Congenital Nonspherocytic Hemolytic Anemia
With an Unstable Hexokinase Variant

By P. G. Board, R. Trueworthy, J. E. Smith, and Kateri Moore

We report a family with a new hexokinase variant that gives rise to nonspherocytic hemolytic anemia in one apparently homozygous family member. The variant enzyme has a normal pH optimum, normal reaction kinetics, and normal electrophoretic properties, but has reduced activity and is apparently inactivated rapidly as the affected erythrocytes age.

Because erythrocyte hexokinase (HK) has a comparatively low activity, and because it catalyzes the first reaction of the glycolytic sequence, it may play a major role in the regulation of glycolysis. Of all the glycolytic enzymes, HK is the most influenced by erythrocyte age. It has been estimated that the mature erythrocyte may have no more than 2%-3% of the HK activity originally present in the reticulocyte. The similarity between the half-life of erythrocyte HK and the half-life of erythrocytes in vivo suggests that the age-related decay of HK activity may be a primary factor limiting erythrocyte survival.

In view of the apparent importance of HK, it may be expected that mutations giving rise to unstable variants or variants with diminished activity may severely restrict the metabolic capacity of erythrocytes. Perhaps because of the potential severity of the deleterious effects associated with diminished HK activity, surviving subjects with HK deficiency are extremely rare. Only eight cases from five families have been reported. Among those families, at least four biochemically different HK variants have been detected.

Our investigation of congenital, nonspherocytic, hemolytic anemia in a young boy has led to the delineation of another example of inherited erythrocyte HK deficiency.

MATERIALS AND METHODS

Case Report

B.V. was referred to the Pediatric Hematology Clinic at the University of Kansas Medical Center in April 1975 at 33 mo of age because of anemia, reticulocytosis, and x-ray evidence of gallstones. Previous admissions to a local hospital had revealed hemoglobin concentrations of 8.6-9.4 g/dl and reticulocyte counts of 6.7%-8.5%. Serum bilirubin totaled 6.1 mg/dl; direct 3.8 mg/dl. Bone marrow examination revealed erythroid hyperplasia. Coombs test, hemoglobin electrophoresis, and incubated osmotic fragility were normal. Tests for glucose-6-phosphate de-
hydrogenase and pyruvate kinase deficiency, Ham’s test, heat stability, hemoglobin F level, intravenous pyelogram, sugar-water test, and hepatitis-associated antigen were all normal.

When he was first seen at the Medical Center, further history revealed that he had experienced neonatal jaundice and was treated with phototherapy. Neither B.V. nor his mother had any history of transfusion therapy.

Physical examination revealed hepatosplenomegaly: the liver was palpable 4 cm below the right costal margin and the spleen was palpable 4 cm below the left costal margin. Based on standards for his age, the patient was in the 50th percentile for height, 50th percentile for weight, and 100th percentile for head circumference.

In subsequent studies his hemoglobin ranged between 8.2 and 10.2 g/dl with reticulocyte counts ranging from 5% to 7.6%. Peripheral smear revealed 2+ hypochromia, 1+ poikilocytosis with a few burr cells, occasional schistocytes, ovalocytes, and two nucleated red blood cells per 100 white cells. Bromsulphthalein was 10%, SGOT 28 and SGPT 45 Karmen units/ml, and alkaline phosphatase 10.3 Bessey Lowry units.

31Cr survival studies revealed a half-life of 15 days, compared with a normal range of 25–35 days. Spleen/liver index at the T1/2 was 1.4 with selective sequestration suggested only at a level greater than 2.3. 31Cr survival with normal transfused cells was 24 days. Haptoglobin was 3; lactate dehydrogenase was greater than 3000 Wroblewski units, with elevations primarily in fractions 1 and 2. IgA was slightly elevated with a normal IgG and IgM. Urine hemoglobin was absent. Autohemolysis was normal. Both mother and patient were A, Rh negative, DU negative.

Other normal values included erythrocyte sedimentation rate, cholesterol, triglycerides, partial thromboplastin time, prothrombin time, complement, vitamin B12, and folate. Family history revealed a high incidence of cholelithiasis in both the maternal and paternal families. The mother’s family was of English extraction, the paternal grandfather of German extraction, and the paternal grandmother of Finnish extraction.

Because of evidence suggesting a hemolytic anemia based on an intracorpuscular defect unassociated with an obvious hemoglobinopathy or morphologic defect in the red cells, the patient was further evaluated by examination of glycolytic enzymes and erythrocyte metabolism.

