Aberrant Regulation of Complement by the Erythrocytes of Hereditary Erythroblastic Multinuclearity With a Positive Acidified Serum Lysis Test (HEMPAS)

By Akira Tomita and Charles J. Parker

Susceptibility to hemolysis in acidified serum is a pathognomonic feature of hereditary erythroblastic multinuclearity with a positive acidified serum lysis test (HEMPAS, congenital dyserythropoietic anemia type II). The purpose of the studies reported herein was to determine if aberrant regulation of complement contributes to the susceptibility of HEMPAS erythrocytes to acidified serum lysis. The results of these experiments have demonstrated that regulation of both the C3 convertase of the alternative pathway and the membrane attack complex of complement by HEMPAS erythrocytes is aberrant. However, these abnormalities are not a consequence of quantitative or functional deficiencies of the erythrocyte complement-regulatory proteins, decay accelerating factor (DAF, CD55), or membrane inhibitor of reactive lysis (MIRL, CD59).

PATIENTS WITH hereditary erythroblastic multinuclearity with a positive acidified serum lysis test (HEMPAS, congenital dyserythropoietic anemia type II) suffer from a moderate to severe anemia. The disease is inherited in an autosomal recessive fashion, and the anemia is a consequence of dyserythropoiesis and ineffective erythropoiesis. The peripheral blood erythrocytes are usually normocytic, but anisocytosis and poikilocytosis are prominent manifestations of the disease. The bone marrow shows abnormal erythroblasts with binuclearity, multinuclearity, and abnormal lobulations. Late erythroblasts seem to have a double plasma membrane when examined by electron microscopy because of an excess of endoplasmic reticulum.

A defining feature of HEMPAS is the susceptibility of the erythrocytes to hemolysis in acidified serum. Sensitivity of erythrocytes to acidified serum lysis is characteristic of only one disease (paroxysmal nocturnal hemoglobinuria [PNH]) other than HEMPAS. The erythrocytes of PNH are hemolyzed in acidified serum because they are deficient in a complement regulatory protein, decay accelerating factor (DAF, CD55). Our recent studies show that glycophorin A (GPA), the major erythrocyte sialoglycoprotein, is a complement regulatory protein. Analysis by radioimmunoprecipitation suggested that GPA on HEMPAS erythrocytes is abnormally glycosylated. Further analysis indicated that the abnormality involves the O-linked oligosaccharide moiety. Together, these studies show that complement regulation by HEMPAS erythrocytes is abnormal and that constituents other than DAF and MIRL participate in controlling complement activation on the erythrocyte membrane. Additionally, these studies suggest that the glycosylation defect that is characteristic of HEMPAS involves GPA.

From the Department of Medicine, Division of Hematology/Oncology, University of Utah, and the Veterans Affairs Medical Center, Salt Lake City, UT.

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Address reprint requests to Charles J. Parker, MD, Hematology/Oncology Section (111c), VA Medical Center, 500 Foothill Dr, Salt Lake City, UT 84148.

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MATERIALS AND METHODS

Erythrocytes. Whole blood from healthy volunteers, a patient with HEMPAS, and a patient with PNH were stored at 4°C in Alsever’s solution. The HEMPAS erythrocytes were provided by Dr J.M. Schwartz, Albert Einstein College of Medicine-Montefiore Hospital, New York, New York.
covered, the cells that had been incubated in acidified serum were resuspended to
scribed above. The remainder of the experiment was performed as de-
GVB+, pH 6.4, containing 5 mmol/L MgCl₂ (GVB⁺, pH 6.4).

**Serum.** Normal human serum (NHS) was prepared from whole
blood obtained from a volunteer donor with blood type AB. Acidified
serum containing 2.5 mmol/L MgCl₂ and 8 mmol/L EGTA was prepared by mixing 1 volume of a stock solution containing 25 mmol/L MgCl₂ and 80 mmol/L EGTA (Sigma Chemical Co), pH 6.4, with 9 volumes of serum that had been titrated to pH 6.4 with 1 mmol/L HCl. The mixture was incubated for 5 minutes at 37°C and kept on ice before use. Serum prepared in this manner does not support activation of the classical pathway of complement. ¹⁶ NHS-EDTA was prepared by mixing 1 volume of 100 mmol/L EDTA with 9 volumes of NHS and incubating the mixture for 5 minutes at 37°C. In some instances, NHS-EDTA was titrated to pH 6.4 with 1 mmol/L HCl before use. This reagent served as a complement blank in the acidified serum lysis experiments described below.

**Activated cobra venom factor complexes (CoFBb).** The methods
used to prepare CoFBb have been described in detail previously. ¹⁵ Anti-C3 was radiolabeled with ¹²⁵I as described previously. ¹⁵ ¹²⁵I-anti-C3 was calculated by using a previously described formula.

