Red Cell Membrane and Cation Deficiency in Rh Null Syndrome

By Samir K. Ballas, Margaret R. Clark, Narla Mohandas, H. F. Colfer, M. S. Caswell, M. O. Bergren, H. A. Perkins, and S. B. Shohet

A 52-yr-old multiparous white female was found to have Rh null blood type. She had macrocytic anemia, with reticulocytosis (15%-20%), of long duration. Although stomatocytes in peripheral blood were numerous and osmotic fragility was increased, suggesting increased cell water, the RBC cation content, and thus cell water, was decreased. Cell dehydration was confirmed by an increased proportion of high density RBC on Strathten density gradients. The deformability of RBC from four gradient subpopulations was measured in the ektacytometer as a function of suspending medium osmolality. Analysis of these measurements showed an abnormal reduction in cell surface area with increasing cell density, thus explaining the increased osmotic fragility of whole blood. This was confirmed by a density-dependent reduction in cell cholesterol content, suggesting membrane instability in vivo. Rh null subpopulations showed a twofold increase in both ouabain-sensitive and -insensitive Na-K ATPase activity and 86Rb transport, even in the dense fraction with the fewest reticulocytes. No membrane protein or glycoprotein abnormality was detected by SDS-PAGE. The associated deficiencies of both membrane surface area and cation content in Rh null cells, as well as increased Na-K pump activity, suggest a pleiotropic functional interrelationship among Rh antigen, membrane stability, and cation regulation.

ERYTHROCYTES FROM Rh NULL individuals lack all Rh-Hr determinants including the Landsteiner-Weiner (LW) factor. Individuals who bear this rare phenotype have an undefined red cell membrane defect associated with variable degrees of stomatocytosis, spherocytosis, increased osmotic fragility, and hemolytic anemia. In addition, Lauf and Joiner found increased rates of Na, K transport and of Na-K ATPase activity, which were higher than could be explained on the basis of reticulocytosis. These associated defects, considered as indications of abnormal membrane function, provide support for the idea that the Rh antigen, whose molecular identity has not yet been firmly established, is an integral part of the red cell membrane. Although the Rh null disease is rare, the estimated incidence being 1/6 x 10^6 individuals, it is, nevertheless, of considerable interest because it indicates that certain blood group antigens may play an important role in the maintenance of normal structure and function of the erythrocyte and its membrane. In an effort to further define the role of the Rh antigen, we have studied selected properties of Rh null red cells.

Rheologic and biochemical measurements showed a reduction in both membrane surface area and intracellular ion and water content, suggesting some role for the antigen in both membrane stability and volume regulation capacity.

MATERIALS AND METHODS

Erythrocyte Preparation

Erythrocytes from the proposita, her relatives, and from healthy volunteer human donors were drawn into heparin or acid-citrate dextrose, cooled to 4°C, and processed within 24 hr of collection. All procedures, unless otherwise noted, were performed at 4°C. Aliquots of cells were washed at least 3 times in buffers, as specified for the various procedures described below, and the buffy coat was removed by aspiration after each wash.

Preparation of Resealed Erythrocyte Ghosts

Erythrocytes, previously washed in 140 mM NaCl, 10 mM Tris-HCl, pH 7.4, were lysed in 40 volumes of ice-cold hypotonic medium containing 7 mM NaCl, 5 mM Tris-HCl, pH 7.4. After hemolysis was complete, the hemolysate was centrifuged at 15,000 rpm for 10 min in a Sorvall RC-5 centrifuge. The supernatant was removed, and the ghosts were suspended in 10 volumes of "resealing buffer," after which they were incubated at 37°C for 1 hr to complete rescaling. The rescaling buffer contained 10 mM Tris-HCl, pH 7.4, to which NaCl was added to adjust the osmolality to 290 mosmole/kg. A subsequent centrifugation at 15,000 rpm for 5 min produced a concentrated ghost suspension for the fragmentation measurement described below. This procedure yielded ghosts impermeable to Dextran, but probably not to small cations, such as Na or K. However, the ghost fragmentation assay only requires rescaling to Dextran in order to produce laser diffraction patterns from the ghosts suspended in Dextran solutions. Thus, it was unnecessary to add chelators, such as EGTA or adenosine triphosphate (ATP), to the "resealing buffer" to insure rescaling to small molecules. When examined by phase-contrast microscopy, the rescaled ghosts had a smooth discoid morphology.

