Blood Group D Antigen Content of Nucleated Red Cell Precursors

By A. Rearden and S. P. Masouredis

The D antigen content of nucleated red normoblasts, basophilic normoblasts, polychromatophilic normoblasts, and orthochromatic normoblasts, respectively, had \( \text{I}^{\text{25l}}\)-anti-D. D antigen first appeared in the approximately, 4, and 7 the quantity of anti-D antigen found on mature red cells. None of the other cell types were found in bone marrow labeled with anti-D.

Maximal anti-D binding occurred on mature red blood cells. Pronormoblasts, and the quantity of anti-D progressively increased during red cell maturation. Various workers have detected D and other common blood group antigens on nucleated red cell precursors in human bone marrow. The agglutination techniques used in these studies, however, are relatively insensitive, and have limited ability either to detect small quantities of antigen, or to provide a quantitative estimate of antigen content. In the present study autoradiography of bone marrow aspirates labeled with \( \text{I}^{\text{25l}}\)-anti-D has been used to detect the first appearance of the D antigen on erythroid precursors and to assess the relative quantity of D antigen on progenitor cells during maturation.

MATERIALS AND METHODS

\( \text{I}^{\text{25l}}\)-Anti-D

The labeled antibody was prepared according to methods published previously.

Bone Marrow Studies

Fifty bone marrow samples were collected from patients in the Pediatric Hematology Clinic, University Hospital, San Diego, Calif., who were having routine marrow sampling as part of a therapeutic protocol. Most samples were obtained from patients with a diagnosis of acute lymphocytic leukemia who were in remission on chemotherapy. Samples were collected in EDTA and centrifuged in a clinical centrifuge for 5 min to obtain the buffy coat. The buffy coat, enriched in nucleated cells, was incubated 30-60 min at 37°C with \( \text{I}^{\text{25l}}\)-anti-D. The sensitized cells were washed four times in antibody-free AB plasma and recentrifuged in microhematocrit tubes; the buffy coat was smeared on precleaned slides. The slides were fixed in absolute methanol for 3 min.

Autoradiography

The slides were coated with NTB-2 emulsion (Eastman-Kodak, Rochester, N.Y.) and processed as previously described. Slides were stained for 20 min with Giemsa (azure B type) made up with 50 volumes of 0.0175 M sodium phosphate buffer, pH 6.8. Nucleated red cells were classified as pronormoblasts, basophilic normoblasts, polychromatophilic normoblasts, and orthochromatic normoblasts, respectively.
normoblasts on the basis of cell size, degree of clumping of the nuclear chromatin, and staining of the cytoplasm.

**Analysis of Silver Grain Counts**

Grain counting was carried out as described previously. Grain counts were not corrected for cell size since even with the smallest cell scored (erythrocyte) more than 90% of the silver grains produced were within the emulsion area occupied by the cell.

Correction for background counts and nonspecific uptake was made by subtracting the grain count for each cell type found on a D-negative marrow that was processed simultaneously from the value for the same cell type in the D-positive marrow. The ratio of background counts to experimental counts varied from 1:50 for red blood cells to 1:2 for pronormoblasts.

Differences in mean grain counts were compared by Student's *t* test, and in relative grain counts by the rank-sum test.

**RESULTS**

Only 15 of the 50 marrows examined were technically suitable for grain counting. Photomicrographs of representative fields are shown in Fig. 1 and illustrate the typical morphology of the red cell precursors as viewed through the nuclear track emulsion.

Table 1 presents data obtained from two sets of D-positive and -negative marrow specimens representative of the marrows studied. Each pair was processed simultaneously so that grain counts on D-positive normoblasts could be corrected as described in Materials and Methods. Grain counts on D-positive
**Table 1. Mean Grain Counts on Marrow Erythroid Cells Sensitized With 125I-Anti-D**

<table>
<thead>
<tr>
<th>Marrow No.</th>
<th>RBC Orthochromatic Normoblast</th>
<th>Polychromatophilic Normoblast</th>
<th>Basophilic Normoblast</th>
<th>Pronormoblast</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 D-pos</td>
<td>7.0 (870)*</td>
<td>5.2 (320)</td>
<td>3.9 (666)</td>
<td>4.6 (150)</td>
</tr>
<tr>
<td>32 D-neg</td>
<td>0.3 (420)</td>
<td>0.3 (168)</td>
<td>0.4 (166)</td>
<td>1.3 (42)</td>
</tr>
<tr>
<td>Corrected</td>
<td>6.7</td>
<td>4.9</td>
<td>3.7</td>
<td>4.2</td>
</tr>
<tr>
<td>49 D-pos</td>
<td>5.0 (183)</td>
<td>3.5 (131)</td>
<td>2.9 (200)</td>
<td>2.3 (45)</td>
</tr>
<tr>
<td>46 D-neg</td>
<td>0.1 (182)</td>
<td>0.0 (10)</td>
<td>0.1 (52)</td>
<td>0.5 (15)</td>
</tr>
<tr>
<td>Corrected</td>
<td>4.9</td>
<td>3.5</td>
<td>2.9</td>
<td>2.2</td>
</tr>
</tbody>
</table>

*Number of cells counted.

erythroid cells were significantly greater than those on D-negative cells ($p < 0.001$). Pronormoblasts had the least number of grains, and grain counts progressively increased during maturation, achieving a maximum value on mature red cells. Intermediate forms followed the pattern shown by marrow 49, in which there was a progressive increase in grain counts that paralleled increasing cell maturity. In some cases, however, as in marrow 30, there was some disparity between grain counts and maturity. D-positive pronormoblasts always had significantly greater mean grain counts than D-negative pronormoblasts. In some preparations, however, D-positive pronormoblasts had zero or only 1 grain, with occasional cells having as many as 8 grains.