To determine the effects of blocking DAF function on the suscep-
tibility to CoFBb-initiated lysis, 30 μL of cells (5 × 10⁹/mL) were incubated at 37°C with 50 μL of GVB-EDTA containing a saturating amount of monoclonal ¹²⁵I-anti-C3 (20 μg/mL). After 30 minutes, aliquots of 100 μL were pipetted into 400 μL polyethylene microtube tubes containing 250 μL of 20% sucrose. After centrifugation for 15 minutes at 12,000g, the tips containing the cells were cut-off, and the radioactivity of the pellet was quantitated in a gamma counter. The radioactivity of the control samples (cells that had been incubated with acidified serum containing 10 mmol/L EDTA) was subtracted to determine specific binding. Binding of ¹²⁵I-anti-C3 was calculated based on the specific activity and a molecular weight of 160 kD.

**Efficiency of binding of nascent C3b to normal and HEMPSA.** The methods for quantitating binding of nascent C3b to human erythrocytes has been previously reported. ¹⁷ Briefly, normal and HEMPSA erythrocytes were washed in GVB-EDTA and resuspended to 1 × 10⁹/mL. Two 1-μL aliquots of each type of erythrocyte were incubated, and the supernate was aspirated as completely as possible without disturbing the cell pellet. To 1 aliquot of each erythrocyte type, 1 mg of purified C3 and 100 μL of CoFBb were added. As a control, GVB-EDTA was substituted for the C3 and CoFBb and added to the other 1-μL aliquots. The samples were incubated at 37°C. After 30 minutes, the cells were washed, and bound C3 was quantitated using radiolabeled monoclonal anti-C3. The radioactivity of the control samples was subtracted to define specific binding.

**Analysis of the susceptibility of erythrocytes to CoFBb-initiated lysis.** Normal, PNH, and HEMPSA erythrocytes were washed in GVB-EDTA and resuspended to 5 × 10⁹/mL. An aliquot of 50 μL of cells was incubated at 37°C with 50 μL of CoFBb and 100 μL of GVB-EDTA containing incremental concentrations of NHS-EDTA. At the same time, the appropriate reagent blanks were also prepared. ¹⁵ After 30 minutes, 3 μL of cold GVB-EDTA were added to the reaction mixtures, the samples were centrifuged, and free hemoglobin was quantitated spectrophotometrically. Based on these results, the percent hemolysis was calculated. ¹⁵

To determine the effects of blocking MIRL or DAF function on the susceptibility of normal and HEMPSA erythrocytes to CoFBb-initiated lysis, 30 μL of cells (5 × 10⁹/mL) were incubated at 37°C with 50 μL of GVB-EDTA containing incremental concentrations of either rabbit anti-DAF or anti-MIRL antiserum. After 15 minutes, the cells were washed in GVB-EDTA and resuspended to 100 μL. Next, the cells were incubated at 37°C with 50 μL of CoFBb and 100 μL of serum-EDTA. After 30 minutes, hemolysis was quantitated as described previously. ¹⁵

To compare the susceptibility to CoFBb-initiated lysis of normal and HEMPSA erythrocytes that had been treated with anti-MIRL, 50 μL of cells (5 × 10⁹/mL) were incubated at 37°C with 10 μL of GVB-EDTA and 10 μL of anti-MIRL antiserum. After 15 minutes, the cells were washed in GVB-EDTA and resuspended to 100 μL. Next, the cells were incubated at 37°C with 50 μL of CoFBb and 100 μL of serum-EDTA. After 30 minutes, hemolysis was quantitated as described previously. ¹⁵

**Fluorescence-activated cell sorter (FACS) analysis.** Normal, HEMPSA, and PNH erythrocytes were washed in GVB-EDTA and resuspended to 2 × 10⁹/mL. Aliquots of 50 μL of cells were incubated on ice with 50 μL of GVB-EDTA containing either a saturating amount of monoclonal anti-DAF or monoclonal anti-MIRL or 50 μg of irrelevant monoclonal murine IgG. After 30 minutes, the samples were washed with cold GVB-EDTA and resuspended to 50 μL. Next, 50 μL of fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG (Sigma Chemical Co) was added, and the samples were incubated on ice. After 30 minutes, the cells were washed and resuspended to 1 mL in GVB-EDTA and subsequently ana-
lyzed by using a FACScan (Becton Dickinson, Research Triangle Park, NC).

**Radioimmunoprecipitation.** The methods used for immunoprecipitation of DAF and MIRL from radiolabeled erythrocytes have been described in detail elsewhere. The samples were electrophoresed using a 6% to 15% linear gradient gel under reducing conditions. The same methods were used to analyze GPA on normal and HEMPAS erythrocytes except that the samples were electrophoresed using a 6% to 12% linear gradient gel. GPA was precipitated using 10 μL of rabbit anti-GPA antiserum.

