Three Cases of Hereditary Nonspherocytic Hemolytic Anemia Associated With Red Blood Cell Glutathione Deficiency

By Akira Hirono, Hideaki Iyori, Isao Sekine, Junichi Ueyama, Hirotane Chiba, Hitoshi Kanno, Hisaichi Fujii, and Shiro Miwa

Three unrelated Japanese patients with chronic nonspherocytic hemolytic anemia were found to have marked deficiency of red blood cell (RBC) reduced glutathione (GSH) (4.4%, 13.1%, and 6.9% of normal, respectively). A panel of RBC enzyme assays showed that one patient had decreased glutathione synthetase activity and the other two were moderately deficient in γ-glutamylcysteine synthetase. Some family members of each patient showed mild deficiency of the respective enzymes. RBCs of these patients also showed a decreased level of glutathione-S-transferase as in previously described GSH-deficient cases. Hemolytic anemia was their only manifestation, and neither 5-oxoprolinemia nor 5-oxoprolinuria, which are usually associated with the generalized type of glutathione synthetase deficiency, was noted in our patients.

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NONSPHEROCYTIC hemolytic anemia with marked deficiency of red blood cell (RBC) reduced glutathione (GSH) is a rare hereditary disorder first described by Oort et al.1 Deficiency of one of the two enzymes in glutathione synthesis, γ-glutamylcysteine synthetase (GC-S) and glutathione synthetase (GSH-S), is known to be responsible for this disorder. GC-S catalyzes the initial and the rate-limiting step of glutathione synthesis. Deficiency of GC-S is extremely rare and only two families with this disorder have been reported so far.2,3 GSH-S deficiency is a more frequent cause of GSH deficiency, and over 20 families with the disorder have been reported.1,4-17 It is noteworthy that there are two distinct types of GSH-S deficiency with different clinical pictures. In the RBC type1,4,7 the enzyme defect is confined to RBCs and the only clinical sign is mild nonspherocytic hemolytic anemia, whereas in the generalized type5-17 the deficiency is also found in tissues other than RBCs,18 and patients show not only hemolytic anemia but also metabolic acidosis with 5-oxoprolineuria and neurologic symptoms including mental retardation. Existence of tissue-specific iso-enzymes,19 enzymological heterogeneity of mutant enzymes,19 and/or different susceptibility of mutant enzymes to tissue specific proteases20 are the possible explanations for the different tissue distribution of the enzyme deficiencies, although the precise mechanism remains to be elucidated.

We report here the first Japanese patients with chronic nonspherocytic hemolytic anemia and marked RBC GSH deficiency caused by GC-S deficiency or GSH-S deficiency of RBCs.

CASE REPORTS

Case 1. A 10-year-old Japanese boy was noted to have jaundice and anemia and was admitted to the National Defense Medical College Hospital for evaluation. He had a history of anemia at birth and received transfusion at 1 month of age, but he had been asymptomatic during the following 10 years. At the time of admission, he was slightly anemic and icteric, and had mild hepatosplenomegaly. There was no evidence of mental or growth retardation, cerebellar dysfunction, or focal neurologic signs. Pertinent laboratory data included the following: RBC count, 3.33 × 10^6/μL; hemoglobin (Hb) concentration, 10.5 g/100 mL; reticulocyte count, 6.8%; serum total bilirubin, 3.0 mg/100 mL, of which 2.3 mg/100 mL was indirect reacting; serum haptoglobin, 80 mg/100 mL. The peripheral blood (PB) film showed marked basophilic stippling in RBCs. Although the proband’s parents came from the same locality, there was no known consanguinity in the family. The patient is now 15 years old and has been asymptomatic since the episode at age 10. The recent laboratory findings included RBC count, 3.73 × 10^6/μL; Hb concentration, 12.0 g/100 mL; reticulocyte count, 7.1%; serum indirect reacting bilirubin, 1.6 mg/100 mL.

Case 2. A 17-year-old Japanese girl was first seen at the Toranomon Hospital for evaluation of anemia. She was noted to have anemia since childhood. At the time of examination, she was slightly anemic, but had no hepatosplenomegaly. She showed no signs of neurologic disorders. Laboratory data included the following: RBC count, 2.94 × 10^6/μL; Hb concentration, 10.2 g/100 mL; reticulocyte count, 7.9%; serum total bilirubin, 1.5 mg/100 mL, of which 0.8 mg/100 mL was indirect reacting; serum haptoglobin, 70.2 mg/100 mL. Her RBC survival time measured by using 51Cr-labeled RBCs was markedly reduced (T½ = 5 days; normal range: 25 to 40 days). The proband’s parents were first cousins. There was no family history of anemia.

