Characterization of the Complement Sensitivity of Calcium Loaded Human Erythrocytes

By Samuel T. Test, Peter Bütkofer, Maggie C. Yee, Frans A. Kuypers, and Bertram Lubin

A deficiency of membrane proteins having a glycosyl-phosphatidylinositol (GPI) anchor is characteristic of the erythrocytes of paroxysmal nocturnal hemoglobinuria (PNH) and is currently believed to be the basis for the enhanced susceptibility to lysis by activated complement observed in these cells. Our recent observation that GPI-anchored proteins are preferentially lost into membrane vesicles shed from normal erythrocytes after calcium loading led us to examine the hypothesis that the remnant erythrocytes might also have increased sensitivity to complement-mediated hemolysis. Indeed, red blood cells treated in such a manner became more sensitive to lysis by antibody and complement or to lysis initiated by activated cobra venom factor complexes (CoFBB). As a consequence of membrane vesiculation, the erythrocytes lost up to ~50% of their immunoreactive decay-accelerating factor and 25% to 30% of their immunoreactive membrane inhibitor of reactive lysis (MIRL). Closer examination of the defect responsible for the marked increase in sensitivity to CoFBB-initiated hemolysis seen in calcium-loaded erythrocytes showed that a complex combination of factors produced the defect. These included a decrease in both functional and immunoreactive MIRL and depletion of intracellular potassium and adenosine triphosphate (ATP). These results suggest the possibility that loss of DAF and MIRL via membrane vesiculation, as well as decreases in intracellular potassium and/or ATP, might contribute to the phenotype of PNH erythrocytes. Further, normal or pathologic red blood cells might develop a PNH-like defect after membrane vesiculation if sufficient decreases in potassium and ATP also occurred.

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OVER TWO decades ago, it was discovered that incubation of human erythrocytes with a variety of chemical agents rendered them highly sensitive to lysis by activated complement.1-3 Red blood cells (RBCs) treated in such a manner were used in model systems of the disease paroxysmal nocturnal hemoglobinuria (PNH), a disease characterized by an enhanced susceptibility of all the formed blood elements to lysis by activated complement.1 The biochemical abnormality responsible for the pathophysiology of PNH has become increasingly better understood in recent years, as a number of cell membrane proteins that function to protect the cell from lysis by complement have been described. Decay-accelerating factor (DAF), a single-chain glycoprotein of M, 70,000, both inhibits formation and induces destabilization of the classical and alternative pathway C3 convertases, C8 binding protein (C8 hp) or homologous restriction factor (HRF) is a 65- to 70-Kd protein that appears to act by preventing C9 polymerization in the nascent lytic pore of the C5b-9 membrane attack complex. Membrane inhibitor of reactive lysis (MIRL) 4 is an 18-Kd membrane protein recently shown to be identical to CD59, p18,9 and HRF20 and also appears to act by interfering with lysis mediated by the membrane attack complex.8 These proteins have in common a glycosyl-phosphatidylinositol (GPI) moiety at the carboxyl terminus of the protein that anchors the protein to the cell membrane.10 It is now believed that this feature is shared by all of the proteins that have been found to be missing from the cells of patients with PNH.11

During studies of the process of membrane vesiculation induced in human erythrocytes by calcium loading, we discovered that at least two GPI-anchored proteins, acetylcholinesterase (AChE) and DAF, were enriched in the released vesicles.12 This suggested that the RBCs remaining after vesiculation might develop a selective deficiency of GPI-anchored complement regulatory proteins and consequently develop a PNH-like defect. Because calcium loading has not previously been shown to induce alterations in the sensitivity of RBCs to complement-mediated lysis, we investigated the effects of such treatment on lysis of human erythrocytes by antibody and complement or by activated cobra venom factor (CoF) complexes (reactive lysis). Changes in the quantities of immunoreactive DAF and MIRL were also assessed to determine if decreases in these proteins were sufficient to explain the observed changes in susceptibility to lysis by activated complement.

MATERIALS AND METHODS

Reagents and Buffers

Purified cobra venom factor (Naja naja kaouthia), CoF), factor B, and factor D were obtained from Cytotech (now Quidel), Inc (San Diego, CA). Purified factor B, purified human MIRL, and activated CoF complexes (CoFBB) were generously supplied by Dr Charles J. Parker (University of Utah School of Medicine, Salt Lake City). CoFBB were also prepared by a slight modification of the method of Holguin et al. Briefly, 100 µL of CoF (200 µg/mL) was incubated with 100 µL of factor B (1 mg/mL), 50 µL of factor D (100 µg/mL), and 50 µL of DGVB containing 5 mmol/L MgCl2 at 37°C. After 60 minutes, 27 µL of 0.15 mol/L EDTA was added and the incubation continued for 5 minutes. The complexes were used immediately or stored at ~70°C. Bovine albumin (fraction V), calcium ionophore A23187, dibutyl phthalate, disopropylfluorophosphate (DFP), histamine dihydrochloride, Sigma 104 phosphatase substrate, valinomycin, and nystatin were from Sigma Chemical Co (St Louis, MO). Valinomycin was prepared as a 10 mmol/L stock in Me2SO (Mallinkrodt, Inc, St Louis, MO). Nystatin was prepared as a 5 mg/mL stock in Me2SO. Other reagents included gelatin (Difco, Inc, Detroit, MI), Tween-20 (Immunologic grade, BioRad Laboratories, Richmond, CA), NP-40 (Calbiochem, 3056-3065

