Red Cell Diphosphoglycerate Mutase. Immunochemical Studies in Vertebrate Red Cells, Including a Human Variant Lacking 2,3-DPG

By Larry Len Peterson

Diphosphoglycerate mutase (DPGM) was purified to homogeneity from human erythrocytes. The enzyme and Freund adjuvant were injected into chickens and yielded a monospecific precipitating antibody. Radial immunodiffusion with this antibody was used to measure the amount of DPGM in hemolysates from human adult and cord red cells. Dog, rabbit, rat, chicken, and goat red cells all had DPGM during the neonatal period, but goat adult red cells had no detectable enzyme. Single bands with no spurs were present on Ouchterlony plates in which human hemolysate was placed adjacent to hemolysates from the other species tested. The amount of human red cell DPGM did not differ between young and old cells separated by centrifugation. Red cells from a patient with a DPGM genetic variant who had erythrocytosis and no detectable enzyme activity contained a reduced amount of DPGM as determined by radial immunodiffusion. The abnormal DPGM differed from normal by immunoelectrophoresis and in stability as measured by the amount of crossreacting material in young versus old erythrocytes.

Chanutin and Curnish and Benesch et al. showed the importance of 2,3-diphosphoglycerate (DPG), the most abundant organic acid-soluble phosphate in human erythrocytes, in the control of oxygen binding to hemoglobin. Since then, investigators have isolated the enzyme that catalyzes 2,3-DPG production, diphosphoglycerate mutase (DPGM). Sasaki et al. isolated homogeneous human erythrocyte DPGM with the aid of isoelectric focusing. Kappel and Hass by a less complex technique obtained relatively large amounts of homogeneous human erythrocyte DPGM. A slight modification of the method of Kappel and Hass was used to isolate human DPGM that served as antigen to obtain DPGM-specific antibody from chickens in our laboratory.

This paper describes the procedure used to produce DPGM-specific antibody and the results obtained by Ouchterlony and radial immunodiffusion and by immunoelectrophoresis. Changes during maturation in several vertebrate species were determined. Preliminary results in a patient with no detectable red cell 2,3-DPG and no DPGM activity are also presented.

MATERIALS AND METHODS

Materials. Outdated human erythrocytes were obtained from the American Red Cross. NADH, NAD⁺, dithiothreitol, dl-glyceraldehyde-3-phosphate (barium free), l-aspartic acid,
the sodium salts of α-ketoglutarate and d-glycerate-3-phosphate (3-PGA), and the pentacyclclohexylammonium salt of glycolate-2-phosphate (CMC, 1.0 meq/g) and carboxymethylcellulose (CMC, 1.0 meq/g) were obtained from Whatman. Agarose was obtained from Sigma and hydroxylapatite (Bio-Gel HTP) from Bio-Rad. Sephadex G-100 superfine was purchased from Pharmacia and enzyme-grade ammonium sulfate from Schwarz/Mann. Freund incomplete and complete adjuvants came from Difco and polyacrylamide from J. D. Baker Chemicals. All other chemicals were of reagent grade.

**Enzyme assays.** All assays were performed spectrophotometrically at 25°C. The DPGM and MPGM assays were done by the method of Kappel and Hass. The nonradioactive 2,3-DPG phosphatase (2,3-DPGP) assay was taken from the work of Sasaki et al. The glutamic oxaloacetic transaminase (GOT) assay was from the work of Beutler. One unit of enzyme activity is defined as that amount of protein that catalyzes the formation of 1.0 μmol product/min under the conditions described.

**Protein concentration** from the DPGM purification procedure was calculated from the absorbance at 280 nm based on the value $A_{280} = 10$.

**DPGM purification.** DPGM was purified by the method of Kappel and Hass except for one modification. The heat treatment, step 6, was omitted because significant DPGM activity was lost.

**The hemoglobin (Hb) concentrations** of the hemolyzates applied to Ouchterlony and radial immunodiffusion plates were determined by the cyanmethemoglobin method described by Miale.

**Ouchterlony.** This technique was as described by Ouchterlony with one exception: the agarose contained NaCl (9 g/dl H₂O), which served to enhance precipitations of the Ag-Ab complex.

