Hemolytic Anemia Due to Pyrimidine-5’-Nucleotidase Deficiency: Report of Eight Cases in Six Families

By E. Beutler, P. V. Baranko, J. Feagler, F. Matsumoto, M. Miro-Quesdada, G. Selby, and P. Singh

We describe the clinical, hematologic, and biochemical findings in eight patients with pyrimidine-5’-nucleotidase deficiency occurring in six unrelated families. In most cases, a profound lowering of red cell pyrimidine-5’-nucleotidase activity, associated with the characteristic change in the u.v. spectrum brought about by increased levels of pyrimidine nucleotides was observed. One patient had an atypical presentation in that no stippled cells were apparent in the peripheral blood film, and the u.v. spectrum of red cell extracts was normal or nearly so. In addition, the one parent of this patient whose red cells were tested was not pyrimidine-5’-nucleotidase deficient. This patient may be a heterozygote for pyrimidine-5’-nucleotidase deficiency with hemolysis due to this or another cause. Several of the patients were found to be mentally retarded, and in one family in which several sibs, two with and one without pyrimidine-5’-nucleotidase deficiency, could be studied, the low IQ values were found only in the enzyme-deficient subjects. A pyrimidine-5’-nucleotidase activity is found in normal fibroblasts and, to the same extent, in the fibroblast of patients with pyrimidine-5’-nucleotidase deficiency. This activity is largely membrane bound, however, and is inhibited by the 5’-nucleotidase inhibitor, α-β-methylene ADP. Accordingly, it appears that fibroblasts are devoid of the type of specific pyrimidine-5’-nucleotidase activity found in normal erythrocytes. We suggest that pyrimidine-5’-nucleotidase deficiency is one of the more common recognized causes of nonspherocytic hemolytic anemia and that in some cases it may produce, in addition to hemolysis, mental retardation.

In 1972, Valentine et al. described a patient with nonspherocytic hemolytic anemia, marked basophilic stippling of the erythrocytes, a decrease in red cell ribose phosphate pyrophosphokinase, and what was believed to be greatly elevated red cell adenosine triphosphate (ATP) levels. Two additional similar kindreds were described in the following year. In 1974, however, the same authors encountered another such patient and now observed that the progress curves in the assay of adenine nucleotides in the red cells from this patient were very slow. This led to their discovery that in reality the erythrocytes contained greatly increased quantities of pyrimidine nucleotides, not adenine nucleotides, and that there was a severe deficiency of pyrimidine-5’-nucleotidase. Since these original reports were published, a number of other patients with pyrimidine-5’-nucleotidase deficiency have been described in various populations.

Since discovery of this defect, we have devised a convenient radiometric method for the assay of pyrimidine 5’ nucleotidase. Application of this technique to the blood of patients with nonspherocytic hemolytic anemia resulted in the identification of nine additional patients with pyrimidine-5’-nucleotidase deficiency in seven families. One of these patients has been reported elsewhere; the present report presents the findings in the additional eight cases, three of whom have undergone splenectomy. We also investigated, for the first time, the activity of this enzyme in cultured skin fibroblasts.

MATERIALS AND METHODS

Assays of glycolytic enzymes and intermediates were carried out using previously published techniques. In order to determine the relative proportion of pyrimidine and purine nucleotides in red cells, 1 volume of whole blood was added to eight volumes of 4% perchloric acid. After neutralization with potassium carbonate, 0.2 ml of the extract was added to 0.3 ml of 1 M glycine buffer, pH 3.0, and 0.5 ml of water. The optical density at 260 nm and 280 nm was read against a water blank. In 10 normal samples, the ratio of OD260 to OD280 was 1.80 ± 0.097 (mean ± 1 SD). Pyrimidine-5’-nucleotidase was assayed using our radiometric method. Hematologic investigations were carried out by standard techniques. Fibroblast cultures were grown from skin biopsies using standard techniques. α-β-methylene ADP was obtained from P-L Biochemicals.