**Treatment of normal and HEMPAS GPA with glycosidases.** GPA was isolated from normal and HEMPAS erythrocytes by using a procedure that has been described in detail elsewhere. Aliquots were radiolabeled with 125I by using IODO-GEN. The specific activity was approximately 2 × 10^8 cpm/μg. The concentration of the samples was adjusted to 100 μg/mL. For treatment with neuraminidase (Vibrio cholerae; Boehringer Mannheim Corp, Indianapolis, IN), with a combination of neuraminidase and O-glycosidase (Boehringer Mannheim Corp), or a combination of N-glycosidase F (PNGase F; Boehringer Mannheim Corp), neuraminidase, and O-glycosidase, 45 μL of 125I-GPA was incubated at 95°C with 5 μL of 1% sodium dodecyl sulfate (SDS). For treatment with PNGase F only, 35 μL of 125I-GPA was incubated at 95°C with 5 μL of 100 mmol/L EDTA, 5 μL of 10% 2-mercaptoethanol, and 5 μL of 1% SDS. After 5 minutes, 6 μL of 10% Nonidet P40 (NP-40; BDH Limited, Poole, England) and 6 μL of 20 mmol/L HEPES (Sigma Chemical Co), pH 7.0, was added to each sample.

For treatment with neuraminidase, 5 μL of 125I-GPA that had been prepared as described above were incubated for 1 hour at 37°C with 5 μL of 200 mmol/L HEPES, pH 7.0, 5 μL of 100 mmol/L calcium acetate, 10 μL of neuraminidase (1 U/mL), and 25 μL of deionized water. For treatment with a combination of neuraminidase and O-glycosidase, 5 μL of 125I-GPA that had been prepared as described in the preceding paragraph were incubated at 37°C with 5 μL of 200 mmol/L HEPES, pH 7.0, 5 μL of 100 mmol/L calcium acetate, 10 μL of neuraminidase (1 U/mL), and 25 μL of deionized water. After 30 minutes, 4 μL of O-glycosidase (25 U/50 μL) were added, and the incubation was continued for 2 hours. For treatment with PNGase F, 10 μL of 125I-GPA that had been prepared as described in the preceding paragraph were incubated for 2 hours at 37°C with 5 μL of 200 mmol/L HEPES, pH 7.0, 5 μL of PNGase F (20 U/100 μL), and 30 μL of deionized water. For treatment with a combination of PNGase F, neuraminidase, and O-glycosidase, 5 μL of 125I-GPA that had been prepared as described in the preceding paragraph were incubated at 37°C with 5 μL of 200 mmol/L HEPES, pH 7.0, 5 μL of 100 mmol/L calcium acetate, 5 μL of PNGase F, and 30 μL of water. After 2 hours, 10 μL of neuraminidase were added, and the incubation was continued for 30 minutes. Next, 4 μL of O-glycosidase were added, and the incubation was continued for an additional 2 hours. In all cases, control samples, in which the enzymes were replaced by deionized water, were prepared in parallel with the treated samples. Subsequently, the samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (reducing condition) and autoradiography.

**Effects of isolated GPA on CoFBB-initiated lysis.** Using methods that have been described in detail elsewhere, the capacity of GPA isolated from normal and HEMPAS erythrocytes to inhibit CoFBB-initiated lysis of guinea pig erythrocytes was compared.

**Statistical analysis.** Unpaired data were compared using the two-tailed Student’s t-test. Differences were considered to be statistically significant if the P value was <.05.

**RESULTS**

**Analysis of the susceptibility of normal, PNH, and HEMPAS erythrocytes to acidified serum lysis.** The enhanced susceptibility of HEMPAS erythrocytes to acidified serum lysis has been attributed primarily to complement activation by a naturally occurring IgM antibody that is present in the plasma of ~30% of the population. Inasmuch as the IgM antibody initiates complement activation via the classical pathway, the contribution of acidification of serum (a process that activates the APC) to the hemolytic process is obscure. In contrast to HEMPAS erythrocytes, PNH erythrocytes are susceptible to acidified serum because they are deficient in membrane proteins (DAF and MIRL) that regulate the activity of the C3 convertase of the APC.