Antibodies

Human serum containing a cold agglutinin of high lytic titer was generously provided by Dr. George Garratty (American Red Cross Blood Services of Greater Los Angeles, Los Angeles, CA). The cold reactive antibody (anti-I) was purified according to the method of Rosse et al.14 and quantitated by radial immunodiffusion for human IgM (The Binding Site, Inc, San Diego, CA). The mouse monoclonal antibodies IA10 and IIH6 to DAF were kindly supplied by Drs. Michael A. Davitz and Victor Nussenzweig (New York University Medical Center, New York). A rabbit polyclonal antiserum to membrane inhibitor of reactive lysis (MIRL) was provided by Dr. Charles J. Parker. Rabbit polyclonal anti-human RBC (IgG fraction) was purchased from Accurate Chemical and Scientific Corp (Westbury, NY). Mn²⁺-purified 125I-donkey F(ab')₂ anti-rabbit IgG was obtained from Amersham Corp (Arlington Heights, IL). Affinity-purified alkaline phosphatase-conjugated goat anti-rabbit IgG was purchased from Caltag, Inc (South San Francisco, CA).

Erythrocytes

Erythrocytes were obtained from the peripheral venous blood of healthy adult donors. The blood was anticoagulated with heparin and subsequently washed three times with 0.9% saline and once with the appropriate buffer (see below).

Calcium Loading of Normal Erythrocytes

Erythrocytes at a hematocrit of ~18% were equilibrated in TBS supplemented with 0.2% glucose and 1 mmol/L Ca²⁺ for 5 minutes at 37°C. Subsequently, the calcium ionophore A23187 was added to the RBC suspension from a 10 mmol/L stock solution in ethanol to give a final concentration of 4 µmol/L. The incubation was continued for 1 hour at 37°C and terminated by the addition of an equal volume of TBS containing 5 mmol/L EDTA to the suspension. Calcium loading causes vesiculation of the cell membrane and the loss of GPI-anchored proteins into shed vesicles could readily be monitored by measuring release of ACHe from RBCs into the cell-free supernatant after low-speed centrifugation.13 ACHe activity was measured according to the method of Ellman et al.13 Shape change of erythrocytes was followed by fixing the cells in 3% glutaraldehyde in 0.9% saline and examination by light microscopy using Zeiss Nomarsky optics.

After incubation with Ca²⁺ and A23187, erythrocytes were pelleted by low-speed centrifugation (5 minutes, 800g, 4°C) and the vesicle-containing supernatant was saved. Subsequently, remnant RBCs were washed at least three times with TBS containing 2 mmol/L EDTA to remove any contaminating vesicles or membrane ghosts. Vesicles from the original supernatant were combined with the wash supernatants and were pelleted by centrifugation (30 minutes, 100,000g, 4°C). Pink ghosts that overlaid the tightly packed vesicle pellet were carefully removed by aspiration. Vesicles were washed three times with large volumes of hypotonic buffer (10 mmol/L Tris, pH 7.50, containing 2 mmol/L EDTA). Both washed remnant RBCs and vesicles were found to be free of contaminating ghosts by light microscopy.

In some experiments, erythrocytes were incubated with Ca²⁺ alone or with A23187 as above except that 60 mmol/L histamine dihydrochloride was added to inhibit transglutaminase-mediated protein cross-linking.15 In other experiments, RBCs were incubated with Ca²⁺ with or without ionophore in 10 mmol/L Tris-HCl, pH 7.40, containing 144 mmol/L KCl, 0.2% glucose, and 0.4 mmol/L quinine sulfate to prevent K⁺ efflux.17

Alterations in Potassium Content of Normal or Treated Erythrocytes

To deplete intracellular potassium, normal erythrocytes were washed and suspended at 3.5 × 10⁶/mL in 70 mmol/L NaCl, 70 mmol/L NaSCN, 10 mmol/L HEPES, pH 7.45. Valinomycin was then added to give a final concentration of 40 µmol/L and the mixture incubated for 45 minutes at room temperature. The incubation was terminated by washing the erythrocytes in DGVB-EDTA. This treatment resulted in a greater than 90% decrease in intracellular K⁺, but had no effect on intracellular adenosine triphosphate (ATP).

Restoration of intracellular potassium in erythrocytes having undergone a decrease in intracellular K⁺ as a consequence of calcium loading was accomplished by exposing the cells to nystatin while suspended in 134 mmol/L KCl, 10 mmol/L NaCl, 10 mmol/L potassium phosphate, pH 7.4, and 27 mmol/L sucrose. Nystatin stock was added to packed erythrocytes in the above buffer to give a final hematoctit of ~5% and a final nystatin concentration of 40 µg/mL. Control cells were incubated with an equivalent amount of Me₂SO. The mixture was incubated for 20 minutes at 0°C, the erythrocytes were pelleted by centrifugation, and then resuspended for an additional 20 minutes at 0°C in fresh buffer containing nystatin. The cells were then washed three times at room temperature in the same buffer without nystatin, but with added 10 mmol/L glucose and 0.1% bovine albumin. Finally, the cells were washed one time in 0.9% saline.

