Hereditary Nonspherocytic Hemolysis
With Erythrocyte Phosphofructokinase Deficiency

By Larry Waterbury and Eugene P. Frenkel

Hereditary nonspherocytic hemolysis associated with abnormal erythrocyte phosphofructokinase activity was demonstrated in a young man. Enzyme activity in the propositus, his mother, and maternal grandmother was approximately 60% of normal controls. There was markedly increased lability of enzyme activity on in vitro storage. Kinetic studies revealed increased sensitivity to adenosine triphosphate inhibition. Erythrocyte adenosine triphosphate levels were depressed. The absence of muscle disease and the presence of normal in vivo lactate production following ischemic exercise differentiated this kindred from those with Type VII glycogen storage disease.

ERYTHROCYTE ENZYME DEFECTS have been identified in many patients with hereditary nonspherocytic hemolysis, and these defects appear instrumental in the pathogenesis of the hemolytic syndromes. Almost every enzyme in the glycolytic and shunt pathways has been implicated. This report describes yet still another erythrocyte glycolytic enzyme defect, deficiency of phosphofructokinase [(PFK) ATP: D-fructose-6-phosphotransferase, E.C. 2.7.1.11], associated with hereditary nonspherocytic hemolysis. PFK catalyzes the conversion of fructose-6-phosphate (F-6-P) to fructose-1,6-diphosphate (F-1,6-P). Adenosine triphosphate (ATP) is a cosubstrate for PFK function, but in excess it is an inhibitor of the reaction. This unique dual action of ATP in the PFK reaction contributes to the enzyme's function as one of the rate-limiting steps in glycolysis (Fig. 1).

CASE REPORT

The propositus, a 23-yr-old white male physician had normal growth and development. At age 15 icterus was noted, his liver was described as four fingerbreadths below the right costal margin, and his spleen was palpable 3-4 cm below the left costal margin. Laboratory studies revealed a hemoglobin of 16g/100 ml, normal white blood count and differential, and a bilirubin of 2.6 mg/100 ml (1 mg/100 ml direct-reacting). The SGOT, thymol turbidity, and cephalin flocculation tests were normal. A presumptive diagnosis of hepatitis was made. Two months later evaluation by a hematologist revealed no hepatomegaly and only a palpable spleen tip on physical examination. His hemoglobin and white blood count were normal. His reticulocyte count was 6%-8%. A direct Coombs test, osmotic fragility, screening test for G-6-PD deficiency, and liver function tests were all normal. A 51Cr-labeled red cell survival study utilizing the patient's own cells revealed a t½ of 14 days. A bone marrow revealed erythroid hyperplasia.

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Since then the patient has been asymptomatic but has continued to have persistent mild icterus, splenomegaly, and normal peripheral hematologic values with a persistent reticulocytosis in the range of 4%-9%. His erythrocyte morphology has been normal, except for polychromatophilia and some fine basophilic stippling.

The propositus has three siblings, two brothers and one sister. His father is of English and German ancestry and his mother of French ancestry. No family member has jaundice, splenomegaly, anemia, reticulocytosis, or an abnormal peripheral smear. The maternal grandmother, while not anemic, did demonstrate anisocytosis and poikilocytosis. The hematologic data on the family are shown in Table 1.

**MATERIALS AND METHODS**

Autohemolysis was measured by the method of Selwyn and Dacie. Hemoglobin electrophoresis on cellulose acetate (pH 8.6) and on starch block was determined by standard methods. Sodium influx using $^{22}$Na was measured by the method of Smithies as modified by Bertles. Heinz body formation after incubation of blood with acetylphenylhydrazine, the heat denaturation test for unstable hemoglobin, the ascorbate-cyanide screening test, and the sugar water test for PNH were conducted using the methods previously described. Erythrocyte in vitro lactate production was determined by a modification of the method of Keitt. Erythrocyte ATP was measured by the firefly method. In vivo lactate production was determined by the technique of Benoit and Watten.

**PFK Assay**

The routine PFK assay was done with EDTA-collected blood, maintained at 4°C. Under these conditions enzyme stability was maintained for at least 12 hr, although assays were done within 4 hr of collection. Heparin or ACD-anticoagulated blood gave comparable results. Since unwashed red cells and red cells washed four times with normal saline gave identical PFK assay results, unwashed cells were used. Whole blood was centrifuged at 3000 g for 20 min at 4°C, and a 1:20 v/v hemolysate was made in cold glass-distilled water using cells aspirated from the bottom of the packed cell column. Completeness of hemolysis was checked by the freeze-thaw technique. Hypotonic lysis was satisfactory for all bloods studied. Hemolysates were spun at 30,000 g for 20 min at 4°C to sediment the erythrocyte stroma, and the supernatant was assayed for PFK activity.

