G-6-PD Manchester: A New Variant Associated With Chronic Nonspherocytic Hemolytic Anemia

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A variant of glucose-6-phosphate dehydrogenase (G-6-PD) designated G-6-PD Manchester, and associated with chronic nonspherocytic hemolytic anemia, was found in an English male. The electrophoretic mobility at pH 7.0 of this G-6-PD variant is the slowest yet described. Substrate specificity is normal but enzyme activity is markedly inhibited by NADPH, and this is thought to account for the severe chronic hemolysis.

The number of different types of glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NADP oxidoreductase, E.C. 1.1.1.49, G-6-PD), which have been described in man is now in the region of 80 different variant enzymes. Many of these are not associated with a hemolytic process, either chronic or under the stress of certain drugs or chemicals. The first case of a G-6-PD variant associated with chronic nonspherocytic hemolytic anemia was reported by Newton in 1958. Since then about 20 such variants have been described.

Detailed studies of individual enzyme variants may reveal the biochemical basis for the shortening of the red cell lifespan in vivo. Thus there may be altered enzyme affinity for the substrate glucose-6-phosphate (G-6-P) or coenzyme, NADP, e.g., G-6-PD Oklahoma, or there may be instability of the enzyme, e.g., G-6-PD Alhambra. The correlation between clinical severity and impairment of enzyme activity, as measured by usual assay methods, is not always close and may sometimes be related to use of unphysiologic concentrations of substrates in the assay system. We now report a new variant, designated G-6-PD Manchester, which gives rise to a severe chronic hemolytic anemia, but where enzyme activity by standard assay is only moderately reduced.

MATERIALS AND METHODS

Blood from the patient and from his mother was collected in ACD, Formula A, and transported and stored at 0°C. Measurements of red cell and leukocyte enzyme activities, and partial purification of the enzyme were performed within 3 days after taking the blood. Characterization of enzymatic properties, electrophoresis, immunologic neutralization, and gel filtration were carried out using the partially purified variant enzyme preparation, with normal enzyme as control.

Blood counts were performed on a Coulter Counter Model S. Standard hematologic techniques were used for assay of pyruvate kinase, reduced glutathione, glutathione stability, and Heinz-body tests. Hemoglobin electrophoresis was performed on starch gel at pH 8.6 and on agar gel at pH 6.0.
Red cells were washed three times with 2 volumes of 0.9% NaCl solution and hemolyzed by mixing with 2 volumes of cold water containing 1 mM EDTA and 1 mM 2-mercaptoethanol. Stroma was eliminated by treatment with toluene. Hemoglobin was assayed by Drabkin’s method. Leukocytes were prepared by the method previously described, and, after suspension in 0.9% NaCl solution, were disintegrated by freezing and thawing three times. After centrifugation at 10,000 g for 15 min, the supernatant was used for enzyme assay.

Erythrocytes were separated into “age” groups, according to their specific gravity, by the two-phase centrifugation method of Danon and Markovsky, “as modified by Kirkman et al.” The effectiveness of this method of red cell fractionation has been well established by reticulocyte counts and estimation of differential labeling after addition of 51Fe. In the present investigation, separation of both the patient’s and his mother’s red cells was controlled by estimation of G-6-PD, 6-phosphogluconate dehydrogenase, and phosphoglycerate kinase levels in the separated fractions. The fractionated red blood cells were hemolyzed by mixing with 5 volumes of water and a few drops of toluene. After centrifugation, the supernatant was used for enzyme assay.

Methods used for enzyme activity measurements, determination of Michaelis constants, and neutralization of the enzyme by antiserum have been previously described. The units of activity of the enzyme are defined as micromoles of NADP reduced per minute at 25°C under the assay conditions. Starch gel electrophoresis was carried out using a phosphate buffer system at pH 7.0, and Tris-EDTA-borate buffer system at pH 8.6. For characterization, the enzyme was partially purified by absorption on DEAE-cellulose. The partially purified enzyme was essentially free from hemoglobin and 6-phosphogluconate dehydrogenase.

CLINICAL FINDINGS

I.J., a 39-yr-old male, of pure English extraction, presented with a history of recent hemipareses. There was no history of neonatal jaundice. A mild icteric tinge was first noticed by his mother when he was 2 mo old. At 2 yr he had an episode of increased icterus, and several similar episodes recurred before he underwent splenectomy at the age of six. Records are not available as to any benefit conferred by splenectomy. He required admission when 10 yr old for increasing pallor and was given a blood transfusion. This episode was not precipitated by preceding infection or exposure to drugs, and there was no history of increased jaundice or anemia after drug therapy at any time. When aged 38 yr he had sudden partial loss of vision in both eyes which lasted for 1 hr only. One year later he had a similar episode of transient loss of vision and in the 3 mo before presentation he had two hemipareses. The first, manifesting as weakness of the right arm and leg, lasted only a few hours. The second episode involved weakness and numbness of the left face, arm, and leg which recovered almost completely over 3-4 wk.