Internal cell potassium was measured by washing 2 × 10⁹ erythrocytes three times in 150 mmol/L choline chloride containing 10 mmol/L MOPS-Tris, pH 7.40. The cell pellet was lysed with 250 µL water and the lysate analyzed for K⁺ content by flame photometry on a Flame Photometer 343 (Instrumentation Laboratory, Inc, Lexington, MA).

Alterations in ATP Content of Erythrocytes

Rapid depletion of ATP²⁰ in normal RBCs was accomplished by incubating the cells for 90 minutes at a hematocrit of ~16% in TBS supplemented with 10 mmol/L inosine and 6 mmol/L iodoacetamide. This treatment resulted in a greater than 90% decrease in intracellular ATP, but had no effect on intracellular K⁺. ATP levels were replenished in remnant RBCs after incubation with Ca²⁺ and A23187 by further incubating the cells for 3 hours at a hematocrit of ~16% in buffer containing 90 mmol/L NaCl, 50 mmol/L Na₂HPO₄, 10 mmol/L HEPES, 5 mmol/L KCl, 1 mmol/L MgCl₂, 0.5 mmol/L EGTA, 10 mmol/L glucose, 10 mmol/L inosine, and 10 mmol/L adenosine.

ATP was determined with a commercially available luciferin/luciferase assay system (Sigma) after extraction of RBCs with ice-cold 0.5% trichloroacetic acid containing 2 mmol/L EDTA or
with perchloric acid by the method of Beutler. Extracts were stored at -20°C until the assay was performed.

Quantitation of DAF and MIRL

DAF immunoassay. DAF was quantitated using a sandwich-type enzyme-linked immunosorbent assay (ELISA) with IA10 as the capture antibody and biotinylated IIH6 as the detecting antibody. Samples were prepared by pelleting 1 mL of erythrocytes (1.25 x 10⁸/mL in PBS) in a 1.5-mL microcentrifuge tube by centrifugation in an Eppendorf microfuge (Brinkmann Instruments Co, Westbury, NY). The cells were then suspended in 1 mL of 5 mmol/L sodium phosphate, pH 8.0 containing 1 mmol/L disodium EDTA and 2 mmol/L DFP, vortexed vigorously, and pelleted as above for 10 minutes at 4°C. The supernatant was removed carefully so as not to disturb the pellet of membrane ghosts and a second similar wash was performed. After removal of the wash supernatant, 120 μL of 1% NP-40 in PBS containing 2 mmol/L DFP was added and the mixture vortexed vigorously. After 60 minutes at room temperature, 1 mL of PBS-BSA-Az containing 2 mmol/L DFP was added to the sample preparation.

Standards consisted of 0 to 80 ng/mL of either purified native human DAF (supplied by Drs Michael Daviz and Victor Nussenzweig) or recombinant human DAF (kindly provided by Dr Ingrid Caras, Genentech, Inc, South San Francisco, CA) in a buffer of composition identical to that of the final sample preparation described previously. Because the protein concentration of the stock preparations was calculated by different methods (Coomassie blue method for native DAF and by amino acid analysis for recombinant DAF), they did not give equivalent results in the immunoassay. By comparing the slopes of the standard curves using both standard preparations in four assays, a conversion factor of 1.565 (±0.05S, mean ± 1 SD) was calculated and used to convert results obtained with the recombinant DAF standard to those obtained with the native DAF standard.

Immun assay plates (Nunc Immunoplate MaxiSorp; Nunc Inc, Newbury Park, CA) were coated overnight at 4°C with 100 μL per well of IA10 anti-DAF (2 μg/mL). The capture antibody was then aspirated and 200 μL/well of PBS-BSA-Az was added and incubated for 1 to 2 hours at room temperature. The plate was then washed with TBS containing 0.1% Tween-20, blotted dry, and 100 μL/well of standards and each unknown sample were added in triplicate. After 90 minutes at 37°C, the wells were aspirated, washed several times with TBS containing 0.1% Tween-20, and blotted dry. Biotinylated IIH6 anti-DAF (1/500 dilution in PBS-BSA-Az) was then added to each well (100 μL/well) and the plate incubated for an additional 90 minutes at 37°C. After aspirating the wells and washing as above, 100 μL/well of streptavidin-alkaline phosphatase conjugate (1/500 dilution in PBS-BSA-Az) was added and incubated for 60 minutes at 37°C. The wells were aspirated and washed six times as above, followed by addition of 100 μL/well of Sigma 104 phosphatase substrate (p-nitrophenyl phosphate disodium, 1 mg/mL in 100 mmol/L glycine, 1 mmol/L MgCl₂, 1 mmol/L ZnCl₂, pH 9.4) and incubation for 30 minutes at room temperature. The plate was then read at 410 nm on a Dynatech MR700 microplate reader (Dynatech Laboratories Inc, Chantilly, VA).