**Radial immunodiffusion.** The procedure came from Fahe and McKelvey, but the agarose contained NaCl (9 g/dl H₂O), which served to enhance precipitation of the Ag-Ab complex. Serum (60 μl) containing DPGM antibody was mixed with 2 ml melted agarose and then poured on a microscope slide: wells were punched after the agarose had cooled.

**Polyacrylamide gel electrophoresis (PAGE)** was carried out by the method described by Davies using 7.5% polyacrylamide gels. Different quantities (5-40 μg) of purified DPGM were loaded. A current 2.5 mA/gel was applied for 1 hr in a water-cooled system.

**Immunization.** Each of seven chickens received 0.5 mg pure DPGM isolated by the method described above. Immunizations were by intramuscular injection of 1 ml of a 1:1 mixture of enzyme in 10 mM phosphate-buffered saline and Freund complete adjuvant. Two weeks later the chickens were reimmunized with the same amount of DPGM and Freund incomplete adjuvant. Blood was withdrawn from wing veins and allowed to clot. The serum was collected by centrifugation at 200 g and stored below 0°C. All subsequent studies were done with undiluted whole serum.

**Old and young cell separation.** Separation of red cells into young and old cells by centrifugation was done according to Murphy’s method.

**Immunoelectrophoresis.** Four microliters of purified DPGM 1 mg/ml or of hemolysate 10 g Hb/dl were added to wells, and electrophoresis was carried out as described by Ouchterlony. A current of 15 mA was applied for 1 hr; the agarose contained 25 mM phosphate buffer pH 8.6. The center slot was filled with 40 μl anti-DPGM serum.

**RESULTS**

**Enzyme purification.** Eight units of whole blood yielded 20 mg homogeneous DPGM with a specific activity of 5.72 U/mg. The yields and specific activities at each step were similar to those described by Kappel and Hass. Sephadex G-100 superfine chromatography of DPGM yielded a single protein peak of constant specific activity. It manifested a single, sharp band when 30 μg was
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loaded on polyacrylamide gels. The homogeneous DPGM had an intrinsic DPGP activity of 0.075 U/mg and MPGM activity of 1.6 U/mg.

Antibody production. All seven chickens were bled before immunization with DPGM, and the sera were tested for nonspecific precipitating antibody to DPGM on Ouchterlony plates; none was detected. One week after the booster shot of DPGM, sera from all seven chickens contained a specific precipitating antibody to DPGM. In an earlier study a similar protocol was used to immunize two rabbits; no antibody was produced. Next, because goats have low levels of DPGM activity,13 an adult goat was immunized and yielded similar negative results.

Ouchterlony plates. Hemolysates prepared from adult chicken, dog, rabbit, rat, rhesus monkey, goat, and guinea pig erythrocytes and from human cord, 9-day-old goat, and chick embryo erythrocytes were tested against adult human hemolysates for antigenic similarity of DPGM. Figure 1 illustrates the single precipitation line found in all of these species. Hemolysates from adult goat erythrocytes contained no crossreacting material to the antibody against human DPGM, but chick embryo and neonatal goat red cells contained the common DPGM demonstrated by this method. No spurs were found in any of these Ouchterlony comparisons of human DPGM with crossreacting material in hemolysates from other species.

Radial immunodiffusion. Table 1 gives the results of the radial immunodiffusion studies carried out on adult and cord blood from humans. Table 2 indicates the results of the young and old cell separation on four different blood samples. As noted by Sass et al.,14 GOT activity is a good indicator of the age of the red cell, with higher activity in younger cells. Table 2 shows that normal young cells have approximately three times more GOT activity than old cells but no difference in DPGM concentration as measured by immunodiffusion. In contrast, crossreacting material to DPGM antibody decreased with cell age in the human variant.