To determine if aberrant regulation of the C3 convertase of the APC also contributes to the enhanced susceptibility of HEMPAS erythrocytes to acidified serum lysis, normal, PNH, and HEMPAS erythrocytes were incubated in acidified normal human serum containing Mg-EGTA. Under these conditions only the APC is activated, because the classical pathway is calcium dependent. Normal erythrocytes were resistant to acidified serum lysis, and only a very small percentage of the HEMPAS erythrocytes hemolyzed under these conditions (Table 1). In contrast, PNH erythrocytes were markedly susceptible to acidified serum (Table 1). However, despite the minimal lysis, HEMPAS erythrocytes bound ~5 times more C3 than normal cells (Table 1). These results indicate that regulation of the APC C3 convertase on HEMPAS erythrocytes is aberrant. The amount of C3 bound to PNH erythrocytes was ~5 times greater than that bound to the HEMPAS cells. There are two plausible explanations for these latter results. First, the mechanism that accounts for the greater deposition of C3 on HEMPAS and PNH erythrocytes may be the same and the PNH erythrocytes may be more severely affected by the abnormality. Second, the mechanism that accounts for the greater deposition of C3 on HEMPAS and PNH erythrocytes may be different.

Activated C3 (C3b) binds covalently to cell surface constituents through formation of an ester or imidoester bond. On human erythrocytes, nascent C3b binds almost exclusively to glycoporphin, and our previous studies have shown that the efficiency with which activated C3 binds to erythrocytes can be influenced by an acquired modification of glycoporphin. One plausible explanation for the greater binding of C3 to HEMPAS erythrocytes is that these abnormally glycosylated cells bind activated C3 more efficiently.

**Table 1. Effects of Acidified Serum on Normal, HEMPAS, and PNH Erythrocytes**

<table>
<thead>
<tr>
<th>Type of Erythrocyte</th>
<th>Hemolysis (%)</th>
<th>C3 Deposition</th>
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<tbody>
<tr>
<td>Normal</td>
<td>0</td>
<td>2,152 ± 245</td>
</tr>
<tr>
<td>HEMPAS</td>
<td>2</td>
<td>10,705 ± 455</td>
</tr>
<tr>
<td>PNH</td>
<td>66</td>
<td>50,484 ± 2,904</td>
</tr>
</tbody>
</table>

Normal, HEMPAS, and PNH erythrocytes were incubated in acidified serum containing Mg-EGTA. Hemolysis and C3 deposition were subsequently quantitated. The amount of C3 that was deposited onto each type of erythrocyte following incubation in acidified serum was quantified by using radiolabeled monoclonal anti-C3. This antibody recognizes an epitope expressed by C3b, iC3b, and C3dg. The values shown are the mean ± SD (n = 5).
the activity of the MAC, thereby protecting the cells from than normal. To investigate this hypothesis, HEMPAS and normal erythrocytes were incubated with CoFBB and isolated C3, and binding of C3b was subsequently quantitated using monoclonal 125I-anti-C3. HEMPAS erythrocytes bound 13,870 ± 2,287 (mean ± SD, n = 5) molecules of anti-C3 per cell compared with 13,956 ± 822 (mean ± SD, n = 5) for normal erythrocytes. These values were not significantly different. Thus, the greater binding of activated C3 to HEMPAS cells during incubation in acidified serum cannot be attributed to greater efficiency of binding of nascent C3b.

DAF is an important regulator of the C3 convertase of the APC, and DAF is deficient on the erythrocytes of PNH. To determine if a functional abnormality of DAF contributes to the greater C3 deposition onto HEMPAS cells during incubation in acidified serum, normal and HEMPAS erythrocytes were treated with anti-DAF. After washing, the cells were incubated in NHS-Mg/EGTA, and hemolysis and C3 deposition were subsequently determined (Table 2). Blocking DAF function enhanced the sensitivity of both normal and HEMPAS erythrocytes to acidified serum (Table 2 compared with Table 1), and C3 deposition onto HEMPAS erythrocytes was significantly enhanced by anti-DAF treatment (Table 2 compared with Table 1). These results demonstrate that DAF is functional on HEMPAS erythrocytes. However, that the C3 deposition onto anti-DAF-treated HEMPAS cells was greater than onto anti-DAF-treated normal cells, indicates that abnormalities of C3 regulatory factors other than DAF contribute to the greater deposition of C3 onto HEMPAS cells (Table 2).

The observation that anti-DAF–treated normal cells bound much less C3 than PNH erythrocytes (Table 2 compared with Table 1) confirms our previous observations that membrane constituents other than DAF control the activity of the C3 convertase when erythrocytes are incubated in acidified serum.

Despite the marked increase in C3 deposition, hemolysis of anti-DAF–treated HEMPAS cells was markedly less than that of PNH cells (Table 2 compared with Table 1). These results indicate that, like normal erythrocytes, HEMPAS erythrocytes express a membrane constituent that inhibits the activity of the MAC, thereby protecting the cells from hemolysis despite the C3 activation. In contrast, C3 activation on PNH erythrocytes results in hemolysis, because the cells are deficient in membrane regulators of the MAC (Table 1).