Ektacytometric Studies

The ektacytometer, a viscosidiffractometer, designed and previously described by Bessis and Mohandas, was used to determine red cell deformability under various experimental conditions. This
devic employs a well-defined laminar shear stress field on erythrocytes, while simultaneously monitoring the extent of cell deformation. The instrument consists of a concentric cylindrical viscometer, in which the outer cylinder can be rotated to produce the desired level of shear stress. A laser beam is directed through the cell suspension, which is contained within a 0.5-mm gap between the two cylinders. The cells are oriented by the shear stress field and diffract the laser beam to produce a coherent diffraction pattern, from which cell deformation is determined, using a photometric image analysis system. A "deformability index" (DI) is obtained, which is equivalent to a measure of the ellipticity of a uniformly deforming cell population. In the standard mode of operation, the DI is recorded continuously as a function of shear rate. For measurement of intact cell deformability, 10 μl of 40% red cell suspension is thoroughly mixed with 3.0 ml of polynvinylpyrrolidone (PVP, mol wt 360,000, 4g/ml, w/v, 32.6 cp viscosity, 290 mosmole/kg, pH 7.4). At 20°C, this suspension produces a maximum shear stress of 170 dynes/sq cm at the maximum applied shear rate of 400 rpm. Numerical values of the maximum deformability index reached, defined as DI max, were used to compare the deformability of different samples.

The ektacytometer was also used to measure whole cell deformability of erythrocytes as a continuous function of the suspending medium osmolality at a constant applied shear stress of 170 dynes/sq cm (Osmotic Gradient Ektacytometry). For these studies, the DI of red cells was continuously recorded as the suspending medium osmolality was linearly increased from 50 to 900 mosmole/kg, as previously described.

Fragmentation Assay

The ektacytometer was also used to evaluate the mechanical stability of cell membranes by determining the relative tendency of red cell membranes to fragment into undeformable spherical vesicles under shear stress. For each measurement, 100 μl of resuspended ghosts, prepared as described above, were thoroughly mixed with 3.0 ml of Dextran solution (mol wt 40,000, 35 g/dl, w/v, 97.5 cp, 290 mosmole/kg, pH 7.4). Samples were brought to the maximum shear stress within 20 sec and maintained at that level until the DI fell to a constant plateau (3–10 min). The DI signal decay was recorded continuously as a function of time, and the time required for it to decay to half its maximum value was designated t1/2, the half-time for fragmentation. The applied shear stress was 575 dynes/sq cm, as calculated from the solution viscosity and the maximum shear rate of 590 sec-1 provided by the ektacytometer.

Separation of Red Cells by Density

Subpopulations of erythrocytes of uniformly defined densities were isolated by centrifuging normal and Rh null cells on discontinuous Stractan II (St. Regis Paper Co., Tacoma, WA) density gradients. The gradients covered a density range from 1.065 to 1.139 g/ml in increments of about 0.004 g/ml.

Active and Passive Influx of 86Rb

Influx of Rb ions was determined by measuring the intracellular accumulation of 86Rb added to the cell suspension medium in the presence or absence of 0.1 mM ouabain, as previously described. In these experiments, erythrocytes were washed and suspended in buffered saline containing potassium and glucose (BSKG), as described before.

Potassium Efflux

Red cells, drawn into heparin, were washed 3 times in a HEPES-buffered (10 mM, pH 7.50) NaCl solution (290 mosmole/kg) containing 5 mM glucose, and the buffy coat was removed after each wash. Washed cells were resuspended at a hematocrit of 10% in the same medium, containing 0.1 mM ouabain to define the ouabain-insensitive component of K efflux. After preincubation at 37°C for 10 min, triplicate aliquots of cell suspension were removed at 15-min intervals, centrifuged in a Beckman microfuge, and the supernatants were reserved for subsequent analysis of K+ by flame photometry. Initial intracellular cation concentrations were determined by hemolysis of cell aliquots washed in isotonic Tris-buffered MgCl2 (10 mM Tris-HCl, pH 7.4, 290 mosmole/kg). First-order K+ efflux rate constants were determined by plotting the log of the declining intracellular K+ concentrations, calculated from the initial and supernatant concentrations, versus time.

To define the chloride-dependent component of the ouabain-insensitive K+ efflux, experiments were performed in which paired samples were run with the addition of 1 mM Piretanide (3-N-pyrrolidino-4-phenoxysulfanoylbenzoic acid). One such experiment was performed on freshly drawn cells, and a second was performed on cells from a separately drawn sample of heparinized whole blood stored for 5 days at 4°C in the presence of 0.5 volume modified Alsever's solution (Gamma Biologicals, Inc., Houston, TX) and 0.5 volume of phosphate-buffered saline containing 5 mM K+ and 10 mM glucose (BSKG). Storage had no effect on the K+ efflux rate constants obtained.

Adenosine Triphosphatase Assays

Hemoglobin-free erythrocyte membranes were prepared from red cells by hypotonic lysis and washing in 7 mM NaCl, 5 mM Tris-HCl, 1.0 mM EDTA, pH 7.4.