CASE REPORTS

Case 1

T.F. was born in October 1964 and was observed to be jaundiced in the neonatal period. She continued to have episodes of jaundice and anemia and was variously diagnosed as having hepatitis and thalassemia. In 1975, the hemoglobin concentration of her blood was 10 g/dl. There was moderate polychromasia and stippled red blood cells on the blood film. The reticulocyte count was 7.1%, bilirubin 1.2 mg/dl direct and 3.5 mg/dl total. No splenomegaly or hepatomegaly was noted. The patient seemed to be mentally slow and was considered to be below normal in intelligence. When seen in December 1977, she complained of intermittent abdominal pain, the liver was felt 2 cm below the right costal margin and the spleen was enlarged to 4 cm below the left costal margin. The hemoglobin was 10.8 g/dl with a hematocrit of 33.4%, red count of 3.16 million, reticulocytes of 12.2%, a white count of 5,000/μl with a normal differential, and a platelet count of 315,000/μl. Prominent basophilic stippling was noted on the peripheral blood smear. The direct reacting bilirubin was 0.9 mg/dl, total bilirubin 4.6 mg/dl, and the serum uric acid 7.7 mg/dl. An abdominal ultrasound scan revealed the presence of multiple calculi in the gallbladder. The spleen was noted to project 18 cm downward from the level of the dome of the
diaphragm. The $^{51}$Cr red cell $T_{1/2}$ was 22 days. The spleen: liver uptake was 4:3:1. In 1977, cholecystectomy was carried out, and a 709-g spleen showing hyperemic pulp with focal areas of extra-medullary hematopoiesis and rather prominent Malpighian corpuscles was removed.

**Case 2**

S.S. is an 8-yr-old white female who was delivered by caesarean section. She was of Hungarian-Italian descent, and her parents were unrelated. Her hemoglobin had ranged from 7.6 g/dl to 10.5 g/dl, and she had been treated with iron on several occasions for "iron deficiency." On physical examination, she had mild pallor, and her spleen was palpable approximately 3 cm below the left costal margin. Mild psychomotor retardation was thought to be present; she was enrolled in a special education class for children with a learning disability. The hemoglobin was 11.0 g/dl, hematocrit 34%, reticulocytes 12.6%, WBC 7600/μl, and the differential was normal. Peripheral blood films showed moderate degrees of polychromasia with basophilic stippling, moderate anisocytosis, mild poikilocytes, and a mild to moderate degree of hypochromia and microcytosis.

**Case 3**

L.L. is a 22-yr-old white male of German-Irish descent. He had been noted to be jaundiced at birth and had scleral icterus throughout life. There was no family history of anemia and none of his sibs had been jaundiced. The patient was considered to be of normal intelligence and had completed a high school education. The spleen was palpable 2 cm below the left costal margin, the hemoglobin was 11.0 g/dl with normochromic and normocytic indices, reticulocytes 12.6%, and white count 6300/μl. Basophilic stippling, poikilocytosis, and a mild to moderate degree of hypochromia and microcytosis were noted on the stained blood film. The osmotic fragility of the red cells, direct and indirect Coombs test, sucrose and acid hemolysis tests, hemoglobin A$_2$ and hemoglobin F levels were reported to be normal. The total bilirubin was 4.5 mg/dl with a direct reacting fraction of 0.8 mg/dl. Plasma γ-globulins were modestly decreased to 0.6 g/dl.

**Case 4**

E.J. is a white female considered to be of probable Polish extraction. Mentally retarded, but able to care for herself, she was admitted for investigation of vague subternal chest pain. The patient had undergone cholecystectomy and splenectomy at age 13, but the reason for performance of these procedures and all of the details of the operation were unknown. The hemoglobin was 9.6 g/dl with a red count of $2.58 \times 10^{12}/μl$, a hematocrit of 30.9%, and a reticulocyte count of 51.8%. The white count was 13,200/μl. The peripheral blood film was reported to have 5%-10% spherocytes, and no comment was made about stippling of the red cells, and we were unable to obtain a blood film for examination. The total bilirubin was 4.9 mg/dl with 0.4 mg/dl direct reacting bilirubin.

After we had established the diagnosis on this patient it was learned that a blood sample from the patient, who was going under another name, had previously been sent to the laboratory of Dr. Henri Frischer, who had also established a diagnosis of pyrimidine-5'-nucleotidase deficiency.

