A New Variant of Glucose-6-Phosphate Dehydrogenase Deficiency Hereditary Hemolytic Anemia, G6PD Cornell: Erythrocyte, Leukocyte, and Platelet Studies

By Denis R. Miller and Michael R. Wollman

A variant of glucose-6-phosphate-dehydrogenase deficiency associated with chronic hereditary hemolytic anemia was discovered in a 9-yr-old white male. The erythrocytes contained 5% of normal enzyme activity, the Km NADP was two to three times normal, the pH optimum was decreased, and the heat stability was markedly decreased. The Km G6PD, electrophoretic mobility (B), and utilization of substrate analogues 2-deoxy-G6P and deamino-NADP were normal. The activity of G6PD in the leukocytes and platelets was 15% and 28% of normal values, respectively, but bactericidal activity and platelet function were unaffected by the deficiency of G6PD.

The heterogeneity of hereditary hemolytic anemia due to erythrocyte glucose-6-phosphate dehydrogenase (G6PD; D-glucose-6-phosphate: NADP oxidoreductase, EC 1.1.1.49) deficiency is now well established. Over 70 variants of the enzyme have been described and of these, 20 are associated with a chronic hereditary hemolytic anemia occurring primarily in whites. Clinically, patients have mild to severe lifelong hemolysis, splenomegaly, and exacerbations incited by infection or ingestion of certain oxidant drugs. The more common G6PD A⁻ and G6PD B⁻ variants occur in blacks and in individuals from the Mediterranean littoral, respectively.

Although the clinical features of the G6PD deficiency syndromes are similar, the variants of G6PD may be differentiated biochemically by evaluation of electrophoretic mobility and determinations of enzyme kinetics, thermal stability, and pH optima. Standardized methodology is now available for the partial purification and biochemical characterization of these new variants of G6PD.

The clinical and biochemical distinctiveness of a new variant of erythrocyte G6PD deficiency associated with a chronic hereditary hemolytic anemia is described in this report. A deficiency of the enzyme in leukocytes, platelets, and skin fibroblasts was also detected, but no abnormalities of bactericidal activity or platelet function were observed.

Materials and Methods

Routine hematologic tests were done by standard techniques. Hemoglobin electrophoresis, thermolabile hemoglobin, and assays of erythrocyte enzymes, glycolytic intermediates, and...
and glutathione and glutathione stability were performed by the methods cited. Red cell glucose utilization, lactate formation, intracellular sodium and potassium content, \( t_k \), and organ sequestration were measured by previously published methods. Erythrocyte G6PD was partially purified without modification as outlined by Motulsky and Yoshida and recommended by the World Health Organization in a normal male, the patient, his mother, and in two patients with known G6PD A and G6PD B. Electrophoresis was performed on starch gels using Tris-EDTA-borate and phosphate buffer systems. Michaelis constants \( (K_m) \) were established for G6P and NADP from the plots of \([S]/V \) versus \([S]\). To determine the \( K_m \) for G6P, the reaction velocity was measured at four to seven different concentrations (0.025-2.0 mM) of G6P at a constant (200 \( \mu M \)) concentration of NADP. For \( K_m \) NADP, G6P was held constant at 2 mM, and the concentration of NADP, confirmed spectrophotometrically, varied between 2.25 and 100 \( \mu M \). The pH optima were determined in a Tris-glycine-phosphate buffer system as proposed by Kirkman et al. The thermal stability and utilization of 2-deoxy-G6P and deamino NADP were measured as described by Beutler, Mathai, and Smith. The inhibition of G6PD by 1 mM N-ethylmaleimide (NEM) was examined at optimal concentrations of NADP and G6P. Prior to the addition of NEM, the concentrations of partially purified G6PD from the patient and the control sample were adjusted to give an optical density change of 0.006-0.012 U/min in the cuvette. All enzyme assays were performed in duplicate at 37°C in a Gilford 2400 recording spectrophotometer. The volume of reactants in the cuvette was 3.0 ml.

Leukocytes and platelets were isolated by differential centrifugation, and G6PD assays of frozen-thawed lysates were performed in the standard assay system. Purification and kinetic studies of leukocyte and platelet G6PD were not done. The patient’s fibroblasts, obtained by punch skin biopsy, were grown in Dulbecco’s modified Eagle’s medium (90%), with fetal bovine serum (10%), and after three subcultures were frozen in glycerol and stored in liquid nitrogen for assay at a later date. Normal control fibroblasts from a 4-yr-old male were obtained from the American Type Culture Collection Cell Repository (Baltimore, Md.) and were assayed upon receipt using frozen-thawed lysates in the standard system.