The Mg2+-dependent adenosine triphosphatase (ATPase) activity was assayed in a medium containing 3.0 mM ATP (disodium salt neutralized to pH 7.1), 20 mM Tris-HCl, pH 7.4, 1.0 mM EGTA, 0.2 mM ouabain, 3 mM MgCl2, 116 mM NaCl, and 16 mM KCl. Ouabain was omitted when the total Na+-K+ ATPase activity was to be determined. The Na+-K+ ATPase was defined as the ouabain-sensitive component, and the ouabain-insensitive component as the Mg2+-dependent ATPase activity. Each assay tube contained 100–300 μg of membrane protein in the presence or absence of 0.2 mM ouabain in a total volume of 2.0 ml. ATP and membrane blanks were run simultaneously. Incubations were at 37°C for 1 hr. The inorganic phosphate (Pi) liberated was measured by the method of Fiske and Subbarow, as modified by Bartlett. Each ATPase activity was expressed as nmole Pi liberated/mg membrane protein/mg.

In some experiments, the Na-K ATPase activity of open membrane preparations was defined as the rate of hydrolysis of adenosine-5'-triphosphate, as previously described.

Membrane Lipid Extraction and Quantitation

Erythrocyte lipids were extracted according to the method of Rose and Oklander using isopropanol-Chloroform. Phospholipid-phosphorus was determined by the method of Fiske and Subbarow, as modified by Bartlett. Cholesterol was measured by the method of Wybenga et al. For analysis of phospholipid classes in red cells, lipid extracts were dried with N2 and the various phospholipid classes were separated by thin-layer chromatography (TLC) on silica gel H plates, using the solvent system described by Skipski et al. Lipid spots on the TLC plates were visualized using iodine vapor, and their phosphorus content was determined.

Analytical Methods

Hemoglobin (Hb), mean corpuscular volume (MCV), and mean corpuscular hemoglobin concentration (MCHC) were determined
by standard methods and the hematocrit (Hct) was determined by centrifugation. Red cell counts were determined using a Coulter Counter Model F. Immunohematologic studies were performed according to standard Blood Bank procedures. Red cell sodium and potassium concentrations were determined by flame photometric analysis of red cell aliquots washed in ice-cold, isotonic Tris-buffered McCl₂ solution (10 mM Trís-HCl, pH 7.4). Membrane proteins were analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE), as previously described. Red cell membrane protein content was determined by the method of Lowry et al.²³

RESULTS

Case History

K.M. is a 52-yr-old white multiparous woman who was transfused in 1958 with 2 U of blood during an operation for oophorectomy for ectopic pregnancy. She was typed, at that time, as blood group O, Rh₀(D) negative. In April 1982, she sustained a fracture of her left humerus and was found to have multiple blood group antibodies in her serum during routine blood bank testing. Comprehensive immunohematologic investigations, described below, were diagnostic of the Rh null phenotype.

The patient is known to have had macrocytic anemia of moderate severity and of unknown etiology for a long time. Serum iron, total iron binding capacity, folic acid, and vitamin B₁₂ levels were all within normal limits. Results of Schilling tests I and II were normal. Her anemia did not respond to therapeutic trials with iron or vitamin replacement.

Serologic Investigations

The patient’s erythrocytes lacked all Rh antigens and the LW factor. Her serum agglutinated all red cells teated, except Rh null cells (two examples), giving 2+ reactions at room temperature, 3+ reactions at 37°C, and 4+ reactions at the antiglobulin phase. The antibody was completely removed by two absorptions with R₁R₁, R₂R₂, and rr cells. It was identified as anti-Rh 29 antibody, which reacts with the total Rh antigens.

Aliquots of the patient’s cells were used to absorb anti-Rh₁(D), anti-rh’(C), anti-rh’’(E), anti-hr’(c), and anti-hr’’(e) antibodies. Eluates were prepared and tested with the appropriate cells. The patient’s cells did not reduce the titer of the antisera, nor was it possible to elute antibody from them after exposure to the antisera.

Taken together, all of the above serologic investigations are diagnostic of the Rh null phenotype. Based on the above and on additional studies, the patient’s erythrocytes typed as group O, Rh null (D−, C−, E−, c−, e−), LW−, M+, N+, S+, s−, U−, Le(a−b−), Pf₁+, I+, Fy(a−b+), Jk(a−b+), K+, k−, K₅(a−b+), Js(a−b+), Vel+ and Lu(b+). Family members studied are indicated in Fig. 1. Her husband typed as group A, Rhₐ(D) positive (R₁R₁, or DCE/ DCE) and her daughter typed as group O, Rhₐ(D) positive (R₁R₁, or DCE/DCE).