Leukocytes were collected for assay by the method of Valentine and Tanaka in ACD, containing added PVP for erythrocyte sedimentation. Assays were done on freeze-thawed WBC lysates and reported per $10^{10}$ white blood cells.

**Table 1. Representative Blood Values and Mean Red Blood Cell PFK Activity in Seven Family Members**

<table>
<thead>
<tr>
<th></th>
<th>Hemoglobin (g/100 ml)</th>
<th>Hematocrit (%)</th>
<th>Reticulocytes (%)</th>
<th>Mean RBC PFK Activity (U/ml RBC)</th>
<th>$p$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propositus (12)*</td>
<td>16.9</td>
<td>47</td>
<td>9.0</td>
<td>2.9 (2.7-3.1)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Mother (4)</td>
<td>13.4</td>
<td>38</td>
<td>0.3</td>
<td>2.7 (2.55-2.9)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Maternal grandmother (4)</td>
<td>11.9</td>
<td>37</td>
<td>0.6</td>
<td>3.1 (2.8-3.3)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Father (2)</td>
<td>16.2</td>
<td>48</td>
<td>0.4</td>
<td>4.9 (4.9-4.95)</td>
<td></td>
</tr>
<tr>
<td>Sister (2)</td>
<td>14.2</td>
<td>41</td>
<td>0.2</td>
<td>3.9 (3.6-4.2)</td>
<td></td>
</tr>
<tr>
<td>Brother 1 (2)</td>
<td>17.2</td>
<td>47</td>
<td>0.3</td>
<td>5.1 (4.95-5.25)</td>
<td></td>
</tr>
<tr>
<td>Brother 2 (2)</td>
<td>17.2</td>
<td>48</td>
<td>0.3</td>
<td>4.1 (4.0-4.2)</td>
<td></td>
</tr>
<tr>
<td>Normals (25)</td>
<td></td>
<td></td>
<td></td>
<td>4.0-5.8</td>
<td></td>
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</tbody>
</table>

*Number of separate assays are given in parentheses.
Fig. 1. Schematic illustration of phosphofructokinase reaction and subsequent aldolase, triosephosphate isomerase, and α-glycerophosphate dehydrogenase reactions.

The PFK assay used was a modification of the method of Koutras et al. The PFK reaction was coupled with the aldolase, triosephosphate isomerase, and glycerophosphate dehydrogenase reactions following the oxidation of NADH at 28°C in a Beckman DU spectrophotometer (Fig. 1). Strict temperature control was maintained, the cuvette housing temperature being monitored. The assay was conducted using reactants with the following final concentrations: 50 mM Tris phosphate buffer, pH 8.2, 8.0 mM (NH₄)₂SO₄, 5.0 mM MgCl₂, 2.5 mM dithiothreitol, 1 mg/100 ml BSA, 1.0 mM EDTA, 1.0 mM F-6-P, 0.15 mM NADH, aldolase (0.4 EU/assay), triose phosphate isomerase (5 EU/assay), glycerophosphate dehydrogenase (0.2 EU/assay), and lysate containing PFK activity to be measured. (The F-6-P used was the Ba⁺⁺ salt (Boehringer-Mannheim, New York). The Ba⁺⁺ was separated by Dowex exchange chromatography. Undialyzed (NH₄)₂SO₄ enzyme suspensions (Calbiochem, Los Angeles, Calif.) Glass-distilled water was added to a total assay volume of 1 ml. The cuvettes were warmed to assay temperature, and the reaction was begun with the addition of 1.0 μmole (10 μl 0.1 M ATP) neutralized ATP. The reaction was followed in a Beckman DU spectrophotometer through a 1 cm light path, and the average Δ OD/min between 5 and 10 mm after AlP addition was used in the calculation of enzyme activity. One unit of PFK activity was arbitrarily defined as that enzyme activity catalyzing the conversion of 1 μmole F-6-P to F-1,6-P per ml of red blood cells, or 10¹⁰ white blood cells, per min at 28°C.

Special Studies

A stabilized enzyme preparation was used for the kinetic studies, enzyme stability evaluations, and enzyme inhibition studies. Hemolysates were made using dilute Tris phosphate buffer (5 mM, pH 8.0) or imidazole buffer mM, pH 8.0) containing dithiothreitol 2.5 mM, EDTA 0.1 mM, F-1,6-P 0.1 mM, and ATP 0.1 mM. Partial hemoglobin separation was accomplished by (NH₄)₂SO₄ precipitation with resuspension in buffer with stabilizers. These preparations were kept at 5°C.

