The \textit{Dpg} Gene: An Intracorpuscular Modifier of Red Cell Metabolism

By Nancy A. Noble and Gerald Rothstein

The genetic locus designated \textit{Dpg} has two alleles in outbred Long-Evans rats. Genotype at this locus affects quantities of red cell 2,3-diphosphoglycerate (DPG) and adenosine triphosphate, as well as activities of two important glycolytic enzymes: phosphofructokinase and pyruvate kinase. Intravascular red cell survival is shortened in low-DPG animals. In order to get closer to the specific action of this locus, we addressed the question of whether the \textit{Dpg} gene acts through intracorpuscular or extracorpuscular factors. Bone marrow transplantation after total body irradiation and \textsuperscript{51}Cr red cell survival after cross-transfusion were the methods used. Because the animals that were used differed in hemoglobin phenotype, donor and recipient cells could be quantified in cross-transplanted animals. Phenotypic markers of \textit{Dpg} genotype were measured in animals 40 to 50 days after transplantation. Values for these markers correlated highly with the percentage of donor and recipient cells present. In vivo survival of low-DPG red cells was significantly shorter than that of high-DPG cells (P < .05), regardless of the genotype of the recipient. From the present studies, we conclude that the action of the \textit{Dpg} gene is exerted by an intracorpuscular factor.

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\textbf{GENETICALLY DETERMINED VARIATIONS in the intra-erythrocytic quantity of 2,3-diphosphoglycerate (DPG) have been ascribed to the influence of a major locus, \textit{Dpg}.\textsuperscript{1} Outbred Long-Evans rat populations possess two alleles at this locus, \textit{D} and \textit{d}, with gene frequencies of 0.75 and 0.25, respectively (unpublished observation, July 1985).\textsuperscript{2} This locus is about two recombination units away from the \textit{Hbb}\textsuperscript{1} locus. Genotype at this locus affects intravascular factors. Bone marrow transplantation after total body irradiation and \textsuperscript{51}Cr red cell survival after cross-transfusion were the methods used. Because the animals commercially obtained Long-Evans rats have been tested, and all have either high or low values for all of these markers.\textsuperscript{3} Also, congenic lines of rats, differing only in the area of \textit{Dpg}, have recently been developed, and their behavior is consistent with the pleotropic effects of one locus (G.J. Brewer, University of Michigan, personal communication, April 1985).

There is considerable interest in understanding how a single genetic locus or closely linked genes can produce these changes, and the mechanism of action of \textit{Dpg} can be generally divided into two categories: (1) extracorpuscular factors and (2) intracorpuscular factors that can influence glycolysis and cellular survival. If a distinction between these can be made, then further investigations as to the nature of the \textit{Dpg} gene can be pursued more efficiently by considering only intracorpuscular or extracorpuscular causes, but not both.

In the present study, we have used bone marrow transplant and cross-transfusion techniques to establish that the action of the \textit{Dpg} gene is dependent on an intracorpuscular factor or factors.

\textbf{MATERIALS AND METHODS}

\textbf{Bone marrow transplantation.} Experimental animals were of two genotypes: \textit{AAdd} and \textit{BBDD}. The \textit{AA} and \textit{BB} refer to genotypes and the \textit{\textsuperscript{15}I\textbeta-globin} locus, which is closely linked to the \textit{Dpg} locus.\textsuperscript{2} Three days before transplantation, 5- to 7-week-old male Long-Evans rats were given drinking water containing 40 mg/L gentamicin, 250 mg/L neomycin, and 1 g/L cefoxitin. One day before transplant, animals received 9.28 Gy (5.8 Gy/min) total body irradiation from a rotating platform in a Gammator containing a cesium source (Isomedix, Parsippany, NJ). Then, animals were housed singly in sterile cages and given sterile food, as well as water containing antibiotics.

Irradiated animals were given 4 to 6 x 10\textsuperscript{6} bone marrow cells flushed from the tibia and femur of single donors into Hanks' balanced saline solution. After collection, the cells were counted electronically, suspended in a volume of 0.6 to 0.9 mL per rat, and immediately injected into the tail vein.