The in vitro bactericidal activity of the patient’s phagocytes was tested by the method of Quie and co-workers using Staphylococcus aureus 502A and Pseudomonas aeruginosa 1024 as the test organisms. Platelet function (platelet aggregation in the presence of ADP, collagen, and epinephrine and platelet factor 3 release) was evaluated by the methods cited.

The \( P_{O_2} \) (the oxygen tension of the blood at pH 7.4 and 37°C when hemoglobin is half saturated) was measured by the mixing technique using an Instrumentation Laboratory model 313 blood gas analyzer, model 181 oximeter, and model 327 tonometer before and after a 2-hr incubation with and without acetylphenylhydrazine, 5 mg/ml.

**Case Report**

This 9-yr-old white boy was referred in January 1972 for evaluation of a chronic hemolytic anemia. Labor was induced 3 wk prior to term because of Rh sensitization (anti-D); his birth weight was 6 lb. Jaundice was noted shortly after birth, and an exchange transfusion was performed on the third day of life. Jaundice persisted until discharge at 3 wk of age. Frequent recurrent infections of the upper respiratory tract were accompanied by fever, pallor, scleral icterus, and dark urine in early infancy. These episodes were often treated with aspirin, sulfisoxazole, and other antibiotics, but increased pallor or jaundice were not observed. He had never eaten fava beans.

Laboratory studies at another hospital revealed: hemoglobin, 12.6 g/100 ml; packed cell volume, 36%; reticulocyte count, 15.7%; total and indirect serum bilirubin, 3.0 and 2.6 mg/100 ml, respectively; direct and indirect Coombs’ test, negative; and normal BUN, total protein, albumen, alkaline phosphatase, SGOT, SGPT, and LDH. A screening test for G6PD deficiency was positive, and he was referred for further evaluation after the standard assay of a crude hemolysate confirmed the deficiency.

His height and weight were at 50% for age, and except for slightly pale mucous membranes and icteric sclerae, no abnormalities were noted on physical examination. The liver and spleen were not enlarged. His intelligence was average by the Wechsler Intelligence Scale for Children.
Family Studies

The family pedigree is presented in Fig. 1. The patient's mother is of Dutch-Irish and German ancestry, and his father is a Cherokee Indian. The maternal uncle (III-13), age 35, was jaundiced in childhood. Two sons (III-4,5) of a maternal great aunt have had a history of persistent jaundice. The only living child of another maternal great aunt has had six children from four marriages. A 12-yr-old son (IV-3) from her third marriage has been jaundiced from birth and has required blood transfusions. A 6-yr-old son (IV-6) from her fourth marriage has been jaundiced since birth and required three blood transfusions following aspirin ingestion. The patient has three half-siblings, a brother and sister from the mother's first marriage and a sister from her fourth (IV-11). The mother (III-12), and a half-sister (IV-9) had G6PD activity in the heterozygous range, and two maternal second cousins (IV-3 and 6) were deficient.

RESULTS

The patient had a well-compensated hemolytic anemia. The hemoglobin was 12.5 g/100 ml; packed cell volume, 35%; red cell count, 4.1 million per cu mm; MCV, 86 cu μ; MCH, 30.5 μg; MCHC, 36%; reticulocytes, 15.2%. Occasional spherocytes and basophilic stippling and moderate poikilocytosis and anisocytosis were seen in the stained smear of peripheral blood. The platelet count was 430,00/cu mm, the white blood cell count was 5700/cu mm, and the differential was normal. No hemosiderin was present in the urine, the serum folate was decreased (2.8 ng/100 ml), and the serum vitamin B₁₂ was normal. The t½ of his erythrocytes was 5 days without evidence of hepatic or splenic sequestration. Electrophoresis and heat stability of hemoglobin were normal, and the activities of all other erythrocyte glycolytic enzymes were increased or normal, commensurate with the reticulocytosis. Haptoglobin was absent, no Heinz
bodies were seen in freshly drawn cells, and the plasma hemoglobin concentration was 4.2 mg/100 ml. Metabolic characteristics of his erythrocytes are presented in Table 1. Glucose utilization, lactate formation, and the intracellular concentrations of 2,3-DPG and ATP were increased. The content of intracellular sodium and potassium in fresh cells and in cells incubated 4 hr at 37°C was normal. Autohemolysis showed a Dacie type I pattern with correction by glucose and ATP. No exaggeration of autohemolysis was seen in the presence of neutralized aspirin, 0.33 mg/ml, or sulfisoxazole, 0.13 mg/ml, when compared to the value obtained with saline additive.

