3α chain is present in all lanes, indicating that unactivated C3 bound nonspecifically to the cells. The band representing a protein with an apparent Mr of 46 Kd seen in all lanes is an autocleavage product that is generated during isolation of C3.

The formation and stability of the amplification C3 convertase of the APC is controlled, at least in part, by erythrocyte DAF. To test the effects of AET treatment on DAF function, normal and AET erythrocytes were incubated with CoFBB and C3. This process results in the deposition of a relatively small number of randomly deposited C3b molecules (EC3b) that can act as the nidus for the subsequent formation of amplification C3 convertases. After incubating the EC3b, with factor B and factor D in the presence of NiCl₂ to form the nickel-stabilized amplification C3 convertase of the APC (C3BBb), C3 convertase activity is assessed by quantitating the amount of C3b bound following a subsequent incubation with C3 (EC3b).
Exposure of normal EC3bBb to C3 resulted in no further deposition of C3b, suggesting that the activity of the C3 convertase was inhibited by membrane regulators of the APC (Fig 3). C3 convertase activity was observed on the AET cells; however, convertase activity on AET cells was only 53% as great as that on anti-DAF-treated normal erythrocytes (Fig 3). Thus, in contrast to MIRL and CR1, the structural (Table 1) and functional (Fig 3) integrity of DAF is only partially disrupted by AET.

As discussed above, CR1 also has decay-accelerating activity. To delineate more precisely the membrane constituents that regulate C3 convertase activity on human erythrocytes, normal and AET cells were incubated with anti-DAF, anti-MIRL, and anti-CR1 either alone or in combination, and the effects of those treatments on C3 convertase activity were analyzed by using the procedure described above. When AET cells were incubated with anti-DAF, C3 convertase activity was much greater than that on either AET cells or on normal cells treated with anti-DAF (Fig 4). Those results confirmed that AET cells have residual DAF activity. The fact that the anti-DAF-treated AET cells had greater convertase activity than anti-DAF-treated normal cells, however, is consistent with the hypothesis that a membrane constituent other than DAF also regulates C3 convertase activity and that the functional activity of that constituent is destroyed by AET.

Inhibition of MIRL function had no effect on C3 convertase activity (Fig 4). Further, when both DAF and MIRL were blocked simultaneously, convertase activity was equivalent to that observed with DAF alone was blocked (Fig 4). Those results are consistent with the concept that MIRL has no direct effect on components of the C3 amplification convertase of the APC.

Inhibition of CR1 function had no effect on convertase activity (Fig 4). However, when both DAF and CR1 were blocked simultaneously, C3 convertase activity was equivalent to that observed when DAF function on AET cells was completely inhibited (Fig 4). Those results show that DAF can control convertase activity in the absence of CR1. However, by blocking DAF function the regulatory effect of CR1 becomes apparent. The observation that convertase activity on anti-DAF-treated AET cells is equivalent to that on normal cells treated with a combination of anti-DAF and anti-CR1 is consistent with the hypothesis that AET destroys the decay-accelerating activity of CR1 as well as the cofactor activity.

Susceptibility of AET erythrocytes to acidified serum lysis. In the experiments described above, isolated components were used to assess C3 convertase activity. To test the effects of AET treatment on APC activity in a whole serum system, erythrocytes were incubated in acidified serum. The acidified serum used in these experiments contained Mg/EGTA so that classical pathway of complement but not APC activity was inhibited. 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 of the APC (C3bBb) is favored.

Like PNH erythrocytes, AET cells are hemolyzed in acidified serum, but normal erythrocytes are resistant to this process (Fig 5A). By blocking residual DAF activity, the susceptibility of AET cells is markedly enhanced (Fig 5A). Those results confirm that DAF activity is present on AET cells, and that DAF participates in regulation of susceptibility to acidified serum. In contrast to the situation with AET cells, inhibition of DAF activity has only a modest effect on susceptibility of normal erythrocytes to acidified serum lysis (Fig 5A). Those results show that other membrane constituents, in addition to DAF, influence susceptibility to acidified serum and that those constituents are inactivated by AET.