Unknown DAF concentrations were calculated on the basis of the standard curve, which was linear over the range of DAF standard concentrations used (0 to 80 ng/mL). The number of molecules of DAF per erythrocyte was calculated based on a molecular weight of 70,000 daltons for DAF.

Binding of anti-MIRL to erythrocytes. Because we were limited to a single polyclonal antiserum to MIRL, relative losses of immunoreactive MIRL from erythrocytes as a consequence of the treatments described previously were assessed using the anti-MIRL binding assay described by Holguin et al. The procedure was followed exactly except that DGVB-EDTA was substituted for GVB-EDTA.

Hemolysis Mediated by Antibody and Complement

Sensitivity of normal and treated erythrocytes to lysis by antibody and complement was determined by measuring lysis by purified anti-I in the presence of excess complement as described by Rosse et al. Erythrocytes (2 x 10⁸ in 100 μL DGVB⁺) were added to increasing amounts of anti-I from 0 to 10 μg in a final volume of 500 μL DGVB⁺ in 12 x 75 mm tubes. Autologous normal human serum (200 μL) was added and the mixture incubated at 0°C for 30 minutes. The tubes were then transferred to a 37°C water bath and incubated for an additional 60 minutes. The cells were pelleted by centrifugation at 4°C and hemolysis determined from the absorbance at 541 nm of the supernatants. The amount of anti-I required to cause 30% hemolysis was calculated for each experiment by performing linear regression analysis on the linear portion of the lysis curve. The mean value for all experiments was calculated from the values obtained for the individual experiments.

Lysis of RBCs by antibody and complement was also assessed using a slight modification of the complement lysis sensitivity assay of Rosse and Dacie. The final volume was 0.375 mL instead of 7.5 mL and erythrocytes (2 x 10⁸/mL) were mixed 1:1 with a 1/8 dilution of commercial rabbit anti-human RBC (IgG fraction) instead of cold agglutinin before addition of a 50-μL aliquot of the mixture to the final incubation preparation.

Susceptibility of Erythrocytes to Complement-Mediated Hemolysis Initiated by CoFBB

Hemolysis initiated by CoFBB (reactive lysis) was assayed by a slight modification of the technique of Holguin et al. Washed erythrocytes were suspended to 5 x 10⁸/mL in DGVB-EDTA. EDTA was added to autologous normal human serum to give a final EDTA concentration of 15 mmol/L, followed by serial twofold dilutions of the EDTA-serum in DGVB-EDTA. CoFBB (50 μL of a 1/5 dilution in DGVB-EDTA) were added to 50 μL of erythrocytes, followed by 100 μL of a dilution of EDTA-serum. A control for spontaneous hemolysis in the presence of each serum dilution was achieved by replacing the CoFBB with 50 μL of DGVB-EDTA. Spontaneous hemolysis in the absence of serum was measured by incubating 50 μL of erythrocytes with 150 μL of DGVB-EDTA. The mixtures were incubated for 40 minutes at 37°C, followed by the addition of 1 mL of 0.9% saline containing 15 mmol/L EDTA and removal of the erythrocytes by centrifugation. Hemolysis was determined by comparing the absorbance at 541 nm of supernatants with that from a 100% lysis control consisting of 50 μL of erythrocytes incubated with 150 μL of water instead of buffer.

Osmotic Fragility

Osmotic fragility was measured according to the method of Beutler except that washed RBCs suspended to 5 x 10⁸/mL in 0.9% NaCl were substituted for whole blood.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

Analysis of proteins by SDS-PAGE was performed under nonreducing conditions on a 17% to 27% gradient polyacrylamide mini-gel (Integrated Separation Systems, Hyde Park, MA) according to the method of Laemmli. Transfer of proteins to nitrocellulose membrane was performed at 100 V in 25 mmol/L Tris, 192 mmol/L glycine, pH 8.3, containing 20% (vol/vol) methanol for 1 hour at 4°C. Nonspecific binding sites were blocked with TBS, pH
8.0 containing 0.05% Tween-20 and 5% instant nonfat dry milk. MIRL was identified on blots with polyclonal rabbit anti-MIRL followed by alkaline phosphatase-conjugated goat anti-rabbit IgG. Purified human MIRL was used as a standard for comparison. Molecular weights were estimated by reference to low molecular weight standards obtained from Integrated Separation Systems.

**Statistical Analysis**

Results are expressed as the mean ± 1 SE for the indicated number of experiments. Data were analyzed by Student's t-test for paired samples (not significant: \( P > .05 \)).