Analysis of the susceptibility of normal, PNH, and HEMPAS erythrocytes to CoFBB-initiated hemolysis. CoFBB-initiated lysis is a form of reactive lysis in that a C3 convertase is not formed on the cell surface. Rather, C5 is hydrolyzed into C5a and C5b in the fluid phase by activated factor B (Bb), the enzymatic subunit of the CoFBB complex. Nascent C5b binds C6 to form the stable, hydrophilic C5b6 complex. Available evidence indicates that the subsequent spontaneous binding of C7 to C5b6 produces a conformational change in this newly formed trimolecular complex (C5b-7) such that a binding site (associated with the C7 component) becomes exposed.

Because of the extreme lability of the reactive site, the complex must encounter a cell quickly or its capacity to bind is lost. Binding to C5b6 also induces a hydrophilic-amphipilic transition in C7 such that once bound to the cell membrane, the C5b-7 complex becomes integrated into the lipid bilayer. Binding of C8 and multiple molecules of C9 occurs spontaneously and in sequence, thereby forming the potentially cytolytic MAC (C5b-9).

When incubated with CoFBB and serum-EDTA (the complement source), PNH erythrocytes were hemolyzed, but both HEMPAS and normal erythrocytes were resistant to this process (Fig 1). These results are consistent with the hypothesis that like normal erythrocytes, HEMPAS erythrocytes express membrane constituents that regulate the activity of the MAC.

Susceptibility to reactive lysis is controlled at least in part by MIRL. When MIRL function was blocked on normal and HEMPAS erythrocytes by treating the cells with anti-

### Table 2. Effects of Inhibiting DAF Function on C3 Deposition Onto Normal and HEMPAS Erythrocytes Incubated in Acidified Serum

<table>
<thead>
<tr>
<th>Type of Erythrocyte</th>
<th>Hemolysis (%)</th>
<th>C3 Deposition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>5</td>
<td>16,256 ± 3,296</td>
</tr>
<tr>
<td>HEMPAS</td>
<td>12</td>
<td>38,014 ± 3,608</td>
</tr>
</tbody>
</table>

Normal and HEMPAS erythrocytes were treated with anti-DAF. Next, the cells were incubated in acidified serum containing Mg-EGTA, and hemolysis and C3 deposition were subsequently quantitated. The amount of C3 that was deposited onto normal and HEMPAS erythrocytes following incubation in acidified serum was quantified by using radiolabeled monoclonal anti-LC3. This antibody recognizes an epitope expressed by C3b, iC3b, and C3dg. The values shown are the mean ± SD (n = 5).

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![Fig 1. Susceptibility of erythrocytes to CoFBB-initiated hemolysis. Normal (○), PNH (▲), and HEMPAS (◆) erythrocytes were incubated with CoFBB and incremental concentrations of serum-EDTA as the complement source. Hemolysis was subsequently calculated based on hemoglobin release. PNH erythrocytes were susceptible to reactive lysis, but both normal and HEMPAS erythrocytes were resistant.](image-url)
was subsequently quantitated. Each input of antiserum on membrane constituents that regulate the MAC, and that these protects normal and HEMPAS erythrocytes from reactive. These results are consistent with the hypothesis that MIRL (Fig 2).

Blocking MIRL caused both normal and HEMPAS erythrocytes to become susceptible to CoFBb-initiated lysis; however, at each input of serum-EDTA, HEMPAS cells were significantly more susceptible.

To investigate this hypothesis further, normal and HEMPAS erythrocytes were treated with incremental concentrations of anti-MIRL antiserum. After washing, the cells were incubated with CoFBb and serum-EDTA, and hemolysis was subsequently quantitated. Each input of antiserum above 2.5 μL caused significantly greater hemolysis of the HEMPAS erythrocytes (Fig 3). That a plateau phase of the concentration-response curve was observed indicates that maximum inhibitory activity was achieved for both types of erythrocytes. The results of these experiments support the hypothesis that regulation of the MAC on HEMPAS erythrocytes is abnormal.

Expression of DAF and MIRL on normal, PNH, and HEMPAS erythrocytes. To investigate further the possibility that the aberrant regulation of complement by HEMPAS erythrocytes is caused by quantitative abnormalities of either DAF or MIRL, expression of these two proteins was analyzed by FACS (Fig 4). Both the quantity and the distribution of expression of DAF and MIRL seemed normal on the HEMPAS erythrocytes. In contrast, there was a bimodal expression of DAF and MIRL by the PNH erythrocytes with ~80% of the cells being deficient in both proteins (Fig 4).

To determine if either DAF or MIRL is qualitatively abnormal on HEMPAS erythrocytes, the two proteins were analyzed by immunoprecipitation (Fig 5). There was no obvious difference in the electrophoretic mobility of either DAF or MIRL immunoprecipitated from HEMPAS erythrocytes compared with immunoprecipitation of the two proteins from normal cells. These results suggest that neither DAF nor MIRL is abnormally glycosylated. Whereas this experiment does not exclude the possibility that minor abnormalities in primary structure may be present in DAF and MIRL from HEMPAS erythrocytes, the probability that functionally significant mutations in the genes that encode DAF and MIRL occur as part of the pathophysiologic process that defines HEMPAS seems remote.