### Table 1. Activities of Red Cell Enzymes in B.V. and Normal Controls

<table>
<thead>
<tr>
<th>Enzyme*</th>
<th>Patient B.V.</th>
<th>Controls†</th>
</tr>
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<tbody>
<tr>
<td>HK</td>
<td>0.65</td>
<td>1.18–1.29</td>
</tr>
<tr>
<td>PFK</td>
<td>10.1</td>
<td>8.74–9.79</td>
</tr>
<tr>
<td>AK</td>
<td>335</td>
<td>337–560</td>
</tr>
<tr>
<td>PGK</td>
<td>335</td>
<td>242–303</td>
</tr>
<tr>
<td>TPI</td>
<td>775</td>
<td>531–563</td>
</tr>
<tr>
<td>LDH</td>
<td>170</td>
<td>97.0–109</td>
</tr>
<tr>
<td>GPI</td>
<td>100</td>
<td>60.7–66.3</td>
</tr>
<tr>
<td>Aldolase</td>
<td>4.34</td>
<td>2.01–3.99</td>
</tr>
<tr>
<td>GAPD</td>
<td>147</td>
<td>66.3–86.3</td>
</tr>
<tr>
<td>DPGM</td>
<td>5.01</td>
<td>3.75–4.95</td>
</tr>
<tr>
<td>MPGM</td>
<td>31.9</td>
<td>14.2–25.1</td>
</tr>
<tr>
<td>Enolase</td>
<td>10.9</td>
<td>4.94–6.39</td>
</tr>
<tr>
<td>PK</td>
<td>18.9</td>
<td>12.5–15.0</td>
</tr>
<tr>
<td>G-6-PD</td>
<td>10.1</td>
<td>5.74–7.02</td>
</tr>
<tr>
<td>6-PGD</td>
<td>11.6</td>
<td>6.74–7.35</td>
</tr>
<tr>
<td>GR</td>
<td>6.90</td>
<td>5.48–6.60</td>
</tr>
</tbody>
</table>

All values expressed as µmole/min/g Hb at 37°C.

*HK, hexokinase; PFK, phosphofructokinase; AK, adenylate kinase; PGK, phosphoglycerate kinase; TPI, triose phosphate isomerase; LDH, lactate dehydrogenase; GPI, glucose phosphate isomerase; GAPD, glyceraldehyde phosphate dehydrogenase; DPGM, diphosphoglycerate mutase; MPGM, monophosphoglycerate mutase; PK, pyruvate kinase; G-6-PD, glucose-6-phosphate dehydrogenase; 6-PGD, 6-phosphogluconate dehydrogenase; GR, glutathione reductase.

†Range of values obtained from transported controls.
**Procedures**

**Samples.** All blood samples obtained from the patient and family for enzyme determinations were collected in Kansas City, placed on ice, and transported rapidly to the laboratory in Manhattan, Kansas. Control samples were obtained from healthy laboratory personnel and were transported in a similar manner. All enzyme determinations were completed within 10 hr of sample collection. The blood samples were filtered through cotton balls to remove contaminating leukocytes.

**Biochemical determinations.** Previously described methods were used to determine the activity of erythrocyte HK, glucose phosphate isomerase, phosphofructokinase, aldolase, triose phosphate isomerase, glyceraldehyde phosphate dehydrogenase, phosphoglycerate kinase, diphosphoglycerate mutase, monophosphoglycerate mutase, enolase, pyruvate kinase, lactate dehydrogenase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, glutathione reductase, and adenylate kinase.

Lactate production rates were determined in erythrocytes washed three times in 150 mM NaCl and incubated in phosphate-buffered saline pH 7.4 (1 part 150 mM potassium phosphate buffer and 9 parts 150 mM NaCl) containing 10 mM glucose. The packed cell volume of the suspended erythrocytes was adjusted to 30%. Lactate concentrations were determined enzymatically by a previously described method.

Methemoglobin reduction rates were evaluated by established procedures.

Kinetic studies were carried out on hemolysates after dialysis for 6 hr against a stabilizing solution containing 2.7 mM EDTA, 0.7 mM 2-mercaptoethanol, and 10 mM glucose.

Optimum pH was determined by comparing HK activity in hemolysates over a pH range of 5.5-11.5. The pH was adjusted by the addition of Tris, glycine, and phosphate buffers.

Thermal stability studies were carried out on hemolysates (1:10) prepared in a stabilizing solution containing 2.7 mM EDTA, 0.7 mM 2-mercaptoethanol, and 10 mM glucose.

Electrophoresis of HK on starch gel was carried out by the methods described by Kaplan and Beutler and Rogers et al.

**RESULTS**

Initially, in order to determine the nature of the defect giving rise to the hemolytic anemia in the patient, a number of glycolytic and associated enzymes were tested. These data are shown in Table 1. It was evident that many enzymes had elevated activities when compared to values from normal controls. In comparison, the patient’s HK activity was only 50% of the normal level. Further comparison of the HK activity with that of a subject with a similar
The degree of reticulocytosis resulting from the presence of hemoglobin S, (2.9 μmole/min/g Hb) suggested that the patient's erythrocyte HK activity was only 25% of normal when compared on the basis of equivalent erythrocyte age. Repeated determinations of HK activity in the patient's erythrocytes over a period of several months consistently showed a severely diminished level of activity. Lactate production and methemoglobin reduction rates were also diminished in the patient's erythrocytes (Figs. 1 and 2).

Investigation of erythrocyte HK activities in the patient's immediate family were carried out (Table 2). Both parents and a sister of the patient were found to have HK activities that were markedly lower than those of normal controls.