**RESULTS**

**Effect of Calcium Loading on Osmotic Fragility of Erythrocytes**

Calcium loading of erythrocytes induces a rapid change in cell shape from discocyte to echinocyte and finally to microspherocyte as membrane vesicles are lost. The left shift in the osmotic fragility curve for calcium loaded RBCs shown in Fig 1 indicates that these cells are more resistant to osmotic lysis than control erythrocytes. This most likely is the result of cellular alterations other than membrane loss; for example, the decrease in cell volume and/or the cross-linking of cytoskeletal proteins.\(^{20}\)

**Effect of Calcium Loading on Hemolysis Mediated by Antibody and Complement**

The effect of calcium loading by incubation of erythrocytes with \( \text{Ca}^{2+} \) and the ionophore A23187 on the subsequent lysis of those cells by purified anti-I and complement is shown in Fig 2. Such treatment significantly enhanced lysis when erythrocytes were incubated with various concentrations of antibody in the presence of excess complement. The amount of anti-I required to induce hemolysis of 30\% of the erythrocytes in the incubation mixture (this point fell on the linear portion of the lysis curve in each experiment, see Materials and Methods) decreased from 5.27 ± 1.21 \( \mu \)g for RBCs incubated without A23187 to 2.17 ± 0.92 \( \mu \)g for RBCs incubated with \( \text{Ca}^{2+} \) and A23187 (\( P < .025, n = 6 \)).

Calcium loaded RBCs were also examined for lysis by various concentrations of complement in the presence of excess antibody using the complement lysis sensitivity assay.\(^{23}\) In three experiments, the amount of serum (human complement) required to cause 50\% hemolysis (the C'LS \( H_\text{50} \)) decreased from 9.74 ± 0.37 \( \mu \)L for cells incubated with \( \text{Ca}^{2+} \) alone to 3.07 ± 0.27 \( \mu \)L for cells incubated with both \( \text{Ca}^{2+} \) and A23187 (\( P < .005 \)).

**Effect of Calcium Loading on Hemolysis Initiated by CoFBb**

Protection of RBCs from lysis by classical pathway activated complement could be mediated by DAF and/or proteins acting to prevent lysis by the membrane attack complex, eg, C8 \( \text{bp}/\text{HRF} \) or MIRL. However, protection from reactive lysis would be afforded by the latter proteins only. Indeed, MIRL alone has been shown to account for the lack of lysis seen in normal erythrocytes when reactive lysis is initiated by activated CoF complexes.\(^{9}\) These complexes (CoFBb) are generated when activated factor B (Bb) binds to CoF forming a stable C3/C5 convertase.\(^{22}\) Thus, we examined the effects of the various RBC treatments on reactive hemolysis initiated by CoFBb (Fig 3). As expected, control erythrocytes showed only minimal hemolysis in this assay. In contrast, calcium-loaded RBCs were consistently highly susceptible to CoFBb-initiated hemolysis.

**Effect of Calcium Loading on Erythrocyte Immunoreactive DAF and MIRL**

As noted above, normal human RBCs are protected from lysis by activated complement by a number of membrane proteins, including DAF and MIRL. To see if the changes in susceptibility to hemolysis observed in the two assay
brane vesiculation." However, the amount of MIRL lost was less markedly affected than the quantity of DAF, with a population and that a subpopulation of cells having lost all that both proteins were lost uniformly from the entire RBC as the mean.

Table 1. Effect of Calcium Loading on Erythrocyte AChE Activity, and Immunoreactive DAF and MIRL

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AChE (IU/mL RBC)</th>
<th>DAF (molecules/RBC)</th>
<th>MIRL (dpm %anti-rabbit IgG bound)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 4</td>
<td>n = 4</td>
<td>n = 3</td>
</tr>
<tr>
<td>−A23187</td>
<td>12.90 ± 0.77</td>
<td>4,263 ± 144</td>
<td>64,533 ± 11,908</td>
</tr>
<tr>
<td>+A23187</td>
<td>6.01 ± 0.42</td>
<td>2,110 ± 47</td>
<td>46,423 ± 8,569</td>
</tr>
<tr>
<td>(% lost)</td>
<td>(53.5 ± 1.4)</td>
<td>(50.4 ± 1.1)</td>
<td>(27.9 ± 1.6)</td>
</tr>
</tbody>
</table>

Erythrocytes (RBC) were incubated with Ca²⁺ in the absence or presence of ionophore A23187 and then assayed for AChE activity, immunoreactive DAF, or immunoreactive MIRL. Results are expressed as the mean ± SE for the indicated number of experiments.

systems described previously correlated with a decrease in membrane levels of these two proteins, we measured immunoreactive DAF and MIRL in RBCs after calcium loading. The quantity of immunoreactive DAF and MIRL lost as a consequence of exposure to Ca²⁺ and A23187 is shown in Table 1. Exposure to Ca²⁺ and ionophore for 60 minutes resulted in loss of ~50% of membrane immunoreactive DAF, an amount nearly identical to the amount of AChE lost. The amount of immunoreactive MIRL detectable on the surface of erythrocytes after calcium loading was less markedly affected than the quantity of DAF, with a decrease in the range of 25% to 30% following exposure to Ca²⁺ and A23187. Immunofluorescence analysis confirmed that both proteins were lost uniformly from the entire RBC population and that a subpopulation of cells having lost all DAF and MIRL was not generated (data not shown).