Hematologic Studies

The patient’s Hb varied between 8 and 10.8 g/dl, Hct 26%-31%, MCV 100–104 fl, and MCHC 31–35 g/dl. The reticulocyte count ranged from 14% to 20%, and the osmotic fragility of freshly drawn red cells was minimally increased (50% hemolysis at 0.45% NaCl; normal range for this laboratory 0.39%-0.43% NaCl). Serum haptoglobin was reduced to 5 mg/dl, and Hb electrophoresis revealed no abnormalities. Examination of the peripheral smear showed numerous stomatocytes, some spherocytes, and polychromasia (Fig. 2A). Wet preparations, examined by phase-contrast
ABNORMALITIES OF Rh NULL ERYTHROCYTES

Table 1. Cation Concentration and Content of Normal and Rh Null Cells

<table>
<thead>
<tr>
<th>RBC</th>
<th>Experiment</th>
<th>Na' (meq/Liter RBC)</th>
<th>K' (meq/Liter RBC)</th>
<th>Na' + K' (meq/Liter RBC)</th>
<th>Na' (meq/10g Hb)</th>
<th>K' (meq/10g Hb)</th>
<th>Na' + K' (meq/10g Hb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1</td>
<td>8.5</td>
<td>94.8</td>
<td>103.3</td>
<td>0.26</td>
<td>2.68</td>
<td>2.94</td>
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<td></td>
<td>2</td>
<td>8.9</td>
<td>92.1</td>
<td>101.0</td>
<td>0.28</td>
<td>3.04</td>
<td>3.32</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>8.9</td>
<td>98.5</td>
<td>107.3</td>
<td>0.28</td>
<td>3.04</td>
<td>3.32</td>
</tr>
<tr>
<td>Rh null</td>
<td>1</td>
<td>6.6</td>
<td>75.0</td>
<td>92.0</td>
<td>0.19</td>
<td>2.46</td>
<td>2.65</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6.4</td>
<td>92.1</td>
<td>98.5</td>
<td>0.19</td>
<td>2.66</td>
<td>2.85</td>
</tr>
</tbody>
</table>

*For a series of 20 control samples, the following average (SD) cation contents were found: Na' — 0.26 (0.05), K' — 2.83 (0.17), Na' + K' — 3.08 (0.18).

Special Hematologic Studies

Because of reported heterogeneity in the hydration status of stomatocytes, we next determined the cation content of Rh null erythrocytes, as summarized in Table 1. Both Na and K were slightly decreased in Rh null red cells compared to control cells, suggesting modest cell dehydration. This cellular dehydration was confirmed by the observation of increased populations of high-density red cells on discontinuous Stractan density gradients (Fig. 3). Table 2 summarizes the reticulocyte count, the MCHC, and the cation content of five subpopulations of Rh null erythrocytes separated on the density gradient. As indicated, the most dense red cells had a very high MCHC and showed a deficiency of about 25% in total cation content, despite the presence of 9.6% reticulocytes. These cells were, therefore, severely dehydrated. The density distribution of the Rh null cells, shown in Fig. 3, indicates that the majority of the cells are the high-density subpopulations (density >1.095), in which a deficiency of cation content was found.

To provide further information about cation regulation in the Rh null cells, we measured various components of monovalent cation fluxes. In the experiment summarized in Fig. 4, 86Rb influx into red cells was determined in the presence or absence of 1.0 mM ouabain. As indicated, both the total and ouabain-insensitive (passive) influx of Rb was increased in Rh null erythrocytes. The ouabain-sensitive influx of Rb, generally taken to represent the active, pump-mediated influx, was also increased (by 72%) in Rh null cells. Similar results were obtained when Rb flux was determined in one of the dense Rh null Stractan fractions (stopping density = 1.105; see Table 2), as shown in Fig. 4B. In this experiment, control cells had

Fig. 3. Stractan gradient density distribution for control and Rh null erythrocytes. The latter show a shift in cell population to higher density. The inset shows the actual density profile of Rh null (A) and normal (B) erythrocyte subpopulations separated on discontinuous Stractan density gradients.
Table 2. Properties of Rh Null Subpopulations Isolated by Stractan Gradient Centrifugation