No evidence indicating the presence of an HK inhibitor could be obtained in experiments utilizing combinations of the patient's and normal hemolysates.

Maximum HK activity in hemolysate prepared from the patient's erythrocytes was obtained at a pH of 8.5. This value did not differ from the pH optimum for normal control subjects (Fig. 3).

The affinity of the patient's HK for glucose and ATP was evaluated on a number of occasions. Figures 4 and 5 are representative of the data obtained.

### Table 2. Erythrocyte Hexokinase in the Patient's Family and Controls

<table>
<thead>
<tr>
<th>Subject</th>
<th>Hexokinase Activity (μmole/min/g Hb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Five normal controls</td>
<td>1.22 ± 0.115</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>1.07 – 1.37</td>
</tr>
<tr>
<td>Range</td>
<td>2.88</td>
</tr>
<tr>
<td>Hemoglobin S control</td>
<td>0.68</td>
</tr>
<tr>
<td>Patient (B.V.)</td>
<td>0.62</td>
</tr>
<tr>
<td>Father</td>
<td>0.75</td>
</tr>
<tr>
<td>Mother</td>
<td>1.07</td>
</tr>
<tr>
<td>Brother</td>
<td>0.84</td>
</tr>
</tbody>
</table>
The patient’s $K_m$ for glucose was found to be $24.8 \times 10^{-6} M$, which did not differ significantly from the value of $24.9 \times 10^{-6} M$ obtained for the controls. Similarly, the patient’s $K_m$ for ATP, $(1.4 \times 10^{-3} M)$ was not significantly different from that of the normal subjects $(1.0 \times 10^{-3} M)$.

Investigation of the stability of erythrocyte HK at 46°C indicated that the patient’s HK was not as stable as that of normal control subjects (Fig. 6). In addition, storage of blood from B.V. in CPD solution at 4°C resulted in a 25% decrease in HK activity over a period of 8 days. In comparison, HK activity in normal blood decreased by less than 5% under the same storage conditions.

Starch gel electrophoresis of HK from the patient and several control subjects, including the subject with the hemoglobin S–induced reticulocytosis, failed to reveal any abnormal structural variations in the patient’s HK.

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**Fig. 3.** Effect of varying pH on activity of erythrocyte hexokinase. Shaded area, normal range; (○), individual values obtained for B.V.

**Fig. 4.** Relationship between glucose concentration and reaction velocity in normal (●) and hexokinase-deficient B.V. (○) erythrocytes. Intercept on the abscissa (S) is equivalent to $-K_m$. 
DISCUSSION

It is most probable that the hemolytic anemia exhibited by B.V. arose from the diminished level of erythrocyte HK activity. The deficiency was particularly striking when comparison was made with a hemoglobin S control whose erythrocytes were of equivalent age. Valentine et al.5 noted the effect of erythrocyte age on HK activity and stressed the importance of using controls with a similar number of reticulocytes when investigating potential cases of HK deficiency. The elevated activity of the other enzymes studied (Table 1) also resulted from the comparatively young age of B.V.'s erythrocytes. It is evident from the lactate production and methylene blue-stimulated methemoglobin reduction studies that the defect results in a significant reduction in metabolic capacity. In view of this reduced metabolic capacity, it is of interest that the autohemolysis test was normal. One previously reported case of HK deficiency had a markedly abnormal autohemolysis test that was only partially corrected by the addition
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of glucose or ATP. In comparison, the cases reported by Valentine et al. and Keitt had, respectively, only a marginally abnormal and a normal response. It therefore appears that the autohemolysis test does not always clearly indicate the presence of a metabolic defect in cases of HK deficiency.

Previously reported cases of erythrocyte HK deficiency were associated with chromosome abnormalities, abnormal reaction kinetics, or instability. The patient described here presented no evidence of the chromosome and associated abnormalities observed in the cases described by Löhr et al. Similarly, the normal reaction kinetics exhibited by HK obtained from B.V. indicate that this case differs from that described by Necheles et al. Our data suggest that the hemolytic anemia observed in B.V. arose from an HK variant that has diminished activity and is inherently unstable. The data available do not indicate whether the lowered HK activity in B.V.’s erythrocyte was due entirely to the instability of the enzyme, or was also a result of a concomitant decrease in specific activity of the variant enzyme.

This particular case appears to differ from previously reported unstable HK variants. Keitt reported a case of hemolytic anemia associated with a marked lability of HK in the absence of glucose. In contrast, HK from B.V. showed a significant decrease in activity even in the presence of glucose. The present case most resembles the unstable variant reported by Valentine et al. However, subsequent study of that variant indicated that it could be identified electrophoretically. Despite using several buffer and pH systems, we have been unable to detect any abnormal electrophoretic variation in HK from B.V.

Unfortunately, there are insufficient data to enable the unequivocal resolution of the mode of inheritance of this defect. Diminished activity in both parents and in the patient’s sister, together with the absence of obvious hematologic disorders in these subjects, suggest that they may be heterozygous for a variant allele, while B.V. provides an example of the homozygous state. That interpretation is consistent with an autosomal codominant mode of inheritance.

REFERENCES

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