Lack of Rhesus Antigen Expression by Human Committed Erythroid Progenitors

By Ann Rearden and Philip Chiu

The D antigen of the Rhesus blood group, an erythroid-specific cell surface marker, is expressed by all morphologically recognizable human nucleated red blood cell precursors including, in low density, the pronormoblast. The object of the present study was to determine the expression of the D antigen by committed erythroid progenitors. Under conditions that produced complete inhibition of BFU-E and CFU-E by known cytotoxic antisera, no significant inhibition was produced by anti-D. Use of anti-human IgG (rabbit) to increase sensitivity and trypsinization to reveal cryptic Rh determinants were both without inhibitory effect. Erythroid bursts and colonies grew normally in methylcellulose that contained anti-D. The addition of anti-D to day 7 BFU-E did not inhibit their proliferation to mature bursts at day 14. These results suggest that the D antigen is not expressed by human committed erythroid progenitor cells. The D antigen is therefore an erythroid-specific differentiation marker, rather than an erythroid-lineage-specific antigen. The development of expression of the D antigen during erythropoiesis parallels that of band 3 protein, to which anti-D has been reported to bind. Lack of Rh expression by committed erythroid progenitors is consistent with the rarity of red cell aplasia in Rhesus hemolytic disease of the newborn and in idiopathic and drug-induced autoimmune hemolytic anemia in which the autoantibodies have apparent Rh specificity. These results imply that Rh compatibility is not a contraindication to human bone marrow transplantation.

ERYTHROID-SPECIFIC surface antigen expression changes during the course of differentiation from the committed erythroid progenitors, the burst-forming unit/erythroid (BFU-E), and the colony-forming unit/erythroid (CFU-E), to more differentiated, morphologically recognizable nucleated red blood cell precursors and mature erythrocytes. Studies of two erythroid-specific cell surface markers—glycophorin and band 3 protein—have shown that neither marker is expressed by committed erythroid progenitors, whereas both markers appear on morphologically recognizable precursors. Spectrin, a protein that exists on the cytoplasmic side of the membrane, is demonstrable in all morphologically recognizable precursors. Study of the expression of erythroid-specific antigens has enhanced our understanding of the process of erythroid differentiation and the cells regulating erythropoiesis, the function of the antigens themselves, the role of immune-mediated suppression of erythropoiesis in aplastic states, and the origin of neoplastic clones in hematologic malignancies.

The study of blood group antigen expression by committed erythroid progenitors and their progeny may be similarly rewarding. Some blood groups, such as ABH and Li, are not erythroid-specific. The Li antigen system, which has wide tissue and species distribution, has been reported present on committed erythroid progenitors and their in vitro progeny. Conflicting reports have been presented regarding the expression of ABH by early precursors. The Rhesus blood group, however, has been shown to be restricted to higher primates and man and is erythroid-specific. The D antigen is expressed in low density by the pronormoblast (6000–9000 antigen sites per cell) and progressively increases in density with subsequent erythroid maturation, reaching maximal density on the mature erythrocyte (25,000–35,000 sites per cell). Binding of anti-D to nucleated red blood cell precursors and to mature red blood cells is significantly enhanced by prior protease modification, suggesting the presence of cryptic Rh determinants within the membrane that are exposed by cleavage of cell surface glycoproteins. Although Rh antigen synthesis has not been examined, synthesis of all known erythroid membrane proteins has been demonstrated in BFU-E cultures. We therefore chose to study the expression of the D antigen in committed progenitors using in vitro erythropoietic cell cultures.

MATERIALS AND METHODS

Isolation of Mononuclear Cells

Peripheral venous blood was obtained from normal human volunteers. Bone marrow aspirates were obtained from patients of the Pediatric Hematology Clinic, University Hospital, San Diego, as part of a therapeutic protocol. Most patients carried the diagnosis of acute lymphoblastic leukemia in remission. Peripheral blood and bone marrow samples were anticoagulated with 5 U/ml of preservative-free heparin (GIBCO, Grand Island, N.Y.) and immediately diluted with an equal volume of Iscove's modified Dulbecco's medium (IMDM, GIBCO) containing 5 U/ml preservative-free heparin. The cell suspension was layered over a gradient of density...
1.071 g/cm³ (Ficoll-Paque, Pharmacia, Piscataway, N.J.) and the gradient centrifuged at 400 g for 30 min at room temperature. The interface cells were washed twice in IMDM and the cell count adjusted to 2 x 10⁶/ml. Cytocentrifuge preparations were made of an aliquot and stained with Giemsa to determine morphology. Only mononuclear cell preparations containing less than 1% contaminating red blood cells were used.