\textbf{Assay of erythrocye constituents.} All reagents were obtained from Sigma Chemical Co., St. Louis. Blood for enzyme assays was passed through a syringe-barrel column of alpha-cellulose (Sigma-cell) for removal of leukocytes and platelets.\textsuperscript{7} Then, the washed packed cells were subjected to lysis in a 1:15 dilution of water, followed in one minute by the addition of an equal volume of the buffer containing 20 mmol/L KPi, 3 mm MgSO\textsubscript{4}, 1 mmol/L fructose-6-phosphate, 5 mmol/L dithiothreitol, 100 \textmu mol/L EDTA, 0.006-4971/86/0705-0002$03.00/0

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and 20% (wt/vol) glycerol, pH 7.6. Enzymatic assays were carried out according to Beutler, and reactions were started with 0.05 mL of hemolysate. Results were expressed as IU/g hemoglobin. Hemolysate hemoglobin was measured by adding 0.02 mL hemolysate to 1 mL of Drabkin’s reagent and measuring the absorbance at 540 nm. Concentrations of DPG and ATP were measured on perchloric acid extracts of whole blood using the Sigma Chemical Co. kits No. 35-UV and 366-UV, respectively. Red cell and white cell counts were measured electronically, using a ZBI Coulter counter (Coulter Electronics, Hialeah, Fla). Reticulocyte counts were determined by counting at least 1,000 cells on smears stained with new methylene blue.

Red cell survival. Forty-eight male rats 3 months of age were used. Half of these rats were of genotype AADD and half were BBdd. Note that these animals were in the opposite linkage phase from those used for bone marrow transplant. Twelve animals of each genotype were given 31Cr-labeled red cells from the other 12 animals of their own genotype. Twelve other animals of each type were given cells from rats of the other genotype.

One milliliter of blood was removed from each rat by cardiac puncture and 20 μCi Na35CrO4 (ICN, 651 Ci/g) was added to each sample. After incubation for 70 minutes at 37°C, the cells were washed three times with 4 vol of sterile saline and suspended in 0.6 mL of warm saline. Labeled cells were reinfused by cardiac puncture into recipients anesthetized with ether. Subsequent samples for radioactive counting were obtained by clipping the tail and collecting the blood in heparinized calibrated pipettes. 35Cr counts were measured in a gamma counter (Model 5500, Beckman Instruments, Irvine, Calif). Serial counts were obtained over a 19-day period, using the value after 24 hours as 100%. Counts were corrected for 35Cr decay and for chromium elution from red cells of 1.88% per day. A regression analysis of the log cpm/0.1 mL blood at various times was calculated for each animal and red cell half-life calculated from these regressions. Seven of the 42 animals had a great deal of noise in chromium counts until day 4, after which decay was linear. For these animals, day 0 counts were estimated from the linear points and percentage of survival was recalculated.

RESULTS

Marrow transplantation. Expression of the β-globin locus was used as a marker for the donor and recipient cells in cross-transplanted animals. First, the electrophoretic mobility for AA, AB, and BB hemoglobin was determined, using the method of Garrick et al (Fig 1). Variant hemoglobins contain ββ-chains and constitute about 20% of the total hemoglobin. To quantify ββ-chain expression, α2β2 and α2ββ bands were cut from Ponceau S-stained cellulose acetate strips, dissolved in 3 mL acetic acid:acetone (1:1), and quantified by reading their absorbance at 520 nm. In order to determine the proportions of A and B hemoglobins in specimens from animals with transplants, a standard curve was produced by plotting the values from electrophoresis of mixtures of blood of types AA and BB in known quantities. The line is the “best fit” line from a least square regression analysis.

Fig 1. Cellulose acetate electrophoresis of rat hemoglobin phenotypes AA, AB, and BB with variant hemoglobins indicated.