Enzyme inhibition studies utilized antihuman muscle PFK made in rabbits as previously reported. (The antihuman muscle PFK antisera was kindly provided by Dr. S. Tarui, Osaka, Japan, and Dr. L. P. Rowland and Dr. W. J. Bank, Philadelphia, Pa.) The incubations of enzyme with antiserum were conducted in the assay cuvettes in the presence of buffer and dithiothreitol. Five-minute room temperature incubations were found to be more satisfactory than longer incubations at any temperature because of enzyme instability.
Table 2. Summary of Special Studies

<table>
<thead>
<tr>
<th></th>
<th>Erythrocyte PFK Activity Mean ± SEM (U/ml RBC)</th>
<th>Enzyme Stability* (% Original Activity at 48-hr Incubation)</th>
<th>Enzyme Inhibition by Anti-PFK Serum (%)</th>
<th>Mean WBC PFK Activity Mean ± SEM (U/10^10 WBC)</th>
<th>RBC [ATP] Mean ± SEM (µmole/g Hemoglobin)</th>
<th>48-hr Autohemolysis Without Glucose (%)</th>
<th>With Glucose (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propositus (9% reticulocytes)</td>
<td>2.9 ± 0.07</td>
<td>27</td>
<td>0</td>
<td>0</td>
<td>210</td>
<td>3.65 ± 0.25</td>
<td>5.1</td>
</tr>
<tr>
<td>Mother (0.3% reticulocytes)</td>
<td>2.7 ± 0.07</td>
<td>14</td>
<td>20</td>
<td>0</td>
<td>239</td>
<td>3.3 ± 0.10</td>
<td>-</td>
</tr>
<tr>
<td>Paternal grandmother (0.6% reticulocytes)</td>
<td>3.1 ± 0.11</td>
<td>20</td>
<td>35</td>
<td>0</td>
<td>271</td>
<td>4.5 ± 0.24</td>
<td>-</td>
</tr>
<tr>
<td>Normals (25)† (&lt;1% reticulocytes)</td>
<td>4.7 ± 0.08</td>
<td>65-85</td>
<td>40</td>
<td>&gt;30</td>
<td>196-304</td>
<td>5.00 ± 0.109§</td>
<td>0.4-4.5</td>
</tr>
</tbody>
</table>

*Enzyme stability is recorded as per cent original activity remaining after 48-hr storage at 4°C (see text for methods).
†Per cent inhibition by antihuman muscle PFK serum is recorded after various periods of storage of enzyme preparation.
‡Number of individual patients in whom erythrocyte PFK and ATP were studied.
§Four assays of ATP were performed in patients with high reticulocyte counts (10%–20%). Values ranged between 6.0 and 7.9 µmole/g hemoglobin.
to time and dilution. Enzyme dilution and protein concentration were controlled. Normal rabbit serum was used in the control incubations.

The enzyme kinetic studies were conducted utilizing the above described enzyme preparations. The ATP/Mg++ ratio was kept constant at one throughout.

RESULTS

Routine Hematologic Studies

The propositus consistently demonstrated normal hemoglobin, white blood count, and platelet values (Table 1). Reticulocyte counts were persistently elevated to between 4% and 9%. The red blood cell morphology was normal. The osmotic fragility, Coombs test, heat denaturation test for unstable hemoglobin, cellulose acetate, and starch gel hemoglobin electrophoreses, sugar water test, ascorbate-cyanide test, and Heinz body test with acetylphenylhydrazine were all normal. Two $^{51}$Cr-labeled erythrocyte survival studies revealed a T½ of 12 days and 14 days using the propositus' cells (normal T½ in our laboratory being 28–32 days). Donor cells survived normally in the patient. No hematologic abnormality was encountered in his three siblings, his father, mother, and maternal grandmother (Table 1).

Erythrocyte Enzyme Studies

Assay of the following erythrocyte hexose monophosphate shunt and glycolytic enzymes in the propositus revealed normal or increased activity consistent with a young population of cells: glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, glutathione reductase, glutathione peroxidase, hexokinase, glucose phosphate isomerase aldolase, triose phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerokinase, phosphoglycerate mutase, enolase, pyruvate kinase, lactate dehydrogenase, adenosine triphosphatase, as well as acetylcholinesterase. Pyruvate kinase was assayed at several PEP concentrations and was normal. Assays of erythrocyte glucose-6-phosphate dehydrogenase, hexokinase, and pyruvate kinase in the father and mother were normal. (With the exception of the PFK assays erythrocyte enzyme assays on the propositus, his father, and mother were kindly done for us by Dr. Kouichi R. Tanaka, Harbor General Hospital, Torrance, Calif.)