These results confirmed that DAF and MIRL were lost, as we had expected from our previous observations on the preferential depletion of GPI-anchored proteins after membrane vesiculation. However, the amount of MIRL lost (~30%) from calcium-loaded RBCs was far less than the minimum of an 80% loss in MIRL, which was considered necessary to effect any change in susceptibility to CoFBB-initiated hemolysis in PNH RBCs as compared with normal red cells. Nevertheless, calcium-loaded RBCs exhibited a marked increase in reactive lysis (see above). Experiments in which PNH erythrocytes were reconstituted with purified MIRL have shown that reactive lysis initiated by CoFBB specifically measures the function of MIRL, to the exclusion of other complement regulatory proteins. This suggested that calcium loading of erythrocytes resulted in additional cellular alterations that could influence the degree of lysis seen in the reactive lysis assay, but that did not involve changes in complement regulatory proteins other than MIRL.

To examine the possibility that functional alterations in MIRL were resulting in enhanced reactive lysis independent of losses of the protein into membrane vesicles, erythrocytes were incubated under conditions that prevented the formation of vesicles. Because the process of membrane vesiculation induced by calcium loading depends on the efflux of K⁺ from the cells, erythrocytes were incubated with Ca²⁺ and A23187 in Tris buffer containing 0.4 mmol/L KCl and 0.4 mmol/L quinine to inhibit K⁺ loss via the Gardos channel. In two experiments, incubation under these conditions completely prevented K⁺ loss and vesiculation; consequently, there was no decrease in immunoreactive MIRL (or DAF, data not shown). Nevertheless, erythrocytes treated in such a manner were lysed to a maximum of ~22% in the reactive lysis assay (Fig 3). Treatment of RBCs with 0.4 mmol/L quinine in high K⁺ buffer in the absence of A23187 resulted in no increase in susceptibility to CoFBB-initiated hemolysis above that of control cells (data not shown). Thus, it is clear that calcium loading induces both quantitative and functional defects in erythrocyte MIRL.

Effect of Manipulation of Intracellular Potassium and ATP on CoFBB-Initiated Hemolysis

Incubation of human erythrocytes with Ca²⁺ and A23187 is known to produce a variety of biochemical abnormalities simultaneous with vesiculation of the cell membrane. Two of the most prominent defects are the depletion of intracellular K⁺ noted previously and of intracellular ATP. Because the degree of reactive lysis far exceeded that expected on the basis of the experiments described in the preceding sections, we investigated the possibility that K⁺ and/or ATP depletion might contribute to the lytic defect observed in calcium-loaded RBCs.

We first examined the effect of intracellular potassium concentration on reactive lysis. As expected, treatment of erythrocytes with Ca²⁺ and A23187 for 1 hour resulted in a decrease in intracellular K⁺ from 77.9 ± 1.9 mmol/10¹² erythrocytes to 16.1 ± 0.4 mmol/10¹² erythrocytes (mean ± SE, n = 5), a decrease of 79.3% ± 1.0%. Because there was only a minimal effect on intracellular Na⁺ (data not shown), the total cation content decreased and there was a concomitant decrease in MCV from 86.8 ± 0.7 fl to 74.6 ± 0.9 fl (mean ± SE, n = 5). The effect of restoring
the intracellular \( K^+ \) concentration toward control values on the subsequent hemolysis of calcium-loaded erythrocytes is shown in Fig 4. The increase in cell volume that would accompany replenishment of intracellular \( K^+ \) would be expected to cause an increase in osmotic fragility (and consequently osmotic lysis mediated by complement) as a result of a decrease in the surface to volume ratio of the cell. However, when the intracellular \( K^+ \) was restored to 99.0% ± 1.4% of the control value, the mean corpuscular volume increased to 86.0 ± 1.0 fL, but hemolysis in the reactive lysis assay was diminished by about 50%. Less complete restoration of intracellular \( K^+ \) (to 64% of normal) resulted in a lesser degree of correction in the lytic defect (data not shown). In contrast, when normal RBCs were rapidly depleted of intracellular \( K^+ \) (to 7.0% of control values) by exposure to valinomycin, only a small increase in susceptibility to CoFBb-initiated hemolysis was observed (Fig 4). Treatment of normal erythrocytes with valinomycin does not cause vesiculation.\(^{11}\) Thus, it appears that although depletion of intracellular \( K^+ \) minimally affects reactive lysis in normal erythrocytes, it can cause a marked increase in CoFBb-initiated lysis of RBCs following a decrease in immunoreactive MIRL, despite a simultaneous decrease in cell volume.

The effect of intracellular ATP levels on CoFBb-initiated hemolysis was also examined. Exposure of RBCs to \( Ca^{2+} \) and ionophore resulted in a decrease in intracellular ATP to 21.9% ± 6.4% (mean ± SE, \( n = 4 \)) of the control value (1.50 ± 0.21 × 10\(^{-11} \) mol/10\(^8 \) erythrocytes). Repletion of ATP to supranormal (161.7% ± 15.1% of control) values had a partially protective effect in the reactive lysis assay (Fig 5). The incubation conditions used to replete cellular ATP had no effect on the intracellular \( K^+ \) concentration (data not shown), which as noted above were ~20% of normal. Similar to the results observed with \( K^+ \) depletion of normal RBCs, rapid ATP depletion (to <8% of control values) of normal erythrocytes did not make them susceptible to CoFBb-initiated lysis (Fig 5). Thus, a decrease in intracellular ATP may partially explain the increase in reactive lysis seen in calcium-loaded RBCs.

