Evidence That Several High-Frequency Human Blood Group Antigens Reside on Phosphatidylinositol-Linked Erythrocyte Membrane Proteins

By Marilyn J. Telen, Wendell F. Rosse, Charles J. Parker, Marilyn K. Moulds, and John J. Moulds

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired hematologic disease in which abnormal stem cells are believed to give rise to circulating hematopoietic cells, lacking some or all surface membrane proteins anchored to the membrane via phosphatidylinositol (PI) linkage. Because the disease is clonal in origin, many patients have in their blood both normal (or near-normal) and abnormal erythrocytes. The abnormal erythrocytes are unusually sensitive to lysis by complement, because several of the missing PI-linked proteins appear to have important complement regulatory functions. These complement regulatory proteins include decay accelerating factor (DAF), membrane inhibitor of reactive lysis (CD59), and C8 binding protein.

We previously demonstrated that DAF carries the Cromer-related blood group antigens, and that the Inab, or Cromer-null phenotype, lacks antigenic and functional expression of membrane DAF. This study was designed to determine whether high-frequency blood group antigens other than the Cromer-related blood group antigens were also likely to reside on PI-linked proteins. Because phosphatidylinositol-specific phospholipase C is largely ineffective in removing PI-linked proteins from human erythrocytes, we used PNH erythrocytes to explore the relationship of various blood group antigens to PI-linked proteins. We have now demonstrated that several blood group antigens, including Cartwright (Y*Y*), John Milton Hagen (JMH), Holley-Gregory (Hy/Gy*), and Dombrock (Do/Do*), are absent from complement-sensitive PNH erythrocytes and thus are likely to reside on PI-linked membrane proteins.

MATERIALS AND METHODS

Blood from normal donors and patients with PNH was collected steriley into acid citrate-dextrose and stored at 4°C until use (usually within 10 days). Erythrocytes were prepared for testing by washing with phosphate-buffered saline, with removal of the buffy coat. Cells were suspended in saline at approximately 4% (vol/vol) for agglutination testing and at 4 x 10^8/mL (confirmed by automated cell counter) for radioimmunoassays. Erythrocytes from PNH patients without measurable populations of PNH II cells (with partial deficiency of PI-linked proteins) were separated into greater than 95% PNH III cells (extremely complement-sensitive and lacking all known PI-linked complement regulatory proteins) by anti-acetylcholinesterase affinity chromatography, as previously described. When adequate numbers of circulating PNH I cells (with near-normal complement sensitivity and expression of PI-linked proteins) were present, those cells were purified by cobra venom lysis of type III cells, also as previously described.

Murine monoclonal antibodies (MoAbs) used in this study included anti-LFA-3, directed against lymphocyte function-associated antigen-3 (CD58) and provided by Dr Timothy Springer; and antibody H8, with apparent JMH specificity, provided by Dr Robert Knowles. Dr Charles Parker provided rabbit anti-CD59 antisera. Rabbit antisera to acetylcholinesterase and DAF, produced as previously described, were also used.

Human antisera to high-frequency antigens were obtained from the Reference Laboratory of Gamma Biologicals, Inc (Houston, TX), and contained only the specificity being tested. No attempts were made to test ABO mismatched antisera or to use antisera from which other specificities had been absorbed. When only one antisera from a given specificity was used for a particular patient's cells, the most strongly and consistently reactive ABO-compatible antisera was chosen. Antisera that appear to subdivide the JMH blood group were those previously described by Moulds et al; these antisera are all nonreactive with JMH-negative red cells, but demonstrate various patterns of reactivity with cells from the producers of the other antibodies in this category. Taken together, these antibodies define at least two separate antigens that are lacking from JMH-negative erythrocytes.