Table 1. Genotypes and Numbers of Animals in Bone Marrow Transplant Groups

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. Irradiated</th>
<th>Marrow Genotype</th>
<th>Transplant Type</th>
<th>No. Surviving to 40 Days</th>
<th>Percentage Surviving</th>
</tr>
</thead>
<tbody>
<tr>
<td>DD6B(HH)</td>
<td>14</td>
<td>ddAA(QL)</td>
<td>L→H</td>
<td>9</td>
<td>64</td>
</tr>
<tr>
<td>DD6B(HH)</td>
<td>15</td>
<td>DD6B(HH)</td>
<td>H→H</td>
<td>15</td>
<td>100</td>
</tr>
<tr>
<td>ddAA(QL)</td>
<td>18</td>
<td>DD6B(HH)</td>
<td>H→L</td>
<td>10</td>
<td>56</td>
</tr>
<tr>
<td>ddAA(QL)</td>
<td>15</td>
<td>ddAA(QL)</td>
<td>L→L</td>
<td>15</td>
<td>100</td>
</tr>
</tbody>
</table>
in Table 2. However, within 40 to 52 days of transplantation, the hemoglobin, red cell concentrations, MCV, and reticulocyte count had returned to pretransplant values. In cross-transplanted animals, the finding of residual A hemoglobin in the H → L animals or B hemoglobin in the L → H animals indicated that irradiation did not completely eradicate the recipient’s marrow. In an attempt to achieve eradication of the recipient marrow, higher doses of irradiation (9.4, 9.7, or 9.8 Gy) were given, but all animals died within four days, exhibiting gut necrosis at postmortem examination.

In order to determine the effect of cross-transplantation on posttransplant Dpg genotype, the enzymatic activities of PK and PFK and the red cell concentrations of ATP and DPG were measured. Then, to determine the phenotypic contribution of marrow from either the donor or the recipient, the results were plotted according to the proportion of circulating hemoglobin derived from H (B hemoglobin-producing) or L (donor cell phenotype) regardless of the donor or the recipient phenotype (P < .001). PFK activity did not correlate as closely with donor cell phenotype (P < .05), but as can be seen (Fig 3), there was considerable variation within each control group and substantial overlap of values for the two (H → H and L → L) control groups.

Red cell survival. In Table 3, the data are shown for 51Cr-labeled red cell survival in four groups of rats. Of the 48 animals transfused, one died during the procedure and five had low counts 24 hours after transfusion. For the remaining 42 animals, the average cpm/0.1 mL blood 24 hours after injection of labeled cells was 10,000 ± 2,465. The mean and standard deviation of regression coefficients (r²) for animals in each group are high, indicating the expected log-linear decay of label (Table 3). The half-life of L cells in L recipients was 18.7 days; that of H cells in H recipients was 26.8 days. Student’s t tests indicated survival of these cells was similar when they were crossed-transfused into recip-

### Table 2. Hematologic Data 40 to 52 Days Posttransplant

<table>
<thead>
<tr>
<th>Transplant Type</th>
<th>L→H</th>
<th>H→H</th>
<th>H→L</th>
<th>L→L</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>9</td>
<td>15</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>Donor cells in circulation (%)</td>
<td>45 (0–87)</td>
<td>—</td>
<td>—</td>
<td>63 (10–99)</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>14.5 ± 26.0</td>
<td>15.1 ± 1.3</td>
<td>15.0 ± 0.9</td>
<td>15.2 ± 1.1</td>
</tr>
<tr>
<td>RBC (10⁶/μL)</td>
<td>6.5 ± 0.7</td>
<td>6.8 ± 1.3</td>
<td>7.0 ± 0.4</td>
<td>6.8 ± 0.7</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>67.3 ± 6.5</td>
<td>64.8 ± 4.4</td>
<td>64.6 ± 4.5</td>
<td>57.2 ± 4.9</td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>3.4 ± 0.8</td>
<td>3.0 ± 1.7</td>
<td>3.0 ± 1.5</td>
<td>3.2 ± 0.5</td>
</tr>
</tbody>
</table>