Erythrocyte Metabolic Studies

The autohemolysis test, using propositus' erythrocytes, revealed slightly increased 48-hr autohemolysis that was corrected by glucose (Table 2). In vitro studies of lactate production were normal when compared to controls with similar degrees of reticulocytosis. Erythrocyte Na and K concentrations were normal as was the rate of Na influx.

The propositus persistently demonstrated depressed erythrocyte ATP concentrations with a mean of 3.65 μmole/g hemoglobin (3.4–3.9 μmole/g) that was especially striking when matched with controls with a similar degree of reticulocytosis (Table 2). The mother also had reduced erythrocyte ATP levels (3.2–3.4 μmole/g) compared to normal controls, although the grandmother did not (4.2–4.7 μmole/g).
Fig. 2. Effect of storage at 4°C on enzyme stability.

**PFK Activity**

The mean PFK values in 24 controls including 12 patients with a variety of hemotologic abnormalities (multiple myeloma, acute leukemia, CGL, CLL, autoimmune hemolytic anemia) are shown in Table 2. The normal range was between 4 and 5.8 U/ml red blood cells, with a mean of 4.7. PFK activity in the propositus, his mother, and maternal grandmother was significantly lower than the normals (Table 1). The data on the family are shown in Table 1.

Assay of old and young cell populations separated by differential centrifugation revealed no difference in activity for either the propositus or control erythrocytes. PFK activity in normal reticulocytes was not increased. Assays of mixtures of normal and propositus hemolysates revealed no evidence for the existence of a PFK inhibitor. The assays of leukocyte PFK activity for the propositus and his family fell within the normal range (Table 2).

**Enzyme Stability**

Although PFK activity in normals was found to be unstable to time and dilution, much more striking lability of enzyme activity was noted in the propositus, his mother, and grandmother under all conditions of enzyme storage. Maximum stabilization of enzyme activity could be achieved by the addition of low concentrations of dithiothreitol, F-6-P, F-1,6-P, and ATP. Under conditions of maximum stabilization of the enzyme and control of dilution, rapid loss of activity occurred with the propositus enzyme when compared with controls (Fig. 2). Similar instability of enzyme activity was demonstrated in hemolysates from the mother and grandmother (Table 2).
Enzyme Kinetics

As demonstrated previously by Layzer et al., both the K and V_max values for either substrate (F-6-P or ATP) were increased by raising the other cosubstrate concentration. At any given ATP level, the K_m F-6-P values were slightly higher in the propositus than in the normals (Fig. 3). In addition, K_m ATP values were not found to be significantly different from normal controls (Fig. 4). However, marked abnormalities in ATP inhibitory kinetics were demonstrated (Figs. 4, 5). At all F-6-P concentrations examined (between 90 μM and 1 mM) there was a greater inhibition of propositus enzyme activity than of control activity. Figure 3 further depicts that the propositus enzyme has sigmoid kinetics, and the double reciprocal plot in Fig. 5 demonstrates this.
Fig. 5. Double reciprocal plot of effect of F-6-P concentration on PFK activity for propositus and control. ATP concentration was 1 mM.

Fig. 6. Effect of diluted antihuman muscle PFK antiserum on PFK activity of the propositus and control enzyme preparations using fresh hemolysates and 5-min room temperature incubations. Microliters of serum were added to 1 ml total volume.
increased ATP activity as the F-6-P concentration was varied and the ATP concentration held constant. The curve obtained with the propositus enzyme preparation demonstrates increasing ATP inhibition as the F-6-P concentration was decreased. No ATP inhibition of control enzyme activity was observed over this range of F-6-P concentrations.

**Enzyme Inhibition Studies**

As previously demonstrated\textsuperscript{16,17} normal erythrocyte PFK activity is inhibited to around the 60% level on incubation with antihuman muscle PFK antiserum. We found that the degree of enzyme inhibition decreased with in vitro storage. However, after 48 hr of storage at 4°C, greater than 25% inhibition could still be demonstrated using normal enzyme preparations. Enzyme inhibition studies revealed no inhibition of propositus erythrocyte PFK activity (Fig. 6). The inhibition studies using enzyme preparations from the mother and maternal grandmother revealed some enzyme inhibition initially (20% in the grandmother and 35% in the mother) using fresh enzyme preparations. However, inhibition was absent after 24-hr storage, unlike preparations from normal controls that continued to be inhibited (Table 2). This loss of inhibitory response with the enzyme preparations of the mother and grandmother occurred coincident with the greatest loss in their enzyme activity, i.e., during the first 24 hr of storage (Table 2).