**Case 5**

T.C. is a 19.5-mo-old white male who was admitted to the hospital for evaluation of a generalized seizure occurring during a febrile episode. His hematocrit was 30%, although at age 14 mo his hematocrit was 35%. He was presumed to be iron-deficient and was given supplemental iron. Further studies of the child's red cells were initiated when, while being followed for a recurrent ear infection, a hemoglobin of 6.1 g/dl, hematocrit of 16.8%, red count of $2.24 \times 10^{12}/μl$, and a reticulocyte count of 0.1% were noted. The bilirubin was 0.5 mg/dl; serum lead level was 23 μg/dl. No stippled red cells were seen on the blood film. A Denver Developmental Screening
Enzyme activities are expressed as U/g Hb.

Normal red cell activity is 1.36 ± 1.86 U/g Hb (mean ± 1 SD).

Table 2. The Levels of Intermediate Compounds of Red Cells in Six Unrelated Pyrimidine-5'-Nucleotidase Deficient Patients (All Values are Expressed in nmoles/ml Red Cells)

<table>
<thead>
<tr>
<th></th>
<th>T.F.</th>
<th>S.S.</th>
<th>L.L.</th>
<th>E.J.</th>
<th>T.C.</th>
<th>M.S.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose-6-P</td>
<td>72</td>
<td>53</td>
<td>48.3</td>
<td>70.4</td>
<td>55.4</td>
<td>61 ± 16.1</td>
</tr>
<tr>
<td>Fructose-6-P</td>
<td>21</td>
<td>21</td>
<td>19.5</td>
<td>24.3</td>
<td>20.1</td>
<td>22 ± 6.7</td>
</tr>
<tr>
<td>Fructose-1,6 dP</td>
<td>3.4</td>
<td>3.7</td>
<td>4.5</td>
<td>10.5</td>
<td>2.9</td>
<td>3 ± 0.9</td>
</tr>
<tr>
<td>Dihydroxyacetone-P</td>
<td>13</td>
<td>12</td>
<td>13.8</td>
<td>49.6</td>
<td>11.7</td>
<td>18.2 ± 4.6</td>
</tr>
<tr>
<td>2,3-Diphosphoglyceric acid</td>
<td>4,749</td>
<td>7,656</td>
<td>5,975</td>
<td>4,221</td>
<td>7,014</td>
<td>4,171 ± 636</td>
</tr>
<tr>
<td>3-Phosphoglyceric acid</td>
<td>45</td>
<td>54</td>
<td>37.8</td>
<td>45.3</td>
<td>41.6</td>
<td>60.7 ± 11.9</td>
</tr>
<tr>
<td>2-Phosphoglyceric acid</td>
<td>6</td>
<td>7</td>
<td>8.0</td>
<td>16.8</td>
<td>7.3</td>
<td>10.7 ± 3.6</td>
</tr>
<tr>
<td>Phosphoenolpyruvate</td>
<td>14</td>
<td>16</td>
<td>15.96</td>
<td>12.5</td>
<td>17.2</td>
<td>20.1 ± 5.9</td>
</tr>
<tr>
<td>ATP</td>
<td>1,953</td>
<td>1,416</td>
<td>1,695</td>
<td>821</td>
<td>1,683</td>
<td>1,438 ± 99</td>
</tr>
<tr>
<td>Reduced glutathione</td>
<td>3,264</td>
<td>3,750</td>
<td>3,176</td>
<td>3,509</td>
<td>2,869</td>
<td>2,234 ± 354</td>
</tr>
<tr>
<td>Oxidized glutathione</td>
<td>2.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>OD260/OD280</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
</tbody>
</table>

* Sister of S.Sa.
† Ratio indicates presence of large amounts of pyrimidine nucleotides.

Test for gross motor, fine motor, adaptive language, and personal and social relationships was considered to be adequate for the child's age. Electroencephalogram was reported to be normal, and no further seizure occurred. One month after the original sample was obtained, the hemoglobin had risen to 11.9 g/dl, the child having been transfused in the interim, and the reticulocyte count was now 3%.

Case 6

S.Sa. is a 19-yr-old Peruvian female with a long-standing history of anemia, jaundice, and persistent right ankle ulcers. The parents are first cousins once removed. The patient has six living sibs, two of whom have hemolytic anemia with characteristics identical to those of the propositus. Two sibs died, one stillborn and another at 6 mo of age of "intestinal fever."

The patient had undergone a cholecystectomy and splenectomy at age 16, and this did not appear to produce any change in her hemolytic anemia. The hematocrit was 28% and the reticulocyte count 11%. Coarse basophilic stippling of the red cells was evident. Autohemolysis was reported to be moderately increased with partial correction with glucose. Coombs test, hemoglobin electrophoresis, hemoglobin heat stability, and isopropanol stability tests and osmotic fragility were all reported to be normal.