Following incubation in acidified serum, normal erythrocytes have bound relatively small amounts of activated C3. Those results indicate that membrane constituents working in concert with serum factors inhibit C3 convertase activity (Fig 5B). As anticipated, anti-DAF–treated normal erythrocytes have bound greater amounts of activated C3 than untreated normal cells following incubation in acidified serum (Fig 5B). Those results were anticipated because by blocking DAF function on normal erythrocytes, membrane control of C3 convertase activity is at least partially inhibited.

AET cells bound approximately the same amount of activated C3 as anti-DAF–treated normal erythrocytes. Inasmuch as AET cells have approximately 50% of the normal amount of DAF activity, those results suggested...
that membrane constituents, in addition to DAF, control C3 convertase activity and that those constituents are inactivated by AET. Evidence supporting this interpretation was provided by experiments in which residual DAF activity on AET cells was blocked with antibody. Following incubation in acidified serum, anti-DAF–treated AET cells had bound much greater amounts of activated C3 than normal cells treated with anti-DAF (Fig 5B).

**Erythrocyte regulation of APC C3 convertase activity using a whole serum system.** To determine if C3 convertase activity using a whole serum system is controlled by the same membrane constituents that regulate convertase activity when isolated components of the convertase are used, normal and AET erythrocytes were incubated with anti-DAF, anti-CR1, and anti-MIRL either alone or in combination. After washing, the cells were incubated in acidified serum, and C3 deposition was subsequently quantified (Figs 6 and 7).

Consistent with the results shown in Fig 5B, the amount of activated C3 bound to AET cells was less than that bound to anti-DAF–treated erythrocytes (Fig 6). Further, anti-DAF–treated AET cells bound greater amounts of activated C3 than anti-DAF–treated normal cells (Fig 6). As was the case using isolated components (Fig 4), inhibition of CR1 had no effect on convertase activity in the whole serum system. However, in contrast to the experiments using isolated components, simultaneous inhibition of DAF and CR1 did not cause increased deposition of C3 above that seen when the function of DAF alone was blocked (Fig 6). Those results indicate that, in a whole
Fig 6. Effects of DAF and CR1 on C3 deposition following incubation of erythrocytes in acidified serum. The functional activity of DAF, CR1, and MIRL either alone or in combination was inhibited on normal and AET cells, and the cells were incubated in acidified serum. After washing, C3 deposition was quantitated. The bars depict the mean ± SEM (n = 4). In acidified serum, DAF but not CR1 modulates C3 convertase activity.

In a whole serum system, CR1 is not an important regulator of C3 convertase activity. When normal erythrocytes were incubated with a combination of anti-DAF, anti-CR1, and anti-MIRL, C3 deposition was equivalent to that observed with anti-DAF-treated AET cells (Fig 6). Those data suggested the possibility that, in a whole serum system, MIRL influenced the functional activity of the APC C3 convertase. To test that hypothesis, the functional activity of MIRL was inhibited on normal cells (Fig 7). Following incubation in acidified serum, normal erythrocytes that had been treated with anti-MIRL had bound more activated C3 than anti-DAF–treated normal cells (Fig 7). Thus, in a whole serum system MIRL is an important regulator of C3 convertase activity. Normal cells treated with both anti-DAF and anti-MIRL had bound as much activated C3 as anti-DAF–treated AET cells (Fig 7). Those results indicate that DAF and MIRL act in concert to control C3 convertase on erythrocytes when the APC is activated in a whole serum system.

Fig 7. Effects of MIRL on C3 deposition following incubation of erythrocytes in acidified serum. The functional activity of DAF and MIRL either alone or in combination was inhibited on normal and AET cells, and the cells were incubated in acidified serum. After washing, C3 deposition was quantitated. The bars depict the mean ± SEM (n = 4). In acidified serum, MIRL regulates C3 convertase activity.