Binding of human antisera to high-frequency antigens was measured by standard blood tank techniques, using agglutination after incubation with anti-human globulin (Coomb's reagent). Tubes containing cells and sera were numbered, and agglutination was evaluated by an observer blinded to the correlation of numbers with cell and serum samples. In all assays, antisera-detecting antigens expected to be normally expressed by PNH III cells (such as anti-En and anti-Ge2, against well-described transmembrane proteins) were tested simultaneously to ensure that PNH cells would agglutinate appropriately. Known antigen-positive controls, as well as cobra venom-treated random donor erythrocytes, were tested...
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simultaneously to ensure appropriate reactivity of the antisera being used. Binding of murine MoAbs and rabbit polyclonal antibodies was quantitated by radioimmunoassay, in triplicate, as previously described, using 125I-sheep F(ab'), anti-mouse immunoglobulin (Ig) (Amersham Corp, Arlington Heights, IL) or 125I-Staphylococcal protein A, respectively.2,18

RESULTS

Erythrocytes from a total of eight PNH patients were examined for expression of various high-incidence antigens. However, due to presence of ABO isoagglutinins and available quantities of antisera and cells, not all cells were tested with all antisera. PNH I and PNH III cells from patients no. 2 and 6 were tested and demonstrated normal reactivity with numerous antisera to high-incidence antigens, including Rh17, LW, Ku, K22, Vel, Sel, Ok, En, FS, U, Er, I, T, P, C, C1, C3, Ge2, and Ge23. Results using those antibodies, which failed to react with PNH III cells, are shown in Table 1. Erythrocytes from five patients were tested with Cartwright system antibodies. Most Yt cells were nonreactive with PNH III cells, although PNH III cells from one patient did react weakly with some anti-Yt sera. This may have been due to the contamination of the PNH III red cell preparation by small numbers of PNH I cells, or this may represent true variability in the PI-linked protein defect of these PNH III cells. PNH I cells, available from 4 of the 5 patients, expressed Yt normally. PNH I cells from two patients expressed Yt, the rare allele of Yt, and these patients' PNH III erythrocytes failed to react with anti-Yt, suggesting that expression of the products of both Yt alleles is abnormal in PNH III cells.

Antisera to the Holley-Gregory antigens were tested using erythrocytes from five PNH patients. PNH III erythrocytes demonstrated no or markedly reduced reactivity with anti-Hy or anti-Gy; however, PNH I erythrocytes, which were available from four of these patients, did react normally with these antisera.

Numerous JMH antisera were tested for reactivity with PNH erythrocytes. PNH III samples from five patients were tested with several examples of human anti-JMH, and all failed to react. (One antiserum demonstrated weak reactivity with one example of PNH III cells.) PNH I cells, which were available from 4 of these 5 patients, reacted normally with all anti-JMH sera tested. In addition, PNH I and III erythrocytes from one patient were tested using eight antisera that appear to subdivide the JMH specificity (as described in Materials and Methods).13 Again, all of these antisera reacted normally with PNH I cells while failing to react with PNH III cells from the same patient. Finally, monoclonal anti-JMH antibody H8 was tested for reactivity with cells from five PNH patients. As shown in Table 2, antibody H8 showed markedly reduced or no reactivity with cell suspensions containing predominantly PNH III cells, while it demonstrated near-normal reactivity with PNH I cells.

In addition, the rare antiserum Emm,16 which recognizes a high-incidence antigen, was tested with the cells from PNH patient no. 2. This patient's PNH III cells failed to react with the Emm serum, while his PNH I cells reacted similarly to a normal control.

PNH II cells, which lack some but not all PI-linked proteins, were also tested for expression of some of these antigens. Erythrocytes from one patient with greater than 90% PNH II cells gave normal reactions with anti-Yt and anti-JMH (data not shown), suggesting that these antigens most likely do not reside on acetylcholinesterase or DAF, since these two proteins have been shown to be markedly decreased or absent on PNH II erythrocytes.