Protease modification of the mononuclear cell suspension was performed by reaction with 0.25% trypsin in IMDM (Flow Laboratories, McLean, Va.) for 30 min at 37°C. The reaction was terminated by the addition of double volume of cold fetal calf serum that had been heat-inactivated at 56°C for 30 min. Cell viability posttrypsinization, as determined by Trypan blue exclusion, was greater than 99%. Proteolytic activity of the trypsin used was verified in the Azocoll assay (Calbiochem-Behring, La Jolla, Calif.).

Erythroid Progenitor Cell Assays

Erythroid colonies and bursts were cultured according to the method of Iscove et al. In general, mononuclear cells were plated at 2 x 10⁶ cells/ml in 0.8% methylcellulose (Methocel A4M Premium, Dow Chemical, Midland, Mich.) in IMDM with 30% fetal calf serum. The cell viability concentration, as determined from the dose-response curve, was 2.7 U/ml human erythropoietin and 4 U/ml sheep plasma erythropoietin for BFU-E, and 0.7 U/ml human erythropoietin for CFU-E. Cultures were plated in quadruplicate in 35 x 10 mm Lux petri dishes with 20-mm grid (MA Bioproducts, Walkersville, Md.) at 37°C in an atmosphere of 5% CO₂ in air at 100% humidity. Colonies were scored using an inverted phase microscope (Olympus CK) on day 7 for CFU-E and days 10-14 for BFU-E. As characteristic colonies of 300 or more cells, or 3 daughter colonies. To verify the erythroid nature, individual colonies or bursts were scored as hemoglobinized colonies of 8-64 cells and CFU-E (Olympus CK) on day 7 for CFU-E and days 10-14 for BFU-E.

Anti-D IgG Eluate

Anti-D IgG eluates free of contaminating non-Rh cytotoxic activity were prepared from high-titered human anti-D sera by a modification of the acid-stromal elution technique of Jenkins and Moore. Control nonimmune IgG eluates were prepared from autologous human serum and erythrocytes using the same technique. These eluates will be described in greater detail elsewhere. Briefly, anti-D-sensitized red blood cells were converted to stroma using either 0.025% digitonin or 3 mM sodium phosphate, pH 8.0. Elution was performed with 0.1 M glycine-HCl, pH 3.0, for 6 min at room temperature, followed by rapid restoration of physiologic pH with 0.8 M K₂HPO₄, pH 8.2. In most instances, eluates were concentrated to 4 times the volume of the starting serum and stabilized by the addition of 10% heat-inactivated fetal calf serum. Following equilibration by dialysis against Dulbecco's PBS, pH 7.4, eluates were sterilized by passage through an 0.2-μm filter (Nalge, Rochester, N.Y.). Examination by SDS-polyacrylamide gel electrophoresis of eluate to which fetal calf serum had not been added revealed that 11%-25% of the total protein was IgG and the remainder consisted primarily of red blood cell membrane protein (34%-64%) and globin chains (18%-45%). Eluates were free of both albumin and transferrin as determined by radial immunodiffusion on plates that reliably detected 4 μg/ml of albumin and 2.5 μg/ml of transferrin, and were free of protease activity as determined by the Azocoll assay and by radial diffusion into agar gel containing milk casein (Bio-Rad, Richmond, Calif.). In some experiments, an anti-D serum lacking lymphocytotoxic activity was employed.

Anti-Human IgG (Rabbit)

Rabbits were immunized with human IgG prepared from pooled normal donor sera by ammonium sulfate precipitation and DEAE-cellulose chromatography. Initially, 1 mg human IgG in complete Freund's adjuvant was injected intradermally, followed bimonthly by intraperitoneal injections of 0.5 mg IgG without adjuvant. The immune rabbit sera were decomplemented at 56°C for 30 min and absorbed with pools of O, A, and B human red blood cells. Monospecificity of the anti-human IgG sera was verified by immunoelectrophoresis. This antiserum was not cytotoxic to BFU-E.

Anti-D Presensitization Method

Sensitization of mononuclear cells with anti-D prior to plating in the progenitor cell assays was performed by the method used by Fitch and Cline to detect serum inhibitors of myelopoiesis. This method, a modification of the microcytotoxicity assay of Terasaki and McClellan, uses inhibition of in vitro colony formation rather than cytotoxicity as an indirect measure of antibody binding to progenitor cells. In the short incubation method, 2 x 10⁶ mononuclear cells/ml were reacted with a single volume of anti-D eluate at 37°C for 30 min. A single volume of normal rabbit serum (C) (rabbit complement for HLA-DR typing, Pel-Freeze, Rogers, Ark., lot 1422) was added and the incubation continued for 60 min at room temperature. The mononuclear cells were washed twice in IMDM containing 10% heat-inactivated fetal calf serum, and the cells plated in the erythroid progenitor cell assays. In some experiments, incubation times were prolonged to 1 hr at 37°C for the anti-D sensitization step, and to 3 hr at room temperature for the antibody plus complement step. The negative control was IMDM or a nonimmune eluate, each containing 10% heat-inactivated fetal calf serum. The positive control, a serum obtained from a multiply transfused renal dialysis patient, was highly cytotoxic to 100% of the lymphocytes of a 40-member HLA-typed panel. This serum, included in representative experiments, resulted in 100% inhibition of both BFU-E and CFU-E colony formation under the reaction conditions described. It did not result in lymphocytotoxicity or inhibition of BFU-E colony formation when C was omitted. C was not inhibitory to BFU-E.