<table>
<thead>
<tr>
<th>Stopping Density</th>
<th>MCHC (g/dl)</th>
<th>MCV (fl)</th>
<th>Reticulocytes (%)</th>
<th>MCHV/10g Hb</th>
<th>Na⁻</th>
<th>K⁺</th>
<th>Na⁻ + K⁺</th>
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</thead>
<tbody>
<tr>
<td>1.075</td>
<td>---</td>
<td>24.8</td>
<td>123</td>
<td>60.4</td>
<td>0.21</td>
<td>4.19</td>
<td>4.41</td>
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<tr>
<td>1.083</td>
<td>30.2</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>1.085</td>
<td>31.0</td>
<td>28.5</td>
<td>112</td>
<td>34.3</td>
<td>0.18</td>
<td>3.49</td>
<td>3.67</td>
</tr>
<tr>
<td>1.087</td>
<td>32.8</td>
<td>93</td>
<td>101</td>
<td>18.0</td>
<td>0.14</td>
<td>2.61</td>
<td>2.75</td>
</tr>
<tr>
<td>1.092</td>
<td>33.8</td>
<td>95</td>
<td>92</td>
<td>0.5</td>
<td>0.25</td>
<td>2.86</td>
<td>3.11</td>
</tr>
<tr>
<td>1.095</td>
<td>34.9</td>
<td>90</td>
<td>0</td>
<td>0.26</td>
<td>0.27</td>
<td>2.24</td>
<td>2.63</td>
</tr>
<tr>
<td>1.096</td>
<td>35.7</td>
<td>89</td>
<td>0</td>
<td>0.27</td>
<td>0.27</td>
<td>2.24</td>
<td>2.53</td>
</tr>
<tr>
<td>1.101</td>
<td>39.0</td>
<td>90</td>
<td>8.4</td>
<td>0.17</td>
<td>0.17</td>
<td>2.09</td>
<td>2.27</td>
</tr>
<tr>
<td>1.105</td>
<td>44.2</td>
<td>81</td>
<td>9.6</td>
<td>0.21</td>
<td>0.21</td>
<td>1.55</td>
<td>1.76</td>
</tr>
<tr>
<td>1.115</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
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<td>---</td>
</tr>
</tbody>
</table>

*The density of the Stractan layer on which each cell subpopulation rested.

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Fig. 4. (A) ⁸²Rb influx into control and Rh null erythrocytes. Cells are incubated at 37°C in glucose phosphate-buffered saline containing 5 mM K and ⁸²Rb in tracer quantities, in the presence and absence of 0.1 mM ouabain. (B) ⁸²Rb influx into control RBC and one of the Rh null subpopulations separated on Stractan density gradient. The Rh null fraction (MCV 90 fl, retics 8.4%) was that which rested on the Stractan layer with 1.106 g/ml density (see Table 2). MCV of unseparated control RBC ~ 89 fl.

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an MCV of 89 fl and a reticulocyte count of 1.2%, whereas the fractionated Rh null cells had an MCV of 90 fl and a reticulocyte count of 8.4%. Thus, both cell types used had the same volume, although other measurements indicated that the Rh null cells had less surface area (see below). Measurement of ATPase activity in membrane preparations from Rh null cells and their subpopulations showed a twofold increase in both Na-K ATPase and Mg ATPase activities, as measured by colorimetric determination of the rate of inorganic phosphorus released from ATP (Table 3). Although the increased number of reticulocytes would be expected to cause an increase in ATPase activity, the percentage of reticulocytes appeared to have little effect on the ATPase activities in the various Rh null subpopulations. An alternative method of measuring ATPase activity, in which we determined the total and ouabain-sensitive hydrolysis of γ⁻³²P-ATP by unseparated red cell membranes, also showed increased ATPase activity in Rh null membranes. Total hydrolysis of radioactive ATP was 6.45 nmole/mg/min by Rh null membranes versus 3.03 in controls; ouabain-sensitive hydrolysis was 2.45 and 1.26 by Rh null and normal membranes, respectively. It should be noted that the values for the ATPase activities measured by γ⁻³²P-ATP hydrolysis are lower than those shown in Table 3, probably because of differences in the conditions of incubation in the two methods used. Most importantly, the concentration of ATP in the colorimetric method was 3.0 mM (see above), whereas in the γ⁻³²P-ATP hydrolysis method it was less than 1.0 mM.⁶,¹⁷

In order to further investigate the nature of the abnormality in cation regulation in Rh null cells, we determined K efflux in the presence or absence of 1.0 mM piretanide, an inhibitor of Cl⁻ transport and chloride-dependent K⁺ flux.¹⁵ Figure 5 shows that Rh null cells had a slightly increased K efflux in the
The first-order rate constant for K\textsuperscript{+} efflux for Rh null cells was 0.024 hr\textsuperscript{-1}, compared to 0.017 in control cells (41.2% increase). However, addition of 1.0 mM piretanide to the medium reduced the rate constant for K\textsuperscript{+} efflux for Rh null cells to that found for normal cells (Fig. 5).

In order to further elucidate the hydration status and surface area/volume ratio in Rh null erythrocytes and their subpopulations, we studied these cells by the method of osmotic gradient ektacytometry, described previously. The osmotic gradient deformability profiles for normal and Rh null cells are shown in Fig. 6.

The density of the Stractan layer on which each cell subpopulation rested.

Values are mean ± SD. The number of determinations is indicated in parentheses.