Psychometric studies on S.Sa. revealed a Wechsler IQ of 67; two sibs with hemolytic anemia manifested IQ values of 68 and 72, respectively. In contrast, the one unaffected sib who was tested had an IQ of 90.

Table 3. Pyrimidine-5'-Nucleotidase Activity of Family Members of Deficient Patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Patient</th>
<th>Father</th>
<th>Mother</th>
<th>Sibs</th>
</tr>
</thead>
<tbody>
<tr>
<td>T.F.</td>
<td>7.4, 6.8, &lt;5</td>
<td>90.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S.S.</td>
<td>&lt;5, 5 &lt;5</td>
<td>90.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L.L.</td>
<td>6.7</td>
<td>143.2</td>
<td>48.2</td>
<td>203.6</td>
</tr>
<tr>
<td>E.J.</td>
<td>19.9, 13.7</td>
<td>106.6</td>
<td>85.71</td>
<td>138.3</td>
</tr>
<tr>
<td>T.C.</td>
<td>34.6, 46.5</td>
<td>123.5</td>
<td>155.4</td>
<td></td>
</tr>
<tr>
<td>S.Sa.</td>
<td>18.5</td>
<td>86.53</td>
<td>7.5, 8.3</td>
<td></td>
</tr>
</tbody>
</table>

Enzyme activities are expressed as U/g Hb.

Normal red cell activity is 136 ± 18.6 U/g Hb (mean ± 1 SD).

RESULTS

The results of red cell enzyme assays and intermediate levels are presented in Tables 1 and 2. The results of pyrimidine-5'-nucleotidase assays on the red cells of family members are summarized in Table 3. An attempt to purify and characterize the red cell enzyme obtained from T.F. was unsuccessful because of its extreme instability.

Skin fibroblast cultures were obtained from patients T.F., E.J., and L.L. and the pyrimidine-5'-nucleotidase activity of extracts of these fibroblasts was compared with that of control cultures. When fibroblast suspensions were frozen and thawed and centrifuged at 39,000 g for 10 min, large amounts of enzyme activity could be detected in the supernatant. The activity of fibroblasts from T.F. was found to be 1.5 U/g protein, from L.L. to be 1.1 U/g protein, and from E.J. 2.0 U/g protein; control values from two controls were both 2.0 U/g protein. Further studies established, however, that the cytidine monophosphate-hydrolyzing activity found in fibroblasts was not, in reality, pyrimidine-5'-nucleotidase. First of all,
it was established that a 0.6 mM concentration of the 5'-nucleotidase inhibitor α-β-methylene ADP produced over 99% inhibition of fibroblast activity, but failed to inhibit pyrimidine-5'-nucleotidase in crude hemolysates. Secondly, while hemolysates were unable to catalyze the hydrolysis of adenosine monophosphate (AMP) when this purine nucleotide was substituted for cytidine monophosphate (CMP) in the radiometric assay, fibroblasts were able to hydrolyze AMP at the same rate as CMP. Finally, we found that centrifugation of fibroblast extracts at 100,000 g for 60 min resulted in removal of about 90% of the detectable CMP-hydrolyzing activity from both mutant and normal fibroblasts, indicating that most of the enzyme was membrane-associated. The enzyme activity that remained in the supernatant was still virtually totally inhibitable by α-β-methylene ADP, and no difference was found between the residual activity of pyrimidine-5'-nucleotidase deficient patients' fibroblast extracts and extracts of normal fibroblasts even after prolonged high-speed centrifugation. Thus, it appears that cultured fibroblasts do not contain the pyrimidine specific 5'-nucleotidase which is so abundant in erythrocytes.