Together, these results support the hypothesis that the aberrant regulation of complement by HEMPAS erythrocytes is not a consequence of quantitative or functional abnormalities of either DAF or MIRL.

Based on electrophoretic mobility, the band that appears between the 46- and 30-kD markers in all lanes of Fig 5 (including the lanes containing the samples incubated with preimmune IgG) putatively represents the monomeric form of GPA. The difference in mobility between the normal and HEMPAS samples suggests that the HEMPAS protein is structurally abnormal. This interpretation is supported by the results shown in Figs 6 and 7 (see below). The reason why this protein is immunoprecipitated by both immune and nonimmune antiserum and why the homodimeric form does not also appear on the autoradiograph is obscure.

Analysis of expression of GPA on normal and HEMPAS.
COMPLEMENT REGULATION BY HEMPAS RBC

**Fig 4.** FACS analysis of expression of DAF and MIRL. Normal (NL), HEMPAS, and PNH erythrocytes were incubated with irrelevant murine monoclonal IgG (nonimmune) or with monoclonal anti-DAF or anti-MIRL. After washing, the cells were incubated with FITC-conjugated anti-mouse IgG, and the samples were subjected to FACS analysis. Expression of both DAF and MIRL on HEMPAS erythrocytes seemed normal. DAF and MIRL expression on PNH erythrocytes showed a bimodal pattern with ~80% of the cells being deficient in both proteins.

Our recent studies have shown that GPA is a complement-regulatory protein. To determine if GPA is abnormal on HEMPAS erythrocytes, expression of the major sialoglycoprotein was analyzed by immunoprecipitation (Fig 6). HEMPAS erythrocytes showed the increase in the electrophoretic mobility of band 3 that is characteristic of the disease (Fig 6, lane 2 compared with lane 1). Studies by other investigators have shown that the abnormality of band 3 on HEMPAS erythrocytes is a consequence of aberrant glycosylation. The electrophoretic pattern of both the GPA monomer and the GPA homodimer [(GPA),] from HEMPAS erythrocytes also seemed abnormal (Fig 6, lane 2 compared with lane 1). This interpretation was supported by the results of immunoprecipitation analysis of GPA from normal erythrocytes (lane 3) and HEMPAS erythrocytes (lane 4).

To investigate the hypothesis that the abnormal electrophoretic mobility of HEMPAS GPA is a consequence of aberrant glycosylation, GPA was isolated from normal and HEMPAS erythrocytes. After radiolabeling, the samples were treated with glycosidases and subsequently analyzed by SDS-PAGE and autoradiography (Fig 7). After treatment with PNGase F, the mobility of both normal and HEMPAS GPA increased, but the difference in mobility between the two samples persisted (Fig 7A, lane 4 compared with lane 5). Because PNGase F cleaves N-linked glycans, these results indicate that the N-linked oligosaccharide on HEMPAS GPA is normal. Treatment with neuraminidase also equally increased the mobility of normal and HEMPAS GPA (Fig 7A, lane 6 compared to lane 7). These results indicate that a difference in the sialic acid moiety does not account for the abnormal electrophoretic mobility of HEMPAS GPA. However, when treated with a combination of neuraminidase and O-glycosidase, the mobility of HEMPAS GPA was observed to be identical compared with that of normal GPA (Fig 7A, lane 8 compared with lane 9). This combination of enzymes removes Gal$^\beta$-4GalNAc from O-linked glycans. Together, these experiments show that the normal electrophoretic mobility of HEMPAS GPA is a consequence of aberrant O-linked glycosylation.

To confirm that the N-linked carbohydrate was similar on normal and HEMPAS GPA, samples were treated with a combination of PNGase F, neuraminidase, and O-glycosidase (Fig 7B, lanes 8 and 9). Following complete deglycosylation, there was no obvious difference in the electrophoretic mobility of HEMPAS GPA compared with that of normal.
These experiments confirm that the abnormal glycosylation of HEMPAS GPA involves O-linked oligosaccharides.

**Inhibition of CoFBB-initiated lysis by GPA isolated from HEMPAS erythrocytes.** Our recent studies have shown that GPA isolated from normal erythrocytes is a potent inhibitor of the MAC. When compared with GPA isolated from normal erythrocytes, no difference was observed in the capacity of GPA isolated from HEMPAS erythrocytes to inhibit CoFBB-initiated lysis of guinea pig erythrocytes (data not shown). These results indicate that the glycosylation abnormality does not affect the capacity of HEMPAS GPA (in its isolated form) to block the assembly of the MAC.