Fig 8. Effects of MIRL, DAF, and CR1 on susceptibility of erythrocytes to hemolysis in acidified serum. The functional activity of MIRL, DAF, and CR1 either alone or in combination was inhibited on normal erythrocytes. After incubation in 100 μL of acidified serum, hemolysis was quantified. The bars depict the mean ± SEM (n = 3). Susceptibility to acidified serum is controlled primarily by MIRL.

Regulation of susceptibility of erythrocytes to acidified serum lysis. Normal human erythrocytes are resistant to acidified serum lysis, and inhibition of CR1 function has no effect on susceptibility (Fig 8). Following inhibition of DAF function, approximately 20% of the cells were hemolyzed by acidified serum (Fig 8). Simultaneous inhibition of both DAF and CR1 caused a modest increase in susceptibility above that seen when DAF alone was blocked (Fig 8). In contrast, inhibition of MIRL function caused 100% susceptibility to acidified serum (Fig 8). Those results show that susceptibility to acidified serum is controlled primarily by MIRL. In this system, MIRL appears to regulate the activity of both the APC C3 convertase and the MAC.

DISCUSSION

The purpose of these studies was to determine the effects of AET on erythrocyte membrane proteins that regulate complement. The results have shown that approximately...
50% of erythrocyte DAF is inactivated by AET while essentially all of the activity of CR1 and MIRL is destroyed. These observations establish the basis for the similarities and differences between AET and PNH erythrocytes in their interactions with complement.

AET erythrocytes resemble PNH III cells in that, following incubation with activated CoF complexes and human serum (the complement source), the cells are hemolyzed because they fail to regulate formation of the MAC.\textsuperscript{9} A consequence, both AET and PNH III erythrocytes have bound relatively large amounts of C5b-9.\textsuperscript{9} This process is controlled by MIRL, but DAF does not participate.\textsuperscript{9,53} Both PNH and AET cells lack MIRL activity. In the case of PNH III cells, the protein is absent,\textsuperscript{9} while for AET cells, MIRL is chemically inactivated.

Both AET and PNH III erythrocytes are abnormally susceptible to hemolysis initiated by the classical pathway.\textsuperscript{33} However, during this process PNH erythrocytes bind greater amounts of activated C3 than AET cells (although AET cells bind more activated C3 than normal erythrocytes).\textsuperscript{28} PNH III cells are profoundly deficient in both DAF and MIRL,\textsuperscript{18} while AET cells have no functional MIRL but have approximately 50% of normal DAF function. Thus, the difference in C3 binding appears to be due to the fact that the residual DAF activity on AET cells partially controls the classical pathway C3 convertase. The effects of MIRL on the classical pathway C3 convertase have not been determined.

Following incubation in acidified serum (a process that induces activation of the APC), both PNH III and AET cells are hemolyzed,\textsuperscript{17} and both have bound relatively large amounts of activated C3.\textsuperscript{34,44} The studies reported here have shown that, in acidified serum, the activity of the APC C3 convertase is controlled by MIRL as well as by DAF. Thus, the binding of activated C3 to AET cells is due to absence of MIRL function as well as to the partial inactivation of DAF. In experiments that compared binding following incubation in acidified serum, AET and PNH III cells were observed to bind equivalent amounts C3b (not shown). Those results suggest that, in acidified serum in the absence of normal MIRL function, the residual DAF activity on AET cells is functionally insignificant.

Another difference between AET and PNH cells is that AET cells have no CR1 activity while PNH III cells have a partial deficiency.\textsuperscript{33,35} Our studies have shown that CR1 has regulatory activity for the APC C3 convertase (Fig 4). While CR1 was originally isolated based on its capacity to decay accelerate the APC C3 convertase on sheep cells,\textsuperscript{1} we are unaware of previous studies that have shown that CR1 functions in situ to regulate the convertase on human erythrocytes. However, for the inhibitory activity of CR1 to be observed, DAF function must be blocked (Fig 4). The inhibitory activity of CR1 was observed using isolated components of the APC. In the acidified serum lysis assay, however, CR1 had no obvious effect on APC activity even if DAF function were blocked (Fig 5). Thus, in a whole serum system, the inhibitory activity of CR1 may be obscured by the presence of factor H.