DISCUSSION

Pyrimidine-5'-nucleotidase deficiency has been recognized for only a few years. Yet, in the study of only 80 samples from patients with hemolytic anemia, we encountered 5 unrelated patients with this disorder. Four of these cases and those in two even more recently encountered families are presented here. It is apparent that this red cell enzyme defect will rank among the more common of the presently definable causes of nonspherocytic hemolytic anemia. As in previously reported cases, basophilic stippling was prominent in most of the erythrocytes of our patients with pyrimidine-5'-nucleotidase deficiency. Thus, in contrast to most other causes of nonspherocytic hemolytic anemia, red cell morphology provides an important clue to the biochemical pathogenesis in pyrimidine-5'-nucleotidase deficiency. In the case of one subject, T.C., stippled cells were not seen even after careful examination of the Wright-stained smear. This patient's red cells had the highest residual enzyme activity of any of the patients examined. However, a borderline abnormality of the ultraviolet spectrum of an extract of his red cells was present. The enzyme activity of the red cells of his mother was normal and that of the father borderline, and it is presumed that he was either a heterozygote for pyrimidine-5'-nucleotidase deficiency with an unrelated hemolytic anemia or that he had a form fruste of this disorder. In one other patient no stippled cells were reported but the authors did not personally examine the blood film. The levels of red cell enzymes other than pyrimidine-5'-nucleotidase were, in general, normal or, in the case of age-related enzymes, usually somewhat increased. While the activities of the most age-dependent enzymes, hexokinase and glutamic oxaloacetic transaminase, were consistently elevated, the activities of other age-dependent enzymes, particularly glucose-6-phosphate dehydrogenase, pyruvate kinase, and aldolase, were only mildly elevated or actually slightly diminished in some instances. The significance of this finding is not clear, and it might represent a complex epiphenomenon of pyrimidine-5'-nucleotidase deficiency. The levels of metabolic intermediates were in general comparable to those observed with control values obtained from patients with high reticulocyte counts. The low ATP level found in patient E.J. is not readily explained, but since it was found in two samples, it must be presumed to be a valid finding. In those cases in which splenectomy was performed and in which the results could be evaluated, no significant ameliorization of the anemia was observed.

Three of the six index cases manifested mild degrees of mental retardation. One of the other patients had a seizure disorder. Although we are unable to establish a cause-and-effect relationship between the enzyme deficiency and the mental retardation in these families, the incidence is sufficiently high so as to raise the suspicion that such a relationship may exist. Moreover, in the family of patient S.Sa., the IQs of the three sibs with hemolytic anemia range between 67 and 72, while the one unaffected sib tested had an IQ of 90. Mental retardation has not previously been noted as a concomitant of pyrimidine-5'-nucleotidase deficiency. However, in the case of two other well characterized red cell enzyme deficiencies, NADH-diaphorase deficiency and glutathione synthetase deficiency, heterogeneity exists with respect to neurologic involvement. In each of the latter two instances, families with and without mental retardation or neurologic symptoms have been described. In patients with NADH-diaphorase deficiency in whom mental retardation is present, a deficiency of NADH-diaphorase in tissues other than red cells has been documented; in those patients in whom no mental retardation is present, tissue levels appear to be normal. The same pattern will presumably also emerge in the case of glutathione synthetase deficiency, where 5-oxoprolinuria and neurologic disturbances occur in some families but not in others.

A likely explanation for such genetic heterogeneity is the existence of two enzyme subunits, under separate genetic control, with one of these being unique to erythrocytes and the other common to erythrocytes.
and brain. A similar pattern of involvement may also occur because a very unstable mutant enzyme will affect the long-lived non-nucleated red cell more severely than tissues that are capable of carrying out protein synthesis. It is entirely possible that such situations may also exist with respect to pyrimidine-5'-nucleotidase, but the tissues distribution and subunit structure of this enzyme is as yet poorly defined. Fibroblasts are totally devoid of pyrimidine-5'-nucleotidase activity, although they contain high levels of the nonspecific, membrane-associated, 5'-nucleotidase. In contrast, the 5'-nucleotidase inhibitor, \( \alpha \)-\( \beta \)-methylene ADP, failed to affect the cytidine monophosphate-cleaving activity of hemolysates. Apparently, red cells are devoid of the nonspecific 5'-nucleotidase. The question of why red cells lack 5'-nucleotidase is an interesting one. One may speculate that because ATP is of vital importance in red cell metabolism and because red cells are limited to the use of the salvage pathway in their limited capacity to synthesize adenine nucleotides, that the existence of an enzyme capable of hydrolyzing AMP might be harmful to the erythrocyte. Whether or not the enzyme discovered by Valentine et al. is limited to red cells or whether other tissues also contain the enzyme with the capacity to hydrolyze pyrimidine nucleotides without hydrolyzing purine nucleotides, is under active investigation. Preliminary studies indicate that the brain does contain this enzyme.

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