Finally, we examined the possibility that replenition of both ATP and \( K^+ \) might have an additive or synergistic effect in decreasing the reactive hemolysis of cells treated with \( Ca^{2+} \) and A23187. The results from two experiments are shown in Fig 6. In both experiments, restoration of both \( K^+ \) and ATP to control values resulted in a reduction in CoFBb-initiated hemolysis that was approximately equal to the sum of the effects of replenishing each constituent by itself. This suggests that after a sufficient decrease in MIRL, variations in intracellular \( K^+ \) and ATP act independently to produce alterations in RBC susceptibility to reactive lysis.

**SDS-PAGE and Western Blotting for MIRL**

Incubation of RBCs with \( Ca^{2+} \) and ionophore results in the proteolysis of several membrane proteins.\(^{11}\) To determine if proteolysis of MIRL was responsible for the increase in reactive lysis seen in calcium-loaded erythrocytes, we subjected RBC membranes to gel electrophoresis and Western blotting under conditions that would permit detection of proteolytic fragments as small as 5 Kd. There
was no evidence of proteolytic degradation of MIRL in either control or calcium-loaded RBCs (data not shown). Thus, it appears that the immunoreactive MIRL detected in the anti-MIRL binding assay consists solely of intact MIRL and does not include nonfunctional proteolyzed forms of the molecule that have retained immunoreactivity.

**DISCUSSION**

The studies reported here clearly show that calcium loading of normal human erythrocytes induces a membrane defect that causes increased hemolysis when the RBCs are exposed to antibody and complement or to activated cobra venom factor complexes. This occurs despite an overall increase in the resistance of the cells to osmotic lysis. The behavior of calcium-loaded erythrocytes in the complement lysis assays is similar to that previously reported for RBCs modified by treatment with 2-amino-ethylisothiouronium bromide (AET).21 Both methods of treatment produce RBCs that lyse at a degree similar to that for PNH II cells in the complement lysis sensitivity assay and to that for PNH III cells in the reactive lysis assay.28 We previously have reported that incubation with calcium and A23187 causes membrane vesicles to be released from the RBC and that these vesicles are enriched in at least two GPI-anchored proteins, AChE and DAF.23 A decrease in the membrane content of DAF to 50% of normal could affect assays of complement lysis sensitivity, but is unlikely by itself to produce a clinically relevant increase in susceptibility to complement-mediated lysis. This conclusion is based on recent reports of patients with the inherited Inab phenotype.20,21 Patients with this disorder have erythrocytes completely lacking in DAF and yet have no evidence of clinical hemolysis and only minimal to moderate abnormalities in functional assays of complement sensitivity.20,21

The decrease in immunoreactive MIRL in calcium-loaded RBCs was far less than would have been predicted on the basis of the profound degree of hemolysis seen in the reactive lysis assay. Reports of experiments using RBCs from patients with PNH suggest that membrane MIRL must decrease to ~20% of normal values before enhanced lysis is seen when CoFBB complexes are used to produce reactive lysis.23 This suggested that additional factors contributed to the lytic defect seen in calcium-loaded erythrocytes. In addition to vesiculation, RBCs exposed to calcium and ionophore A23187 develop a number of biochemical changes.27 Two of the most prominent changes are a decrease in intracellular potassium and a decrease in total cellular ATP.27 Indeed, potassium efflux is required for membrane vesiculation to occur.27 If K+ efflux was blocked by incubating erythrocytes in high K+ buffer containing quinine, vesiculation did not occur and there was no decrease in immunoreactive MIRL. However, a functional defect in MIRL was apparent in that hemolysis to a maximum of ~20% occurred when these cells were used in the reactive lysis assay (Fig 3). It is unclear how an increase in intracellular Ca2+ (and intracellular calcium-dependent protease activity) could induce a functional defect in a protein anchored to the external membrane surface by a GPI anchor. It is conceivable that an intracellular or inward-facing membrane protein acts as a cofactor for MIRL and would be susceptible to the consequences of cell exposure to Ca2+ and ionophore.

In experiments in which RBCs were incubated with Ca2+ and ionophore under our standard incubation conditions, restoration of the intracellular K+ to normal values resulted in a nearly twofold reduction in the reactive hemolysis of calcium-loaded RBCs. ATP repletion caused a generally smaller correction of the lytic defect. These experiments did not delineate the molecular basis for the observed decrease in lysis, but the additive effect of K+ and ATP repletion (Fig 6) in protecting calcium-loaded RBCs from reactive lysis suggests that variations in these cellular constituents might be acting by different mechanisms to influence the ability of the cell to defend itself against reactive lysis. However, the inability of either rapid K+ or ATP depletion of normal erythrocytes to enhance reactive lysis demonstrates that depletion of either of these constituents by itself does not result in increased susceptibility to reactive lysis. Rapid K+ depletion of RBCs following incubation with Ca2+ and A23187 under conditions that prevented the loss of MIRL by vesiculation (see above) failed to induce a further increase in CoFBB-initiated hemolysis (data not shown). Thus, it appears that the quantitative decrease in MIRL associated with vesiculation...
must occur before K+ depletion has an effect on the susceptibility of calcium-loaded RBCs to lysis in this assay.