Immunoelectrophoresis. A single arc of precipitation was found for purified DPGM and for normal and variant human enzymes. As shown in Fig. 2, the

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Table 1. Quantitation of Red Cell DPGM (Mean ± SD)

<table>
<thead>
<tr>
<th>Type of Blood</th>
<th>No. of Different Bloods Tested</th>
<th>Concentration of DPGM (mg/g Hb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult Human</td>
<td>5</td>
<td>1.00 ± 0.04</td>
</tr>
<tr>
<td>Human Cord</td>
<td>5</td>
<td>1.44 ± 0.08</td>
</tr>
</tbody>
</table>
Table 2. Quantitation of DPGM in Human Old and Young Red Cells

<table>
<thead>
<tr>
<th>Types of Cells</th>
<th>Reticulocyte Count (%)</th>
<th>GOT Activity (U/10^12 RBC)</th>
<th>Concentration of DPGM (mg/g Hb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal subjects*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixed</td>
<td>0.62 ± 0.08</td>
<td>0.95 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>3.55 ± 0.82</td>
<td>1.15 ± 0.07</td>
<td>0.94 ± 0.03</td>
</tr>
<tr>
<td>Old</td>
<td>&lt; 0.10</td>
<td>0.34 ± 0.06</td>
<td>0.95 ± 0.02</td>
</tr>
<tr>
<td>Patient with DPGM variant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixed</td>
<td></td>
<td>0.34</td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>2.00</td>
<td>3.10</td>
<td>0.37</td>
</tr>
<tr>
<td>Old</td>
<td>&lt; 0.10</td>
<td>0.63</td>
<td>0.25</td>
</tr>
</tbody>
</table>

*Normal data are expressed as the mean ± SD for four subjects.

electrophoretic mobility of purified DPGM was the same as that in normal hemolysate and less anodal than that for the DPGM variant.

DISCUSSION

Criteria for purity of the DPGM used as antigen were similar to those used by Kappel and Hass. Isoelectric points of human DPGM and MPGM have been reported as pH 4.9 and 6.2, respectively. Even though these two enzymes were shown to have structural homology by Hass et al., they should have distinguishable arcs of immuno-electrophoresis. The occurrence of single arcs in Fig. 2 suggests that there is no detectable crossreaction of the anti-DPGM described here with MPGM.

Rosa et al. reported that DPGM and DPGP activities have the same electrophoretic mobility in hemolysates from man, rat, horse, dog, and pig and that both are absent from goat hemolysates. A single enzyme protein in human, equine, and day-old chick erythrocytes has been shown to have DPGM, DPGP, and MPGM activities, although the MPGM activity is much less than that of the separate and homologous MPGM enzyme. Further evidence for the monospecific character of the anti-DPGM antibody described here is a sin-

Fig. 2. Immunoelectrophoresis of normal and variant human hemolysate and purified DPGM. H, human hemolysate; P, purified DPGM; V, human variant. Tracking dye was placed in outer wells and confirmed a uniform migration rate.
gle immunoelectrophoretic arc (Fig. 2B) for the genetic variant that was more anodal than that of normal DPGM.

The concentration of DPGM antigen has not been reported previously. Higher amounts were found in human cord blood than in adult samples. This relationship of "fetal" to adult levels held true for all of the vertebrate species tested, including goats, in which no DPGM activity is detectable in adults but is present in 9-day-old goats. The activities of DPGM reported by Chemtab et al.25 as 314.0 and 240.0 mU/ml in fetal and adult human blood, respectively, correspond nicely with the values presented in Table 1.

The occurrence of an antigenically identical DPGM during early postnatal life in all of these vertebrate species is in keeping with the evolutionary model proposed by Coates.23 The ancestral gene for erythrocyte DPGM is probably as ancient as the divergence of amphibian from avian and mammalian species, since all rely on the production of red cell 2,3-DPG at some stage during their development. Figure 1 illustrates the immunologically identical reaction with DPGM for all of the vertebrate species we examined.

Those species, like man, that utilize red cell 2,3-DPG to modulate oxygen delivery during adult life provide an opportunity to study the effect of genetic variants of DPGM. The family reported by Rosa et al.24 suggests that absence of DPGM activity can be compensated by an erythrocytosis in the same way as Hb variants with an increased oxygen affinity. Preliminary results on the proband, who had a \( P_0 \) of 17.3 mm Hg and a red cell mass of 44 ml/kg, indicate that an inactive DPGM is present and that it differs from the normal enzyme in stability and electrophoretic mobility. The proband’s two children have 2,3-DPG levels of 3.53 and 3.27 U/g Hb.

ACKNOWLEDGMENT

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