**DISCUSSION**

The results of the experiments reported herein have shown that complement inhibition by HEMPAS erythrocytes is aberrant. The functional activity of both the APC C3 convertase and the MAC is enhanced on HEMPAS cells; but, unlike PNH erythrocytes, this lack of regulatory activity is not a consequence of quantitative (Fig 4) or functional (Table 2, Fig 1) abnormalities of either DAF or MIRL. Our studies also suggest that, in at least some cases, the glycosylation defect in HEMPAS involves GPA.

Despite the greater activation of the APC C3 convertase on HEMPAS erythrocytes during incubation in acidified serum, significant hemolysis was not observed (Table 1). These results are consistent with other observations that indicate that activation of the classical pathway by antibody is a necessary component of the hemolysis of HEMPAS erythrocytes in acidified serum. Nonetheless, it seems plausible to suggest that abnormal regulation of the C3 convertase of the APC contributes to acidified serum lysis of HEMPAS erythrocytes by amplifying the complement activation that is initiated by antibody.

Even when DAF function is blocked, hemolysis of HEMPAS erythrocytes is relatively modest despite deposition of amounts of activated C3 similar to those found on PNH erythrocytes (Table 2 compared with Table 1). These results indicate that, unlike PNH erythrocytes, HEMPAS erythrocytes express a membrane constituent that regulates the activity of the MAC. This interpretation was supported by experiments that indicated that MIRL is quantitatively (Fig...
COMPLEMENT REGULATION BY HEMPAS RBC

Fig 7. Autoradiograph of $^{125}$I-GPA from normal and HEMPAS erythrocytes treated with glycosidases. (A) Lanes 2, 4, 6, and 8 contain $^{125}$I-GPA from normal erythrocytes. Lanes 3, 5, 7, and 9 contain $^{125}$I-GPA from HEMPAS erythrocytes. Lanes 2 and 3 contain control (untreated) samples. Lanes 4 and 5 contain samples treated with PNGase F. Lanes 6 and 7 contain samples treated with neuraminidase. Lanes 8 and 9 contain samples treated with neuraminidase and O-glycosidase. Lane 1 contains the molecular weight standards. On the left, the mass of the standards is indicated. A difference in the mobility of $^{125}$I-GPA from normal and HEMPAS erythrocytes persists until the samples are treated with a combination of neuraminidase and O-glycosidase, indicating that the abnormality in glycosylation of HEMPAS GPA involves the O-linked oligosaccharide moiety. (B) Lanes 2, 4, 6, and 8 contain $^{125}$I-GPA from normal erythrocytes. Lanes 3, 5, 7, and 9 contain $^{125}$I-GPA from HEMPAS erythrocytes. Lanes 2, 3, 6, and 7 contain control (untreated) samples. Lanes 4 and 5 contain samples treated with a combination of neuraminidase and O-glycosidase. Lanes 8 and 9 contain samples treated with a combination of PNGase F, neuraminidase and O-glycosidase. Lane 1 contains the molecular weight standards. On the left, the mass of the standards is indicated. After removal of the O-linked oligosaccharides (lanes 4 and 5) and after complete deglycosylation (lanes 8 and 9) normal and HEMPAS GPA had the same electrophoretic mobility.

4) and functionally (Figs 1, 2, and 3) normal on HEMPAS erythrocytes. Our previous studies have demonstrated that MIRL is deficient on PNH erythrocytes and that the greater susceptibility of PNH erythrocytes to complement-mediated lysis is primarily a consequence of this deficiency.15,23

Despite the normal MIRL function, regulation of the MAC by HEMPAS erythrocytes is abnormal (Figs 2 and 3). It seems plausible to hypothesize that this defect in MAC regulation contributes to the enhanced sensitivity of HEMPAS erythrocytes to acidified serum lysis. According to this hypothesis, activation of both the classical pathway (by antibody) and the alternative pathway (by acidification of serum) is sufficient to overwhelm the protective effects of MIRL, thereby showing the defective MAC regulation on HEMPAS erythrocytes.

Our recent studies have shown that, in its isolated form, GPA from normal erythrocytes is a potent inhibitor of the MAC,18 and the present study has demonstrated that GPA on HEMPAS erythrocytes is abnormally glycosylated (Fig 7). However, in its isolated form, HEMPAS GPA has normal MAC inhibitory activity (data not shown). There seem to be two plausible interpretations of these results. First, the defective regulation of the MAC on HEMPAS erythrocytes may not be a consequence of the glycosylation abnormality of GPA. According to this hypothesis, membrane constituents other than GPA that are abnormal on HEMPAS erythrocytes account for the MAC regulatory dysfunction. Alternatively, the MAC regulatory defect may be caused by aberrant glycosylation of GPA, but the effect may be indirect. According to this hypothesis, abnormal glycosylation of proteins that have complement regulatory activity (eg, GPA) could modify their conformation or distribution (or both), thereby affecting their functional integrity. Support for this hypothesis is suggested by studies by Fukuda et al24 that have shown that band 3 is unevenly distributed on HEMPAS erythrocytes and that the clustering seems to be a consequence of the abnormal glycosylation of the molecule that results in an increase in hydrophobicity.