Two examples of JMH-negative and three examples of Yt(a−) cells were obtained and tested for reactivity with various antibodies to known PI-linked erythrocyte membrane proteins (Table 3). JMH-negative cells, which are

Table 1. Reactivity of Antiserum to High-Incidence Antigens With PNH Erythrocytes.

<table>
<thead>
<tr>
<th>Patient No. and Cell Type</th>
<th>Yt</th>
<th>Hy</th>
<th>Gy</th>
<th>JMH</th>
<th>JMH-var</th>
<th>Do</th>
<th>Do</th>
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<tbody>
<tr>
<td>1 PNH I</td>
<td>+10</td>
<td>NT</td>
<td>+4</td>
<td>+3</td>
<td>+7</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>PNH III</td>
<td>+1</td>
<td>NT</td>
<td>+1</td>
<td>+3</td>
<td>+7</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
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<td>+1</td>
<td>O1</td>
<td>+4</td>
<td>O1</td>
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<td>+7</td>
<td>NT</td>
<td>NT</td>
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<td>3 PNH I</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>NT</td>
<td>NT</td>
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<td>PNH III</td>
<td>O1</td>
<td>O1</td>
<td>O1</td>
<td>O1</td>
<td>O1</td>
<td>NT</td>
<td>NT</td>
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<tr>
<td>4 PNH I</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
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<td>O1</td>
<td>O1</td>
<td>NT</td>
<td>NT</td>
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<tr>
<td>5 PNH III</td>
<td>O1</td>
<td>NT</td>
<td>+2</td>
<td>+1</td>
<td>+2</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>6 PNH I</td>
<td>NT</td>
<td>NT</td>
<td>+2</td>
<td>+1</td>
<td>+2</td>
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<td>NT</td>
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<tr>
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<td>O2</td>
<td>O1</td>
<td>O1</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

Abbreviation: NT, not tested.

*JMH-var, antibodies that appear to recognize at least two antigens within the JMH complex, as described in Materials and Methods.
†In most instances, these antisera gave 2+ reactions with normal cells; + indicates a reaction equal in strength to that of normal controls; Dec, a reaction of decreased strength; and O, absence of any agglutination either after incubation with the serum alone and at antiglobulin phase.
erythrocytes was tested with rabbit anti-CD59 and reacted normally. Thus, these two rare phenotypes are unlikely to

specific activity (cpm/pg protein) of the anti-mouse Ig antibody used in
each particular assay. For most experiments, "normal" represents the
mean of ≥3 normal controls tested simultaneously.

Abbreviation. NT, not tested.

Table 2. Reactivity of MoAb H8 With PNH Erythrocytes

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Cell Type</th>
<th>Specific cpm Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>PNH III</td>
<td>Patient Normal Controls * % Normal Controls</td>
</tr>
<tr>
<td>4</td>
<td>PNH I</td>
<td>1,203 1,649 73%</td>
</tr>
<tr>
<td>5</td>
<td>Unsep ( &gt;80% PNH III)</td>
<td>515 1,663 31%</td>
</tr>
<tr>
<td>6</td>
<td>PNH III</td>
<td>0 224 0%</td>
</tr>
<tr>
<td>7</td>
<td>PNH III</td>
<td>35 876 4%</td>
</tr>
<tr>
<td>8</td>
<td>Unsep ( &gt;90% PNH III)</td>
<td>130 2,745 5%</td>
</tr>
</tbody>
</table>

*Values obtained with the normal controls varied according to the
specific activity (cpm/μg protein) of the anti-mouse Ig antibody used in
each particular assay. For most experiments, "normal" represents the
mean of ≥3 normal controls tested simultaneously.
†Unsep, unseparated, these patients had almost entirely PNH III cells,
as determined by standard complement lysis sensitivity assay.

presumed to lack the moiety bearing all JMH-related antigens,
generated normal amounts of LFA-3 and acetylcholinesterase, as measured by reactivity with monoclonal and polyclonal antibodies, respectively. In addition, H8 monoclonal anti-JMH failed to react with purified acetylcholinesterase or DAF by solid phase radioimmunoassay. Yt(a−) cells, which bear the rare Yt antigen Yt a, also expressed normal amounts of acetylcholinesterase and LFA-3 when similarly measured. In addition, one example of Yt(a−) erythrocytes was tested with rabbit anti-CD59 and reacted normally. Thus, these two rare phenotypes are unlikely to arise from deficiency or marked abnormality of acetylcholinesterase or LFA-3.