**In Vivo Lactate Production**

Measurement of in vivo lactate production in the propositus demonstrated a normal rise in the venous lactate value following ischemic exercise. The resting level of 5 mg/100 ml rose to a level of 25 mg/100 ml at 5 min.

**DISCUSSION**

Since 1965, a few patients have been described by Tarui and co-workers\textsuperscript{16,21} and Layzer et al.\textsuperscript{17,18} with a McArdle syndromelike defect characterized by muscle fatigue, cramping muscle pain with exercise, and intermittent episodes of dark urine associated with an absence of muscle PFK. The defect has been classified as Type VII glycogen storage disease. All patients described have demonstrated the absence of the normal rise in venous lactate levels that follows ischemic exercise. Another feature is the presence of only 50% normal erythrocyte activity but virtual absence of muscle PFK activity in the affected individuals. Although there is no inhibition of erythrocyte PFK in affected patients, rabbit antihuman muscle PFK antiserum was able to inhibit RBC PFK by approximately 40%. These data have led both groups of authors to suggest that there are at least two PFK isoenzymes and that erythrocyte PFK is a composite, one of the isoenzymes being of the muscle type.

The results of the antibody inhibition studies with our propositus resemble those patients with the muscle PFK defect previously described. However, our patient is notably different from those previously reported. He has had no muscle symptoms or weakness in spite of strenuous exercise. Furthermore, ischemic exercise resulted in increased in vivo lactate production (from a resting value of 5–25 mg/100 ml). Comparison of enzyme kinetics and stability
and erythrocyte ATP levels with the previous cases is not possible, since such
data have not been reported.

Recent evidence supports Tarui’s hypothesis that erythrocyte PFK is a com-
posite of isoenzymes, one of which is the muscle component accounting for
approximately 50% of normal red blood cell PFK activity.17,18 Our studies
implicate the muscle isoenzyme as the defective component in this family. The
propositus enzyme activity is not inhibited by antihuman muscle PFK anti-
serum, and the inhibition is only transiently observed with the enzymes ob-
tained from the mother and maternal grandmother. The rapid deterioration
of mother and maternal grandmother enzyme activity with storage corresponds
chronologically with the loss of enzyme inhibition by antiserum, suggesting
the presence of an unstable defective muscle isoenzyme.

The association of congenital nonspherocytic hemolysis with a glycolytic
defect occurring at the PFK step is of particular interest because of this
enzyme’s unique function in glycolysis. The PFK reaction is one of the two
irreversible reactions in glycolysis utilizing ATP as cosubstrate. Recent data
suggest that at physiologic or decreased erythrocyte pH the PFK reaction is
the rate-limiting step in erythrocyte glycolysis.19 Of particular importance in
this regulation is the dual effect of ATP as inhibitor, as well as cosubstrate, in
the reaction. As shown by Layzer et al.,18 erythrocyte PFK normally functions
under inhibitory conditions in that at the usual erythrocyte F-6-P concentra-
tion (around 0.020 mM) ATP is present in inhibitory concentrations. The
significance of this regulatory mechanism on erythrocyte glycolysis is sub-
stantiated by the present studies. Although total erythrocyte PFK activity is
only moderately depressed in the propositus, the increased sensitivity of the
propositus enzyme to ATP inhibition would predictably result in the lowered
erythrocyte ATP concentration observed.

It is difficult to explain the absence of muscle symptoms in the propositus.
Classical Type VII glycogen storage disease patients have essentially no muscle
PFK activity. In contrast, our data in this family suggests the presence of de-
fective, but partially functional, muscle PFK activity. Our propositus con-
sistently demonstrated PFK levels of approximately 65% of normal compared
to levels of approximately 50% in patients with Type VII glycogen storage
disease. The presence of decreased in vitro enzyme stability and altered ATP
kinetics best favors the interpretation of an altered functioning enzyme rather
than complete absence of muscle PFK. The lack of enzyme inhibition by anti-
human muscle PFK antisera could be explained by altered enzyme structure, as
well as complete absence of muscle PFK. Therefore, it is likely that there is
limited altered muscle PFK activity in the propositus, possibly sufficient to
protect him from muscle symptoms. Unfortunately, our patient refused a
muscle biopsy that might have clarified this issue.

It is impossible on the basis of the small pedigree available to be certain of
the inheritance. The involvement of three successive generations suggests that
it is a single unit character, but the pedigree data do not allow one to dif-
ferentiate a sex-linked from autosomal (with variable expressivity) inheritance.
ACKNOWLEDGMENT

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REFERENCES

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