The basis of the aberrant regulation of the APC C3 convertase on HEMPAS erythrocytes also remains to be established definitively. Whereas our previous studies have demonstrated that GPA is the primary binding site for activated C3,20 there is no direct evidence that GPA regulates the activity of the APC.

However, compelling evidence indicates that the carbohydrate moiety of the cell greatly influences the activity of the APC C3 convertase25 and the MAC.26 Based on these observations, it is conceivable that the aberrant regulation of complement on HEMPAS erythrocytes is a direct consequence of the glycosylation defect. According to this hypothesis, specific protein inhibitors of complement (eg, DAF and MIRL) function normally on HEMPAS erythrocytes.
cytes, but the intrinsic regulatory properties of the carbohydrate moiety are rendered dysfunctional by the glycosylation defect.

Our studies have shown abnormalities in regulation of both the C3 convertase of the APC and the MAC. Studies by Rosse et al have shown that during incubation with normal human serum and an IgM cold agglutinin that binds equally to normal and HEMPAS erythrocytes, the HEMPAS cells bind much greater amounts of activated C4 (C4b).13 The mechanism that accounts for this greater binding of C4b to HEMPAS erythrocytes has not been identified: however, Isenman and Young27 have shown that GPA is the primary acceptor for nascent C4b. Together with the studies presented herein, those results suggest that modifications of GPA may influence the functional activity of multiple elements of the complement cascade.

Our finding that the electrophoretic mobility of GPA on HEMPAS erythrocytes is abnormal (Fig 6) suggested that, in at least some cases, the glycosylation defect that is characteristic of HEMPAS involves membrane constituents other than bands 3 and 4.5. Fukuda et al have presented evidence that the HEMPAS defect is caused by a deficiency of N-acetylgalcosaminyItransferase II.28 This enzyme seems to be required for the biosynthesis of lactosaminoglycans. GPA is heavily glycosylated (~60% carbohydrate by weight). Fifteen of the 16 carbohydrate residues are O-linked sugars consisting primarily of tetrasaccharides, although a small content of trisaccharides and pentasaccharides has also been observed.29 GPA also contains a single N-linked sugar. Whereas the exact structure of the N-linked sugar is controversial,29 there is no evidence that GPA normally contains lactosaminoglycans. Further, our studies suggest that the N-linked oligosaccharide on HEMPAS GPA is normal (Fig 7).

However, Adamany et al31 identified a mother and daughters whose erythrocytes contained N-acetyllactosamine units O-glycosidically linked to GPA. Thus, in certain cases, the O-linked carbohydrate moiety of GPA can be modified by the addition of lactosaminoglycans, and our studies show that the abnormality in glycosylation of GPA on HEMPAS erythrocytes involves the O-linked moiety (Fig 7). In HEMPAS, lipids glycosylated with poly-N-acetyl lactosamine accumulate abnormally on the erythrocytes, apparently because modification of bands 3 and 4.5 with lactosaminoglycans does not occur. Conceivably, the abnormal glycosylation of GPA on HEMPAS erythrocytes is a manifestation of this same epiphenomenon. However, arguing against that hypothesis is the observation that, based on electrophoretic mobility, HEMPAS GPA seems to be underglycosylated compared with normal GPA. A definitive understanding of the basis of the aberrant glycosylation of GPA on HEMPAS erythrocytes may be obvious when the precise structure of the O-linked carbohydrate moiety becomes known. That information may provide new insights into the molecular basis of HEMPAS.

MIRL contains a single N-linked complex oligosaccharide that seems to be required for functional activity.32 Like GPA, DAF is heavily glycosylated and contains both N- and O-linked polysaccharides.6 Apparently, the glycosylation defect of HEMPAS does not involve either DAF or MIRL because the electrophoretic mobility and functional activity of these two proteins are normal (Figs 1 and 5). These observations are consistent with the hypothesis that the genetic abnormality that underlies HEMPAS does not give rise to a global disturbance of glycosylation. Thus, the aberrant glycosylation of GPA on HEMPAS erythrocytes seems to be a specific manifestation of the glycosylation defect that is characteristic of the disease.

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Aberrant regulation of complement by the erythrocytes of hereditary erythroblastic multinuclearity with a positive acidified serum lysis test (HEMPAS)

A Tomita and CJ Parker