DISCUSSION

This study was designed to determine whether high-incidence blood group antigens other than Cromer-related antigens were also likely to reside on PI-linked proteins. Identification of such antigens was sought eventually to make feasible the study of polymorphisms of PI-linked proteins, as well as possibly to identify functionally important PI-linked proteins not yet recognized. By definition, high-incidence

blood group antigens occur in greater than 99.9% of all individuals; antisera directed against such antigens are thus rarely encountered. Nevertheless, such antigens often reside on highly conserved molecules, whose uniformity from one person to another may be required by their functional importance. Thus, the investigation of the biochemical make-up of high incidence antigens, along with the ability to identify serologically those individuals with rare variant forms, can offer insight into the structure of functionally important erythrocyte membrane proteins.

PNH erythrocytes were chosen as the system in which to investigate these antigens for several reasons. First, expression of antigens by PNH I but not by PNH III cells demonstrates that these antigens are lacking due to an acquired rather than hereditary defect (ie, it is not the lack of these molecules that predisposes to the acquisition of PNH). Second, presence of antigen-positive and antigen-negative cells from the same individual ensures that antigens are not absent due to a rare allele producing a molecule not recognized by the chosen antisera. Finally, PNH erythrocytes rather than phosphatidylinositol-specific phospholipase C-treated erythrocytes were used, because previous studies demonstrated that human PI-linked proteins are only minimally sensitive to digestion by this enzyme. The blood group antigens we identified as lacking from complement-sensitive PNH III erythrocytes are diverse from the serologic as well as biochemical points of view, and would be expected to reside on several different proteins. For example, anti-JMH fails to react with cells exposed to trypsin or papain, while anti-Yta antisera often react with such cells unless they have been treated with protease in the presence of a reducing reagent. Dombrock antisera likewise recognize factors that appear resistant to digestion by papain and other proteases. Also, although cells completely lacking the JMH and Hy/Gy determinants have been identified (in less than 1 per 1,000 individuals), no such null phenotypes have been demonstrated for the Dombrock and Cartwright blood groups. Thus, these four blood groups each most likely reside on a unique protein.

The fundamental defect of PNH erythrocytes has now been clearly shown to be the failure to express PI-linked proteins. Therefore, it is most likely that the antigens we identified as missing or reduced from PNH erythrocytes reside on such proteins. Although PNH erythrocytes have been shown to bear a reduced amount of sialic acid, this reduction has not been demonstrated to interfere with expression of blood group antigens, including such sialic acid-dependent determinants as M and N on glycoporphin. Furthermore, all the antigens demonstrated in this study as lacking from PNH III erythrocytes are sialidase-resistant antigens. The possibility also remains that one or more of the antigens we identified reside on a protein whose expression is reduced by some other mechanism that results from the complement regulatory defect; eg, such a down-regulation may occur in the expression of the erythrocyte complement receptor (CR1). However, such a mechanism is unlikely to account for the multiple antigenic defects we identified.

Further work with the appropriate antisera will be required in each case to identify the proteins that bear the
Yt<sup>a</sup>/Yt<sup>b</sup>, JMH, Hy/Gya, and Do<sup>a</sup>/Do<sup>b</sup> antigens. It is anticipated that such work will lead to characterization of polymorphisms of these proteins and, perhaps, to identification of heretofore unknown PI-linked membrane proteins.

ACKNOWLEDGMENT

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