The concentration of glutathione in fresh cells was decreased, and marked instability of GSH (−67%) occurred after 2 hr incubation with acetylphenylhydrazine.

The characteristics of the partially purified G6PD are presented in Table 2. These results, representing the average of two separate studies, are compared to normal G6PD evaluated by similar methods in this laboratory and to other
variants occurring in whites with chronic hereditary hemolytic anemia and normal electrophoretic mobility reported in the literature.²

The activity of G6PD in the patient’s erythrocytes was 5% of normal. The $K_m$ G6P was normal (55 $\mu M$), but the $K_m$ NADP (11.7 $\mu M$) was two to three times the normal value. Electrophoresis on starch gel with Tris-EDTA-borate buffer, pH 8.6, and with phosphate buffer, pH 7.0, was identical to the B, or normal, variant. The enzyme was extremely labile. After 20 min incubation at 42°C, 95% of the activity was lost. The pH optimum with Kirkman’s triple buffer system was 1.0 pH units below the values obtained in normal patients. The utilization of 2-deoxy glucose-6-phosphate and deamino NADP were normal (3.2% and 51%, respectively). Marked inhibition of G6PD activity by the sulfhydryl inhibitor NEM was observed; whereas a 44% inhibition of normal enzyme activity occurred with the addition of 1 mM NEM, and a 98.4% inhibition of the patient’s enzyme was noted.

The activity of G6PD in the patient’s leukocytes (Table 3) was only 15% of normal, but his phagocytes had normal bactericidal activity against S. aureus and P. aeruginosa. The bacterial count per cubic centimeter decreased by nearly 2 logs after an incubation of 2 hr.

Platelet G6PD activity was 28% of that in an equal number of platelets from a normal individual (Table 3). Platelet factor 3 release and platelet aggregation with ADP, epinephrine, and collagen were normal. No measurable G6PD was found in the patient’s fibroblasts using G6P or deoxy-G6P as substrate, whereas normal fibroblasts contained 0.958 U/mg cell protein. In the presence of 1 mM NEM, G6PD activity was 0.625 U/mg cell protein, a decrease of 34.8%. With deoxy-G6P, no activity was present in the normal fibroblasts.

The oxygen affinity of the patient’s hemoglobin was decreased ($P_{50}$, 33.8 mm Hg; normal, 27.0 ± 1.0 mm Hg) (Table 4). Following 2 hr incubation with acetylphenylhydrazine, the methemoglobin increased from 1% to 80%, and the $P_{50}$ decreased to 8.25 mm Hg. In the control sample a 10% decline in the $P_{50}$ was observed after incubation with APH when the concentration of methemoglobin was 50% (Table 4).

### Table 3. Activity of G6PD in Leukocytes, Platelets, and Fibroblasts

<table>
<thead>
<tr>
<th></th>
<th>Patient</th>
<th>Normal Mean (Range)</th>
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</thead>
<tbody>
<tr>
<td>Leukocytes, U/10^{10} WBC</td>
<td>32.1</td>
<td>214 (156–271)</td>
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<tr>
<td></td>
<td>per cent normal</td>
<td>15</td>
</tr>
<tr>
<td>Platelets, U/10^{10} platelets</td>
<td>1.1</td>
<td>3.9 (2.5–5.3)</td>
</tr>
<tr>
<td></td>
<td>per cent normal</td>
<td>28</td>
</tr>
<tr>
<td>Fibroblasts, U/mg protein</td>
<td>0</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>per cent normal</td>
<td>0</td>
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</tbody>
</table>

### Table 4. Oxygen Affinity and Methemoglobin Formation in the Presence of Acetylphenylhydrazine

<table>
<thead>
<tr>
<th></th>
<th>Patient</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MetHb (%)</td>
<td>$P_{50}$ (mm Hg)</td>
</tr>
<tr>
<td>Fresh Cells</td>
<td>&lt; 1</td>
<td>33.8</td>
</tr>
<tr>
<td>2-hr control</td>
<td>2.0</td>
<td>31.1</td>
</tr>
<tr>
<td>2-hr, APH</td>
<td>79.6</td>
<td>8.25</td>
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</table>
DISCUSSION

The distinguishing clinical and biochemical characteristics of this patient's moderately severe, well-compensated chronic hemolytic anemia include (1) no evidence of hepatic or splenic sequestration, (2) markedly decreased activity of the partially purified G6PD, (3) normal Michaelis constant ($K_m$) for G6P, (4) a two- to threefold increase in $K_m$ NADP, (5) striking thermal instability, (6) normal (B) electrophoretic mobility, (7) normal 2-deoxy-G6P and deamino NADP utilization, (8) decreased pH optimum, and (9) almost total inhibition by the sulfhydryl inhibitor, NEM.