Even though the average amount of CR1 on PNH erythrocytes is less than normal, because the normal range for CR1 expression is extremely wide, the absolute number of CR1 molecules on PNH erythrocytes usually falls within the normal range.\textsuperscript{33,35} Further, rigorous studies have shown that CR1 function on PNH cells is normal.\textsuperscript{44} Together, those observations suggest that the partial deficiency of CR1 does not make an important contribution to the aberrant interaction of PNH erythrocytes with complement, especially in a whole serum system.

The observation that anti-MIRL-treated normal erythrocytes bound large amounts of activated C3 following incubation in acidified serum was unexpected because previous studies had shown that MIRL inhibits complement-mediated lysis by blocking formation of the MAC.\textsuperscript{9} Unlike DAF and CR1, the effects of blocking MIRL function on C3 convertase activity were not observed when purified components were used (Fig 4). The simplest interpretation of those results is that MIRL requires a serum cofactor to regulate APC C3 convertase activity. Studies aimed at investigating this hypothesis are being undertaken.

A number of different reagents have been used to induce the PNH phenotype in normal erythrocytes.\textsuperscript{69-92} Our studies suggest that the sulfhydryl compounds used for this purpose (eg, AET, glutathione, cysteine, and dithiothreitol) work by disrupting intrachain disulfide bonds that are critical for the structural and functional integrity of membrane constituents that regulate complement. AET was used in these studies because of the consistency and reproducibility of its effects, and the fact that in previous investigations complement interactions with AET and PNH cells have been compared.\textsuperscript{13,38} The conditions for AET treatment were selected based on the results of studies by Sirchia and Dacie\textsuperscript{37} that showed treatment with an 8% solution for 9 minutes at 37°C produced a cell that resembled PNH III cells in complement lysis sensitivity assay. Incubation for shorter periods of time produced cells that were less sensitive to complement, suggesting that the functional activity of the membrane regulatory proteins had been less completely disrupted.

DAF and CR1 share a considerable amount of structural homology.\textsuperscript{35} Both proteins are comprised largely of a series of contiguous short consensus repeats of approximately 60 amino acids with each repeat containing four cysteines. The fact that AET completely inactivates CR1 but not DAF suggests that in situ, tertiary structural characteristics of DAF protect some disulfides from reduction by AET.

Although not structurally related to DAF and CR1, the derived amino acid sequence shows that MIRL has 10 cysteine residues.\textsuperscript{14,20,21} Clearly, other membrane constituents that depend on intrachain disulfides for their structural and functional integrity will also be affected by AET. For example, erythrocyte acetylcholinesterase (a GPI-linked protein that is deficient in PNH) is partially inactivated by sulfhydryl reagents.\textsuperscript{9,50}

PNH III erythrocytes are also deficient in HRF/C8bp, but the relationship of this deficiency to the enhanced susceptibility to complement-mediated hemolysis is not entirely clear. Recently, Young et al\textsuperscript{34} reported that HRF/C8bp is immunochemically related to C8 and C9 through...
cysteine-rich domains. Thus, it is possible that AET may also disrupt the structural and functional integrity of HRF/C8bp by disrupting intrachain disulfides. Further studies are required to confirm this hypothesis.

The studies reported here have demonstrated that, while there are similarities between PNH and AET cells, important differences also exist. Accordingly, it would be inappropriate to imply that normal erythrocytes are transformed into PNH cells by treatment with AET. Nonetheless, AET cells have provided interesting insights into the mechanisms by which membrane constituents regulate the activity of complement.

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