Table 3. Comparison of ATPase Activities in Control Cells, Rh Null RBC, and Their Subpopulations

<table>
<thead>
<tr>
<th>Stopping Density</th>
<th>Reticulocytes</th>
<th>Na\textsuperscript{+} - K\textsuperscript{+} ATPase Activity (nmol Pi/mg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ouabain-Sensitive</td>
</tr>
<tr>
<td>1.085</td>
<td>34.3</td>
<td>(2) 13.7 ± 0.17</td>
</tr>
<tr>
<td>1.095</td>
<td>18.0</td>
<td>(2) 13.5 ± 0.15</td>
</tr>
<tr>
<td>1.105</td>
<td>8.4</td>
<td>(2) 15.4 ± 0.20</td>
</tr>
<tr>
<td>1.115</td>
<td>9.6</td>
<td>(2) 12.7 ± 0.34</td>
</tr>
<tr>
<td>Unseparated RBC</td>
<td></td>
<td>(2) 13.1 ± 3.10</td>
</tr>
<tr>
<td>Rh null</td>
<td>15.6</td>
<td>(2) 6.3 ± 1.44</td>
</tr>
<tr>
<td>Control</td>
<td>0.7</td>
<td>(2) 13.1 ± 3.00</td>
</tr>
</tbody>
</table>

Fig. 5. K\textsuperscript{+} loss from freshly drawn normal and Rh null erythrocytes. Cells were incubated for 1 hr at 37°C in 10 mM HEPES (pH 7.5), 5 mM glucose, and NaCl to bring the final osmolality to 290 mosmole/kg, in the absence or presence of 1.0 mM piretanide. The values were corrected for the very small amount of hemolysis that occurred during the incubation.

Fig. 6. Osmotic deformability profile for normal and Rh null erythrocytes. The ektacytometric deformability index (DI) was recorded as a continuous function of suspension osmolality. The profiles are characterized by the following features: (1) 0 min, the osmolality at which the DI reaches a minimum in the hypotonic arm of the curve, (2) the maximum value attained by DI, which occurs near isotonic osmolality for normal cells, and (3) O', the osmolality at which DI equals half the normal maximum on the hypertonic arm of the curve. The shaded area represents the range for normal control cells.
volume ratio under isotonic conditions. This could have shifted to higher osmolality, which was consistent with increased numbers of high density cells and of reduced potassium and total cation content indirectly implicated a reduction in membrane surface area. The deformability profile provided more direct evidence in that the maximum DI value was reduced, an effect seen in surface-deficient red cells. However, the abnormally gradual slope of the hypertonic arm of the profile indicated that the Rh null sample consisted of a heterogeneous mixture of cells covering a broad MCHC range, and it was also possible that cellular heterogeneity could have been responsible for the reduced DI maximum.

In order to resolve this question, we also obtained osmotic gradient deformability profiles for the Rh null subpopulations isolated on density gradients. As shown in Fig. 7, the two most dense, dehydrated subpopulations had the same O min as the more hydrated cells and were thus osmotically fragile despite their reduced water content. In addition, these dense cells showed even more striking reductions in the maximum DI than the whole blood. Taken together, these observations provided further support for a process of concomitant loss of membrane area and intracellular cation content. Further, direct measurement of red cell phospholipid and cholesterol content showed that unseparated Rh null cells, despite their high proportion of lipid-rich reticulocytes, did not contain more cholesterol per cell than normal cells (Table 4). Analysis of density-isolated cells showed that the high-density cells were preferentially reduced in cholesterol content. Although it is true that normal cells also show a density-associated reduction in cholesterol, the most dense 9% of Rh null cells, which contained 7% reticulocytes, were lower in cholesterol than the most dense 2% of normal cells, which contained no reticulocytes. As expected, the low-density, gradient-separated cell populations had elevated cholesterol content because of their very high reticulocyte content. Analysis of the phospholipid subclasses revealed no abnormality in the phospholipid composition of unseparated Rh null cells or their subpopulations (Table 5).

To determine whether the presumed instability of the Rh null membrane could be detected as an increase in mechanical fragility, we measured their vulnerability to shear-induced fragmentation. These measurements showed that Rh null membranes fragmented at the same rate as control membranes. Analysis of membrane proteins by SDS-PAGE showed no detectable quantitative abnormality in either membrane protein or glycoprotein composition of Rh null cells.