Due to variations in radioactivity of the labeled antibody and exposure time of the autoradiographs, the mean grain counts of individual marrow specimens could not be mathematically averaged to obtain a mean value for the series. A more meaningful comparison was the relative mean grain count obtained by assigning a value of 1 to the counts on the mature red cell (Table 2). When this was done the data showed a progressive increase in anti-D labeling from the pronormoblast to the red cell. The degree of variability about the mean value expressed as the coefficient of variation ranged from 10% to 35%, indicative of the inherent limitations of the method. Pronormoblasts bound about 25% of the anti-D bound to red cells; maturation to basophilic normoblasts added a further 20%, to polychromatophilic 15% more, to orthochromatic 10% more, and finally to mature red cells the additional 30%. Differences between the nucleated precursors were all significantly different at $p < 0.03$ except for the difference between the polychromatophilic and orthochromatic normoblasts.

Other cell types found in the marrow, such as lymphocytes, megakaryocytes, plasma cells, and myeloid cells did not label above background.

**DISCUSSION**

Only erythroid precursors in human bone marrow label with 125I-anti-D, and the labeling progressively increases from the earliest recognizable red cell

**Table 2. Relative Mean Grain Counts of D-positive Normoblasts**

<table>
<thead>
<tr>
<th>RBC</th>
<th>Orthochromatic Normoblast</th>
<th>Polychromatophilic Normoblast</th>
<th>Basophilic Normoblast</th>
<th>Pronormoblast</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.71 ± 0.24</td>
<td>0.61 ± 0.06</td>
<td>0.46 ± 0.12</td>
<td>0.26 ± 0.05</td>
</tr>
</tbody>
</table>

Values were based on seven marrow specimens, corrected for background and expressed as mean ± 1SD.
precursor, the pronormoblast, reaching a maximum on the mature red cell. This progressive increase in antibody binding reflects either a biosynthetically produced increase in intrinsic D antigen or membrane alterations during maturation that affect antibody binding. Changes in sialic acid, surface glycoproteins, or orientation of the D-antigen–containing moiety could significantly affect antibody binding without necessarily indicating a change in the intrinsic D-antigen content.

Little is known about the surface properties of human normoblasts. Cationized ferritin, a positively charged particle that reacts primarily with sialic acid, binds similarly to all the developmental stages of human erythroid cells, indicating that surface sialic acid does not vary appreciably with maturation. Changes in the distribution patterns as well as a decrease in the number of receptors for concanavalin A (Con A) and *Ricinus communis* agglutinins occur during maturation of chick erythroblasts. The observations indicate that there is extensive remodeling of membrane glycoproteins during early cell differentiation. Similar results have been obtained with Con A binding to human bone marrow cells. Nucleated red cells bind approximately equal amounts of Con A, but reticulocytes and mature red cells take up greatly decreased amounts. These studies indicate that surface glycoproteins vary during human normoblast maturation, but the extent of this variation and its effect on anti-D binding remain to be determined. Although the anti-D binding studies show that D antigen first appears on the pronormoblast and subsequently increases in quantity, reaching its peak on the mature red blood cell, the data cannot rule out the possibility that the quantity of D antigen is constant in red cell precursors and that only its expression in terms of antibody binding varies with maturation. Irrespective of which interpretation proves to be correct, the results obtained indicate that there are significant changes in membrane properties during erythroid maturation.

Although there is a progressive increase in anti-D labeling that is associated with red cell maturation, it is not possible to conclude unequivocally that the labeling directly correlates with the morphologically defined erythroid precursors. Optimal conditions for cytologic identification do not prevail in examining cells which have been manipulated during antibody sensitization and washing and then are viewed through the photographic emulsion. As a result there was some difficulty in identifying the degree of normoblast maturity and some cells may have been misclassified. Anti-D binding to reticulocytes was not studied, and it remains to be determined if they have a value between that of the nucleated precursors and the mature red cells.

The quantity of D antigen immunologically available on erythroid precursors can be estimated by comparing their mean grain counts to those found on mature red cells, using the red cell as a standard. The quantity ranges from 25% on pronormoblasts to 70% on orthochromatic normoblasts. The D content also can be expressed as the number of D-antigen sites present on the membranes of the nucleated precursors. The number of D sites on peripheral red blood cells of various Rh genotypes has been determined using *I*–anti-D binding and ultrastructurally with immunoferritin conjugates with a range of 25,000–35,000 sites per cell. On this basis, pronormoblasts would have 6000–9000 D sites and intermediate normoblasts 11,000–26,000 sites.
A possible reservation in generalizing these findings relates to the patient population from which the marrows were obtained. Either chemotherapy or the underlying leukemic disease may have affected the expression of the D antigen in erythroid precursors. Rarely, active leukemia may alter the reactivity of the D antigen, and it is not known whether chemotherapy has any effect on this antigen. Grossly, there were no detectable changes in either the blood group or type in any of the patients studied.

The maturation of erythroid cells is accompanied by dramatic changes involving loss of nuclear material, loss of mitochondria, cessation of RNA synthesis, and the global consequences of these changes. Similar changes involving membrane composition and structure undoubtedly occur during maturation. There are changes in number and membrane distribution of Con A receptors, and the results of the present study indicate that erythroid maturation is associated with changes in reactivity of the D blood group antigen.

The reduced reactivity of the D antigen on erythroid stem cells may have survival value in the immune hemolytic anemias. Normoblasts can be coated by antibody in cases of erythroblastosis fetalis and in autoimmune hemolytic anemia, in which the antibody frequently has an Rh specificity. It would appear that immune hemolysis preferentially affects mature red cells with higher antigen content and partially spares the progenitor cells in order to maintain cell production.

ACKNOWLEDGMENT

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REFERENCES


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