Although rapid ATP depletion of normal erythrocytes has no effect on lysis by activated complement, slow ATP depletion causes membrane vesiculization and might be expected to affect complement lysis sensitivity. Indeed, slow ATP depletion over 48 hours resulted in losses in DAF and MIRL comparable with those seen in calcium-loaded RBCs, and the cells showed enhanced lysis in both of the functional assays (Test ST; Büttikofer P, Yee MC, unpublished observation, 1990).

The ability of the RBC to regulate intracellular K+ recently has been implicated as a potentially critical factor in protecting the cell from lysis by activated complement. In that study, it was concluded that the protective effect most likely resulted from activity of the calcium-activated potassium (Gardos) channel following insertion of the membrane attack complex into the cell membrane and a subsequent rise in intracellular Ca++. However, this mechanism would have been incapable of operating under the conditions of reactive lysis used in the present experiments. The buffer in which the RBCs were suspended contained no added Ca++ and had a high (15 mmol/L) concentration of EDTA. Thus, the intracellular K+ content of calcium-loaded erythrocytes must influence reactive lysis by another mechanism.

It appeared that proteolyzed forms of MIRL were not generated as a consequence of calcium loading. However, it should be noted that the polyclonal antibody to MIRL used for these experiments is capable of reacting with MIRL only after electrophoresis under nonreducing conditions. Because MIRL is a single polypeptide chain with a large number (10) of cysteine residues and thus is thought to contain a high degree of intrachain disulfide bonding, it is possible that electrophoresis under nonreducing conditions would fail to detect proteolytic degradation occurring at points between the cysteine residues of individual disulfide bonds. Thus, we cannot completely exclude the presence of function-altering proteolysis of MIRL in calcium-loaded RBCs. We also cannot exclude the possibility of proteolytic degradation of an as yet undiscovered membrane cofactor which may be necessary for the normal function of MIRL (see above).

In addition to proteolysis of membrane proteins, calcium loading has been shown to induce protein cross-linking, presumably via the action of an endogenous transglutaminase. The cross-linking reaction can be inhibited by adding a competitive substrate, e.g., histamine, to the reaction mixture. It is unlikely that cross-linking of either DAF or MIRL contributed to the enhanced complement sensitivity of calcium-loaded erythrocytes in our experiments, as the presence of 60 mmol/L histamine dihydrochloride during incubation with Ca++ and A23187 had no effect on the subsequent lysis of RBCs in the complement lysis assays (data not shown).

Although protection of erythrocytes from reactive hemolysis has also been shown to be a function of C8 bp/HRF, reconstitution of PNH erythrocytes with MIRL by itself is sufficient to correct the defect in lysis initiated by CoFBb complexes. Thus, it is unlikely that abnormalities in C8 bp/HRF account for our results seen when CoFBb complexes were used to initiate reactive lysis. Taken together, our results suggest that the abnormal susceptibility to complement-mediated lysis seen in RBCs following incubation with calcium and A23187 results from a complex combination of factors including decreases in immunoreactive and functional DAF and MIRL, potassium and ATP depletion, and potentially other as yet undetermined cellular alterations.

Our results have potential implications with regard to the pathogenesis of certain disease states. PNH currently is believed to be characterized by a general absence of GPI-anchored proteins from the membranes of all blood cells. The precise mechanism by which this defect arises has not been defined. Studies of PNH cells have shown that DAF present on erythroid progenitors is lost during erythropoiesis in vitro. In light of recent descriptions of membrane vesiculization occurring during the maturation of reticulocytes in vitro, our results suggest that such a process, or a similar defect in cell membrane modeling during maturation, might lead to the selective loss of GPI-anchored proteins and contribute to the final phenotype of mature PNH cells. Further, our results suggest that any situation which would lead to a decrease in either cellular potassium or ATP could enhance the degree of lysis seen when complement is activated. At the least, they suggest that studies of PNH cells should be performed on freshly isolated cells or on cells stored under conditions that maintain normal levels of K+ and ATP. This caveat is underlined by a recent report showing increased Ca++ accumulation after ATP depletion in PNH RBCs compared with normal cells. Thus, PNH erythrocytes might vesiculate even more than normal RBCs under conditions leading to ATP depletion and consequently suffer a greater loss of GPI-anchored complement regulatory proteins.

Finally, a variety of hematologic disorders have been shown to be characterized by membrane vesiculation. The results reported here suggest that vesiculation alone would be unlikely to lead to an increased susceptibility to complement-mediated lysis in the RBCs of patients with these disorders. However, in those cases where the disease is characterized by additional biochemical defects, such as the K+ and ATP depletion seen in the densest population of erythrocytes in sickle cell anemia, vesiculation might lead to a biologically significant alteration in sensitivity to activated complement and contribute to the hemolysis seen in the disease. This possibility is currently under active investigation in our laboratory.

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