For the antiglobulin cytotoxicity assay, employing anti-human IgG (rabbit), the procedure was modified as follows: following incubation with anti-D eluate, cells were washed twice in IMDM and reacted with a single volume of anti-human IgG (rabbit) for 30 min at 37°C. The C' was then added as described above.

Addition of Anti-D to Erythroid Progenitor Cell Cultures

Unsensitized mononuclear cells were grown in the presence of anti-D, either by incorporation of anti-D into the methylcellulose at a final concentration of 10% (v/v) prior to plating (day 1) or by
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Comparison of mean colony counts by the Student’s t test revealed no statistically significant inhibition of BFU-E or CFU-E by anti-D in any of the experiments (Table I). The coefficient of variation of quadruplicate plates, however, varied between 3% and 71%, with a mean of 28%. Because of this variability, minor degrees of inhibition by anti-D would not have been detected.

DISCUSSION

The expression of the Rh antigen by committed erythroid progenitor cells was investigated by two methods; namely, sensitization of mononuclear cells with anti-D prior to plating in erythroid progenitor cell assays, and secondly, the incorporation of anti-D into the tissue culture medium in which previously unsensitized progenitor cells were grown. The limitations of the first method, namely, presensitization of cells, have been discussed by Fitchen et al.26 This method is relatively insensitive and does not detect antigens present in low density; it does not discriminate between direct effects on the progenitor cells and effects on auxiliary cells involved in hematopoiesis, and in most instances, is complement-dependent, requiring both an antibody that is complement-fixing and a cell that is susceptible to complement-mediated lysis. The requirement for complement is not absolute; complement-independent inhibitors of myelopoiesis24 have been demonstrated by this method. Rh antibodies, in general, occur in the complement-fixing IgG subclasses; however, these antibodies do not fix complement when bound to the D antigen as expressed by the mature red blood cell. Such antibodies may fix complement, however, when bound to cell surfaces that allow mobility of the D antigen, for instance, red cell stroma.27 It is possible that a similar mobility of the D antigen may exist in the plasma membrane of nucleated red blood cell precursors and, hence, permit complement fixation by anti-D.

We attempted to increase the sensitivity of the presensitization assay by several modifications. Primary sensitization of cells by antibody was carried out

Table 1. Effect of Anti-D on Committed Erythroid Progenitors

<table>
<thead>
<tr>
<th>Colonies/10⁶ Mononuclear Cells*</th>
<th>Control</th>
<th>Anti-D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FCS</td>
<td>Eluate</td>
</tr>
<tr>
<td>Peripheral blood BFU-E</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Presensitization</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Short incubation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rh negative</td>
<td>47 ± 32</td>
<td>51 ± 15</td>
</tr>
<tr>
<td>Rh positive</td>
<td>30 ± 13</td>
<td>24 ± 9</td>
</tr>
<tr>
<td>Long incubation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rh negative</td>
<td>188 ± 30</td>
<td>160 ± 42</td>
</tr>
<tr>
<td>Rh positive</td>
<td>195 ± 70</td>
<td>215 ± 128</td>
</tr>
<tr>
<td>Unmodified</td>
<td>180 ± 25</td>
<td>240 ± 5</td>
</tr>
<tr>
<td>Trypsinized</td>
<td>225 ± 50</td>
<td>190 ± 20</td>
</tr>
<tr>
<td>Anti-IgG (rabbit)</td>
<td>194 ± 32</td>
<td>179 ± 14</td>
</tr>
<tr>
<td>Addition to culture</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1, eluate</td>
<td>200 ± 35</td>
<td>230 ± 40</td>
</tr>
<tr>
<td>Day 1, IgG fraction (2000)</td>
<td>125 ± 15</td>
<td>140 ± 10</td>
</tr>
<tr>
<td>Day 7, eluate</td>
<td>290 ± 25</td>
<td>265 ± 35</td>
</tr>
<tr>
<td>Bone marrow BFU-E</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Presensitization</td>
<td>220 ± 95</td>
<td></td>
</tr>
<tr>
<td>Addition to culture</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1, eluate</td>
<td>2,112 ± 88</td>
<td></td>
</tr>
<tr>
<td>Bone marrow CFU-E</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Presensitization</td>
<td>530 ± 80</td>
<td></td>
</tr>
<tr>
<td>Addition to culture</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1, eluate</td>
<td>220 ± 45</td>
<td>200 ± 10</td>
</tr>
<tr>
<td>Rh positive</td>
<td>2,036 ± 543</td>
<td></td>
</tr>
<tr>
<td>Rh negative</td>
<td>745 ± 380</td>
<td>725 ± 305</td>
</tr>
</tbody>
</table>