### Table 3. 51Cr-Labeled Red Cell Survival

<table>
<thead>
<tr>
<th>Transfusion Type</th>
<th>(donor cell type)</th>
<th>N</th>
<th>X ± SD</th>
<th>T₀½</th>
</tr>
</thead>
<tbody>
<tr>
<td>L→L</td>
<td></td>
<td>9</td>
<td>0.97 ± 0.05</td>
<td>18.74 ± 3.40</td>
</tr>
<tr>
<td>L→H</td>
<td></td>
<td>10</td>
<td>0.96 ± 0.05</td>
<td>16.10 ± 4.51</td>
</tr>
<tr>
<td>H→H</td>
<td></td>
<td>11</td>
<td>0.94 ± 0.05</td>
<td>26.83 ± 9.78</td>
</tr>
<tr>
<td>H→L</td>
<td></td>
<td>12</td>
<td>0.97 ± 0.02</td>
<td>24.77 ± 5.99</td>
</tr>
</tbody>
</table>

Fig 3. Values for phenotypic markers of Dpg genotype: (A) PK and (B) PFK activities and (C) DPG and (D) ATP levels. Open and closed circles are the means with the standard deviation for the L → L and H → H control rat groups, respectively. Diamonds and squares are individual values for the H → L and L → H cross-transplantation groups, respectively. Lines drawn are from the regression analyses using values for H → L and L → H animals only.
patients of differing phenotype. Survival was significantly lower for L cells than for H cells, regardless of recipient phenotype ($P < .05$).

**DISCUSSION**

Different alleles at the *Dpg* locus are responsible for alterations in the PFK and PK activities of red cells and in concentrations of the glycolytic intermediates, DPG or ATP. That the changes in glycolytic intermediates are secondary to in vivo differences in PFK activity seems clear, since in vivo glycolytic intermediate levels reflect a metabolic block at the PFK step of glycolysis in low-DPG red cells. However, the primary mechanism responsible for the enzymatic alterations is still unknown.

In a broad sense, the *Dpg* gene might act in one of two ways. First, it may be postulated that an extracorpuscular, perhaps a soluble factor in the plasma, causes the glycolytic alterations by its effect on the red cell. For example, studies of uremic patients have shown that plasma phosphate concentrations modify red cell metabolism. Magnesium deficiency in rats results in shortened red cell survival and decreased DPG and ATP levels. Glucocorticoids, catecholamines, and aldosterone are among hormones that modulate DPG metabolism. Another broad category is the consideration of an intracorpuscular product of the *Dpg* gene that might modify glycolysis.

Recent work from this laboratory suggests that the allelic products of the *Dpg* locus act during reticuloocyte maturation; reticulocytes from *DD* and *dd* rats do not differ with respect to markers of *Dpg* genotype. As these reticulocytes mature, however, differences characteristic of genotype become apparent. Because the quantity of immunologically detectable PFK does not differ in mature *DD* and *dd* cells, synthesis and degradation of PFK is probably not affected by genotype at this locus. Rather, the PFK present may be altered, perhaps by a secondary modification during reticuloocyte maturation, to yield an enzyme with lower specific activity in mature red cells.

The same mechanism by which PFK loses activity in mature red cells may alter the specific activity of PK. Thus, the *Dpg* gene product could be a factor produced only in reticulocytes that acts on both enzymes. Simultaneous alterations in the values for the four markers of genotype at *Dpg* are consistent with the hypotheses that *Dpg* is either a single genetic locus or a cluster of closely linked genes affecting red cell metabolism and survival. Structural loci for PK and PFK are not linked in humans, but until they are mapped in the human genome, it is conceivable that part of the survival difference observed is due to different elution rates from *DD* and *dd*.

Several things suggest that this is not the case. First, these *Dpg* containing hemoglobins constitute only about 20% of the total hemoglobin present (Fig 1). Second, in a previous study, autologous transfusion of $^{51}$Cr-labeled L or H cells was done using animals of all four possible genotypes (ie, *ddAA*, *ddBB*, *DDAA*, and *DDBB*). Although L cells had shortened survival compared with H cells, there were no differences within groups that could be attributed to different hemoglobin types.

From the present studies, we conclude that the action of the *Dpg* gene is best explained by an intracorpuscular factor.

**ACKNOWLEDGMENT**

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**REFERENCES**


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NA Noble and G Rothstein