DISCUSSION

The immunohematologic data establish this patient as another example of Rh null disease. Our findings confirm, and, to an extent, expand the data described in previously reported cases. The fact that the proposita transmitted the R, DcE gene complex to her daughter (Fig. 1) indicates that the mode of inheritance in this case is due to a suppressor, not an amorphic, gene. The negative findings of the absorption and elution experiments indicate that the expression of Rh determinants is completely suppressed in this patient. The technique used detects the weak Rh variant (D's) and the weakest subtype of blood group A, and even at this level of sensitivity, no Rh or Hr
ABNORMALITIES OF Rh NULL ERYTHROCYTES

Table 4. Membrane Lipid Composition of Normal Cells, Rh Null RBC, and Their Subpopulations

<table>
<thead>
<tr>
<th>Stopping Density*</th>
<th>Percent of Total Cells</th>
<th>Reticulocytes (%)</th>
<th>Cholesterol (g/10^12 Cells†)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Rh Null</td>
<td>Control</td>
</tr>
<tr>
<td>1.083</td>
<td>3.9</td>
<td>—</td>
<td>7.5</td>
</tr>
<tr>
<td>1.087</td>
<td>35.5</td>
<td>—</td>
<td>0.8</td>
</tr>
<tr>
<td>1.092</td>
<td>33.5</td>
<td>30.8</td>
<td>0.5</td>
</tr>
<tr>
<td>1.096</td>
<td>21.1</td>
<td>—</td>
<td>0.5</td>
</tr>
<tr>
<td>1.101</td>
<td>4.4</td>
<td>24.2</td>
<td>0.5</td>
</tr>
<tr>
<td>1.106</td>
<td>—</td>
<td>20.9</td>
<td>—</td>
</tr>
<tr>
<td>1.115</td>
<td>—</td>
<td>15.5</td>
<td>—</td>
</tr>
<tr>
<td>1.168</td>
<td>1.6</td>
<td>8.6</td>
<td>0.5</td>
</tr>
<tr>
<td>Unseparated RBC</td>
<td>1.0</td>
<td>13.4</td>
<td>1.29 (0.04)</td>
</tr>
</tbody>
</table>

*The density of the Stractan layer on which each cell subpopulation rested.
†Values shown are the average of two determinations. The range of variation is given in parentheses.

Determinants could be detected. Similarly negative studies from elution experiments were reported in other cases of Rh null disease.1,28,29 It must be pointed out that the clinical and hematologic picture in Rh null disease is the same regardless of whether the mode of inheritance is due to suppressor or amorphic gene. Thus, it appears that the absence of the Rh antigen binding site itself results in the membrane defect.

Rh<sub>mod</sub> is a different RBC abnormality, described in two North American families,30 in which expression of Rh antigens is depressed, but not absent, because of the influence of a suppressor gene. This disorder was also accompanied by stomatocytosis and hemolytic anemia, which required splenectomy.30 This variant was ruled out in the present case by the absorption and elution studies described.

Another point to be noted is that the patient's erythrocytes were found to be U-negative, although they are S+. This aberrant U blood group behavior accompanying Rh null disease has been noted before.3,31,32 The absence of the Rh-Hr determinants seems to cause attenuation of the Ss and U antigens. In other words, Rh null cells type falsely negative for the U antigen. The exact mechanism leading to this aberrant antigenic interrelation is not well understood. The proposed mechanism is homozygosity for a gene whose normal allele produces components of the erythrocyte membrane that serve as a common substrate for the action of the Rh-Hr genes and of the genes of the MNSs and U systems.1

The clinical picture of our patient is similar to previously reported cases of Rh null disease.1,7,25,26 She had anemia of unknown etiology of long duration before the diagnosis was established. Other causes of anemia were looked for and ruled out. Unlike most of the previously reported cases, this patient presented with macrocytic anemia due, most likely, to the high reticulocyte count. Stomatocytes and spherocytes were noted in her peripheral blood.

The present studies, like those of previous investigators,6,33,35 found evidence for increased rates of both active and passive cation transport in Rh null cells. In our patient, these cation flux abnormalities were associated with apparent in vivo loss of cations and water from the cells. Although the extent of dehydration was substantial, as indicated by a marked increase in cell density for virtually the entire cell population, the observed leakage of K from the cells was only modestly increased. Thus, the dehydration may not simply be due to an increase in K permeability, as was proposed for other disorders involving red cell dehydration.36

One alternate (speculative) possibility is that an increased number of red cell Na pump sites, as was found in another Rh null patient by Lauf and Joiner,6

Table 5. Distribution of Phospholipids in Rh Null Subpopulations

<table>
<thead>
<tr>
<th>Stopping Density*</th>
<th>SM</th>
<th>PC</th>
<th>PI + PS</th>
<th>PE</th>
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<tbody>
<tr>
<td>1.085</td>
<td>27.1</td>
<td>26.3</td>
<td>17.4</td>
<td>29.2</td>
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<tr>
<td>1.095</td>
<td>25.9</td>
<td>27.1</td>
<td>18.8</td>
<td>28.3</td>
</tr>
<tr>
<td>1.105</td>
<td>28.1</td>
<td>29.5</td>
<td>15.4</td>
<td>27.0</td>
</tr>
<tr>
<td>1.115</td>
<td>26.1</td>
<td>28.3</td>
<td>16.2</td>
<td>29.4</td>
</tr>
<tr>
<td>Unseparated RBC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rh null</td>
<td>(4) 25.0 ± 1.96</td>
<td>(4) 28.6 ± 0.39</td>
<td>(4) 17.3 ± 1.34</td>
<td>(4) 29.1 ± 2.55</td>
</tr>
<tr>
<td>Control</td>
<td>(4) 27.0 ± 0.40</td>
<td>(4) 28.9 ± 0.18</td>
<td>(4) 16.2 ± 0.82</td>
<td>(4) 28.0 ± 0.84</td>
</tr>
</tbody>
</table>