*Unless otherwise specified, mononuclear cells from Rh-positive donors.
†Numbers in parentheses refer to the antiglobulin titer against an OR,r cell.
at 37°C rather than room temperature. Previously, it has been demonstrated that the rate of binding of anti-D is enhanced at 37°C. Furthermore, it was postulated that incubation at 37°C may enhance mobility of the D antigen, as has been demonstrated for HLA antigens. In addition, the duration of both the primary antibody sensitization and the secondary reaction of antibody plus complement were increased substantially. Similar incubation times have been shown to increase the sensitivity of lymphocytotoxcity testing for HLA antibodies. The antiglobulin cytotoxicity assay was used to enhance sensitivity, induce mobility of the Rh antigen, and increase the likelihood of complement fixation. This method has been shown by Pellegrino and coworkers to be effective in developing cytotoxicity by non-complement-fixing antibodies to HLA antigens. Furthermore, protease modification of the mononuclear cells by trypsin was employed to reveal cryptic Rh determinants. Trypsinization conditions were chosen to optimize the effect on binding of anti-D to peripheral blood BFU-E and were significantly dissimilar to those chosen by Rowley et al., which enhanced the number and size of erythroid bursts from bone marrow and fetal liver. We did not note any significant increase in burst size or number with the trypsination conditions used.

The second method used, growth of committed erythroid progenitors in antibody-containing media, has been shown to result in inhibition of erythroid colony formation in a complement-independent fashion in some cases of antibody-mediated aplasia. For this reason, committed progenitor cells were grown in the presence of anti-D. Even at very high anti-D titer (2000), there was no inhibition of erythroid colony formation, nor was there any morphologically discernible difference in either the gross appearance of the erythroid colonies or their microscopic morphology when compared to those grown in non-antibody-containing medium. Although macrophages were present in the cultures, there was no evidence of engulfment of anti-D-sensitized erythroid colony cells.

Continuous presence of antibody in culture may have produced negative results by antigenic modulation. To test this hypothesis, BFU-E were grown in antibody-free medium until day 7, at which time anti-D was added by diffusion. Burst growth continued unaltered through the 14th day.

These culture conditions closely mimic the situation encountered by the fetus who suffers from Rhesus hemolytic disease of the newborn. The ability of committed erythroid progenitors to proliferate normally in the presence of anti-D suggests a negligible role for immune suppression of erythropoiesis in the pathophysiology of the anemia of Rh hemolytic disease. Marrow hypoplasia has been reported in neonates suffering from severe HDN; however, this hypoplasia may result from an effect of anti-D on more mature normoblasts, or from nonspecific effects secondary to the peripheral destruction of mature erythrocytes. It is unlikely that such hypoplasia results from the direct action of anti-D on committed erythroid progenitors. Similarly, lack of expression of Rh on committed erythroid progenitors is consistent with the rarity of red cell aplasia in cases of idiopathic and drug-induced autoimmune hemolytic anemia in which the autoantibody has apparent specificity within Rh. The Rh complex is, therefore, an unlikely candidate for the target antigen in cases of antibody-mediated suppression of erythropoiesis.

Our results suggest that the D antigen is not expressed by the committed erythroid progenitors BFU-E or CFU-E, but rather is first expressed by the earliest morphologically recognizable erythroid precursor cell, the pronormoblast. Expression of the D antigen progressively increases with erythroid maturation, reaching a maximum on the mature red blood cell. The D antigen is, therefore, an erythroid differentiation marker, rather than an erythroid-lineage-specific antigen as defined by Berridge. This sequence of development, with lack of expression by BFU-E and CFU-E and increasing expression during subsequent erythroid differentiation, parallels that already described for band 3 protein. This parallel development is compatible with the concept that anti-D binds to band 3 protein.

The therapeutic implication of these findings is that Rh incompatibility between donor and recipient is not a contraindication to human bone marrow transplantation. Successful engraftment of marrow from an Rh-positive donor can be expected in a sensitized Rh-negative recipient, in spite of the presence of anti-D at the time of transplantation. Reduction in anti-D titer by plasmapheresis and reduction of erythrocyte contamination of the marrow infusate would be of value, reducing the risk of transfusion reaction at the time of transplantation and of subsequent hemolysis of Rh-positive red cells produced postengraftment.

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