The patient had no brothers or sisters and only one child, a daughter aged 3. His mother had only one brother, who was not available for study. There was no family history of anemia, jaundice, or gall bladder disease.

Examination showed scleral icterus. The sole neurologic abnormality was slight weakness of the left facial muscles. There was no other abnormality on physical examination. He was normotensive, blood pressure 125/80 mm Hg, and there were no arterial bruits.

RESULTS

Laboratory studies on the propositus included hemoglobin 12.4 g/100 ml, reticulocyte count 20.4%, total bilirubin 3.4 mg/100 ml, indirect 2.9 mg/100 ml, direct 0.5 mg/100 ml. A G-6-PD screening test (Sigma Corporation) showed no decolorization at the end of 6 hr, indicating a deficiency of G-6-PD. Estimation of reduced glutathione showed a level of 41.4 mg/100 ml (normal 60-90 mg/100 ml). A glutathione stability test showed a reduction of glutathione by 36% after incubation with acetyl phenylhydrazine (reduction in normal individuals is less than 20%). No Heinz bodies were present in native blood, but after incubation with acetylphenylhydrazine, 96% of red cells showed five or more Heinz bodies (normal < 32.5%). Hemoglobin electrophoresis on starch gel and agar gel was normal. Pyruvate kinase assay showed a level of 1.56 U (normal 1.05-2.79 U, expressed as μmole NADH oxidized/ml red cells/min at 37°C). A blood count on the
propositus' mother showed a hemoglobin of 13.7 g/100 ml and reticulocytes were 1.9%.

G-6-PD activity in the red cells from the propositus and his mother was 20%--25% and about 70% of normal, respectively. This presumably indicates that the mother is heterozygous for Gd Manchester and normal Gd B*. The younger red cells from the subject had slightly higher G-6-PD activity (1.8 U/g Hg) than had the older red cells (1.4 U/g Hg). By contrast, younger red cells from normal individuals have two to three times higher activity than do the older red cells. This is probably due to the fact that the subject had severe hemolytic anemia, and only relatively young cells were present. The G-6-PD activity of the leukocytes of the propositus was only 15% of normal and that of his mother's leukocytes was 66% of normal.

The variant enzyme showed slower anodal electrophoretic mobility than the normal B* enzyme on starch gel electrophoresis using a Tris-EDTA-borate buffer at pH 8.6 (90% of mobility of B*), and using a phosphate buffer at pH 7.0 (37% of mobility of B*) (Fig. 1). The variant has the slowest electrophoretic mobility at pH 7.0 among about 80 known G-6-PD variants thus far reported. In order to find out the reason for the extremely slow mobility at pH 7.0, the molecular size of the variant enzyme was estimated by gel filtration with Sephadex G-200. The normal enzyme B*, dissolved at higher concentration (> 0.1 mg/ml), is predominantly tetrameric (mol wt = 220,000) at pH 6.0 and predominantly dimeric above pH 8.0. In a more dilute solution, such as a hemolysate or the enzyme solution used for the starch gel electrophoresis, the normal enzyme is predominantly dimeric at pH 6.8–7.0, and the enzyme was eluted at the same position as beef heart lactate.

Fig. 1. Electrophoresis of G-6-PD in starch gel with phosphate buffer pH 7.0. Current 8 v/cm for 4 hr at 4°C. 1: G-6-PD Manchester 2: Normal G-6-PD (B*).
dehydrogenase (mol wt, 130,000) from a Sephadex G-200 column. Using a calibrated Sephadex G-200 column, the molecular size of the variant enzyme was estimated as 200,000-250,000 at pH 6.8-7.0. Therefore, the extremely slow electrophoretic mobility of the variant enzyme could be attributed to the fact that it is predominantly tetrameric even in dilute solution at pH 7.0. Similar unusual association characteristics have been found in G-6-PD Tel Hashomer \(^{17}\) and G-6-PD Capetown, \(^{18}\) which also have very slow electrophoretic mobilities (55%-70% of normal).

Michaelis constants of the variant enzyme were similar to those of the normal enzyme, i.e., 64 \(\mu M\) for G-6-P and 6 \(\mu M\) for NADP. The utilization rate of 2-deoxy G-6-P as substrate was 3.5% of G-6-P (normal > 3%). The utilization rate of deamino NADP by the variant enzyme (79% of NADP) was slightly higher than that of the normal enzyme (55%-60% of NADP).