Other G6PD variants associated with chronic hereditary hemolytic anemias, normal electrophoretic mobility and varying abnormalities of kinetics, thermal stability, pH optima, and in the utilization of substrate analogues are presented in Table 2. A complete listing of G6PD variants has been published by the World Health Organization. Although technical and methodologic artifacts may be a partial explanation for the abundance of G6PD variants, the G6PD mutant found in this patient appears to be unique and differs from all previously reported variants associated with chronic hemolytic anemia. Accordingly, this variant is designated G6PD Cornell.

Schlegal and Bellanti proposed that there was a relationship between G6PD lability and/or deficiency and increased susceptibility of deficient males to infection. Despite the marked deficiency of G6PD in this patient's leukocytes, he was not unusually susceptible to infections. Similar degrees of G6PD deficiency in the leukocytes of other patients with the red cell defect have been reported, but decreased bactericidal activity has not been observed unless there is more marked (< 5%) or total absence of G6PD in the leukocytes. The metabolic and bactericidal defects in this group of severely affected individuals resemble those seen in the phagocytes of patients with chronic granulomatous disease. The availability of alternate pathways of intracellular bacterial destruction probably permits the partially G6PD-deficient phagocyte to function normally.

Approximately 15%-25% of glucose metabolism in the resting platelet flows through the pentose phosphate shunt, and this metabolic pathway appears to be coupled with lipid synthesis through the generation of NADPH. Deficiency of G6PD in the platelets of patients with a deficiency of the red cell enzyme has been reported previously, but abnormalities of platelet function or de novo lipid synthesis have not been described. The partial deficiency of G6PD in the patient's platelets had no observable adverse effect clinically or in vitro as measured by platelet aggregation and the release of platelet factor 3. An alternate means of generating NADPH, the citric acid cycle, is present in platelets and no doubt explains their resistance to G6PD deficiency.

The pathophysiologic mechanism of hemolysis and shortened erythrocyte survival in G6PD deficiency appears to be related to the decreased generation of NADPH and reduced glutathione. The formation of mixed disulfide linkage between glutathione and globin chains of hemoglobin results eventually in denatured, precipitated Heinz bodies. The primary site of erythrocyte destruction is in the microcirculation of the spleen where Heinz bodies are removed by phagocytic reticuloendothelial cells resulting in cells with increased membrane
rigidity, enhanced spheroidicity, decreased deformability, altered membrane function, and shortened survival. However, paradoxically, most patients with hereditary hemolytic anemia due to G6PD deficiency fail to respond to splenectomy. The lack of splenomegaly and significant splenic sequestration in our and other, but not all, reported patients with G6PD deficiency suggests that erythrocyte entrapment and destruction must also be occurring in the liver and the bone marrow.

The significant decrease in oxygen affinity of hemoglobin (increased $P_{50}\text{O}_2$) observed in this patient was multifactorial. Increased levels of 2,3-DPG and a high MCHC would increase the $P_{50}$ and shift the oxyhemoglobin dissociation curve to the right. Other factors, such as base excess and intracellular pH, would also modulate the value for $P_{50}$. Despite a $P_{50}$ of 33.8 mm Hg and a hemoglobin of 12 g/100 ml, erythropoiesis, as approximated by the absolute reticulocytosis of 615,000/cu mm, was increased six- to tenfold above normal. Thus, increased oxygen delivery to the tissues failed to dampen red cell production in the presence of severe, but compensated, hemolysis and a markedly shortened $t_\frac{1}{2}$ of $^{51}\text{Cr}$-labeled cells.

The dissociation between relatively mild clinical symptoms and marked aberrations in cell survival, intracellular enzyme activity, kinetics, and stability emphasize the marked heterogeneity of the syndromes associated with G6PD deficiency. Yoshida has recently demonstrated that the physiologic activity of G6PD, under simulated intracellular conditions, is a more important determinant of the severity of hemolysis than is in vitro activity. Variants of G6PD associated with chronic hemolytic anemia have a high $K_m$ NADP or a low inhibition constant ($K_i$) for NADPH, whereas the nonhemolytic variant enzymes are characterized by a low $K_m$ for NADP and a high $K_i$ for NADPH. To provide a more meaningful characterization of G6PD variants, a revision of the current standards for defining variants is warranted. The revised methods should take into account simulated in vivo conditions and the inhibiting effect of enzyme products and other intermediate metabolites.

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