*The density of the Stractan layer on which each cell subpopulation rested.
†SM, sphingomyelin; PC, phosphatidylcholine; PI + PS, phosphatidylinositol and -serine; PE, phosphatidylethanolamine.
‡Values are mean ± SD. Number of determinations is indicated in parentheses.
could contribute to a reduction in total cation content. That is, because the Na-K pump expells three Na ions for the uptake of two K ions, a high level of pump activity would tend to depress both Na and total cation levels. The fact that our patient's red cells consistently contained subnormal amounts of Na is consistent with this possibility. The patients studied by Lauf and Joiner had both normal Na and water content. However, variations in the red cell permeability to Na might account for differences in hydration status, as the steady-state ion and water content of the cell represents a dynamic balance of active and passive ion fluxes. It should be noted that, in our patient, reticulocytosis may have contributed to the increase in ouabain-sensitive ion transport. However, normal reticulocytes do not have reduced cation content; thus, reticulocytosis per se cannot explain the reduced cation content in this patient.

A second possibility is that red cell dehydration in our patient could be the result of abnormal Na-K cotransport. Our finding that the slight increase in K efflux was reduced to normal by piretanide, an inhibitor of the chloride-dependent cotransport process, strongly suggests an abnormality in this component of cotransport. However, because the possible role of cotransport in red cell volume regulation has not yet been defined, and because we have measured the cation flux in only one direction, the implications of this observation are not clear.

One point of interest is that substantial numbers of reticulocytes were found throughout the density gradients, indicating that even very young cells had become dehydrated. Thus, it appears that the process that causes dehydration affects cells randomly and does not represent a progressive, cumulative change over the cell's lifespan.

In addition to a reduction in red cell cation and water content, the present study provides unequivocal evidence for a loss of membrane surface area by these Rh null cells. Both unseparated and density-separated cells had increased osmotic fragility, as determined from the ektacytometric osmotic gradient profiles. In addition, the density-separated cells showed a progressive reduction in lipid content, which culminated in a lipid content below that of normal cells in the denser fractions, even though the reticulocyte count remained substantially elevated. The decreased osmotic fragility of these dense cells, whose reduced water content should have made them osmotically resistant, indicates that the reduction of lipid in the relatively more mature cell populations went beyond a simple maturational remodeling. It therefore appears that absence of the Rh(D) antigen rendered the membrane unstable in some way. Smith et al. have observed differences in the fluorescence of a membrane probe and in labeling of membrane sulfhydryl groups in Rh null cells that they interpret as indications of abnormal membrane lipid–protein interactions. Such an alteration could possibly be a secondary consequence of membrane loss and reorganization. As Schmidt has pointed out, Rh null disease is similar in many respects to hereditary spherocytosis. The present evidence for membrane loss in vivo extends these similarities. In hereditary spherocytosis, one or several defects in spectrin are thought to be responsible for membrane instability. The biochemical nature of the Rh antigen has not yet been defined with certainty, and at present, there is no information about its interaction with other membrane components, such as spectrin or other possible components of the membrane skeleton.

Although several studies agree that the Rh(D) antigen is an integral membrane protein, estimates of its molecular weight vary from 7,000 to 180,000. It is believed to be a lipoprotein of low molecular weight. More recently, Victoria and colleagues have shown that anti-Rh(D) IgG binds to band 3 glycoprotein of the human red cell, suggesting a band 3 localization for the Rh antigen. SDS-PAGE analysis of Rh null membranes from our patient was similar to previous studies in showing no detectable abnormalities in either the amount or the molecular weight localization of the band 3 protein. Thus, even if the Rh(D) antigen is localized on band 3, the Rh null defect does not suppress overall protein synthesis of band 3. The Ss and U antigens, which also showed abnormal expression in our patient, are carried on glycoporphin B. PAS staining of the SDS-PAGE gels showed no abnormality in either glycoporphin A or B. The multiple abnormalities of Rh null cells suggest a functional interrelationship among Rh antigens, membrane stability, and cation regulation, or, perhaps, a fundamental alteration in membrane organization. Firm identification and characterization of the Rh antigens may well provide useful insights into structural requirements for membrane permeability and stability.

ACKNOWLEDGMENT

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