In the quantitative neutralization test, using antiserum to normal G-6-PD, \(^{13}\) equal amounts of antiserum neutralized fewer units of the variant enzyme (2.4 U by 1 ml serum) than that of normal enzyme (4.2 U by 1 ml serum). Assuming that the serologic activities of both variant and normal enzymes are similar, i.e., that approximately equal amounts of both enzyme proteins are absorbed by antiserum, the specific enzyme activity of the variant enzyme would account for about 55% of the normal enzyme. On the other hand, assuming that the serologic cross-reactivity of the variant enzyme for the antiserum used is lower than that of the normal enzyme, the specific enzyme activity of the variant enzyme could be higher than 55% of the normal enzyme.

The variant enzyme had a slightly biphasic pH-activity profile, having pH optima at pH 7.0 and pH 9.5. Thermostability of the variant enzyme, measured at pH 7.3 and at 37\(^\circ\)C, was much lower (75% inactivation at 60 min) than that of the normal enzyme (less than 10% inactivation).

G-6-PD Manchester was not associated with very severe red cell enzyme deficiency (20%-25% of normal), and the Michaelis constants for G-6-PD and NADP were in the normal range, suggesting that the affinity of the variant enzyme for the substrate and coenzyme is not defective. Since there are several G-6-P variants which are associated with a more severe red cell deficiency without resulting in a hemolytic process, it is necessary to explain the severe chronic hemolysis of our subject. In order to resolve this problem, inhibition of the normal and the variant enzymes by NADPH was measured under physiologic conditions (at pH 7.3 and 37\(^\circ\)C). It was found that the variant enzyme was much more strongly inhibited by NADPH (Fig. 2).

**DISCUSSION**

It is evident that G-6-PD Manchester may be easily distinguished from the 80 or so G-6-PD variants thus far reported. Comparison of Michaelis constants, degree of substrate specificity, pH optima, and, in particular, electrophoretic mobility readily shows G-6-PD Manchester to be a new variant. The extremely slow electrophoretic mobility of G-6-PD Manchester at pH 7.0 could be attributed to its unusual association property as demonstrated by gel filtration with Sephadex G-200. The red cell enzyme activity of the variant enzyme, when measured at near saturation of both substrate and NADP but in the absence of NADPH, showed re-
Fig. 2. Inhibition of glucose-6-phosphate dehydrogenase by NADPH. The reaction mixture contained 0.05 M Tris-Cl, pH 7.3, 4 mM MgCl₂, 0.1 M KCl, 0.10 μM NADP, 60 μM D-glucose 6-phosphate, and various concentrations of NADPH. Measurements were carried out at 37°C. Relative activity is given taking the activity without NADPH as 100. Continuous line-dot: Normal G-6-PD(B). Dash-triangle G-6-PD Manchester.

In many G-6-PD variants associated with red cell enzyme deficiency, enzyme activity in nucleated cells, such as leukocytes, is not as deficient as that of the red cells. For example, red cell enzyme activity of Gd Mediterranean is less than 5% of normal, but leukocytes from Gd Mediterranean subjects have about 30% of normal leukocyte enzyme activity. Leukocyte enzyme activity of Gd A⁻ is close to normal, although Gd A⁻ red cells have only about 15% of normal red cell activity. The G-6-PD activity from the subject's leukocytes was at least as deficient as that of his red cells, although it is possible that the variant enzyme might have been inactivated during the extraction of the enzyme from leukocytes.

The majority of reports of chronic hemolysis associated with G-6-PD variants have not included neurologic complications. However, Westring and Pisciotta described a 5-yr-old boy with bilateral cataracts and an ill-defined neurologic disease including mental retardation, nystagmus, and generalized seizures. There have been two reports of optic atrophy associated with G-6-PD deficiency and chronic hemolysis. The first report was of a man who developed optic atrophy and generalized seizures in middle age. In the second report, three males in an affected family had associated G-6-PD deficiency, optic atrophy, and color blindness, thought to be due to mutations of more than one gene on the affected X chromosome. Since, in at least some variants of G-6-PD, low levels of enzyme activity have been shown in tissues other than the red cells, it has been suggested that neurologic abnormalities might be due to deficient enzyme activity in neural cells.

Our patient had evidence of low leukocyte G-6-PD levels, and it seems possible that other tissues including neural cells might also be affected. However, although the etiology of his neurologic symptoms is not yet apparent, they appear to be thromboembolic in nature. The contribution of enzyme deficiency in neural cells to the symptomology of this patient can only remain a matter for speculation.
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