Case 3. The patient was a 4-year-old Japanese boy. He was born at 36 weeks and weighed 2,362 g. He was admitted to Fuji City Central Hospital because of low birth weight. At 6 days of age, his Hb concentration was decreased to 5.9 g/100 mL, and he received transfusions. He also showed severe neonatal jaundice and needed phototherapy. During the next 2 months, he had consistent hemolytic anemia and repeated transfusion was required. His hemolysis then became well compensated and he had no hemolytic episode until age 3. The patient was admitted to Daisan Hospital of the Jikei University School of Medicine at 7 months of age for evaluation of hemolytic anemia. At the time of admission, his only clinical findings were compensated hemolytic anemia with the Hb concentration of 11.0 g/100 mL and a reticulocyte count of 3.2%. He showed no other signs or symptoms including neurologic abnormalities. His blood was positive for Heinz body formation and several target cells...
The inorganic phosphate released during each enzymatic reaction was deproteinized in equal volumes of 0.6 N perchloric acid. After centrifugation an aliquot of the supernatant was neutralized with potassium hydroxide and subjected directly to acid hydrolysis. The enzyme activities, IU/g Hb; GSH levels, mg/100 mL RBC; 5-oxoproline levels, nmol/mL. Abbreviation: ND, not determined.

MATERIALS AND METHODS

Substrates and enzymes were purchased from Boehringer-Mannheim (Mannheim, Germany) except for γ-glutamylcysteine, which was obtained from Kohjin (Tokyo, Japan). 5-Oxoproline was from Wako Pure Chemical Industries (Osaka, Japan). All the other reagents were of analytical grade.

Heparinized blood was obtained from the patients, their several family members and normal healthy adults after obtaining their informed consent. After separation from plasma and a buffy coat, RBCs were purified by cellulose filtration.

RBC enzyme activities and GSH concentration were assayed by standard procedures. GC-S activity and GSH-S activity were assayed by the method of Konrad et al with slight modifications. The reaction mixture for GC-S assay consisted of 30 μL of 1 mol/L Tris-HCl buffer, pH 8.0, containing 5 mmol/L EDTA and 0.1 mol/L MgCl₂ (buffer A); 30 μL each of 100 mmol/L cysteine, 100 mmol/L glutamate, and 100 mmol/L adenosine triphosphate (ATP); 30 μL of H₂O₂; and 150 μL of hemolysate. The reaction mixture for GSH-S assay consisted of 30 μL of buffer A; 30 μL each of 100 mmol/L glycine, 100 mmol/L γ-glutamylcysteine, and 100 mmol/L ATP; 30 μL of H₂O₂; and 150 μL of hemolysate. The blank consisted of 30 μL of buffer A, 30 μL of 100 mmol/L ATP, 90 μL of H₂O₂, and 150 μL of hemolysate. After 30 minutes of incubation at 37°C, the inorganic phosphate released during each enzymatic reaction was measured with subtraction of the blank value.

Plasma and urine 5-oxoproline was determined by the method of Marstein et al with slight modifications. Briefly, samples were deproteinized in equal volumes of 0.6 N perchloric acid. After centrifugation an aliquot of the supernatant was neutralized with 5 N potassium hydroxide and subjected directly to acid hydrolysis. The concentration of 5-oxoproline in the sample was estimated by the enzymatic determination of glutamic acid formed after acid hydrolysis. Recovery of added authentic 5-oxoproline was greater than 80%.

RESULTS

Table 1 summarizes the levels of GSH and the activities of GSH-related enzymes. The marked deficiency of GSH in RBCs was observed in all the probands, and mother of case 1 also showed moderately reduced GSH level. Enzyme assays clearly indicated that the depletion of GSH was caused by the deficiencies of enzymes involved in the GSH synthesis. In case 1 and case 2, the GC-S activity was 5.6% and 23.3% of that in the normal controls, respectively. On the other hand, in case 3 the GSH-S activity was 3.1% of that in the normal controls. Mothers of cases 1, 2, and 3 and the father and a brother of case 3 also showed mild deficiency (48.9% to 75.6% of normal) of the corresponding enzymes. It is noteworthy that the three patients had low activity of glutathione-S-transferase (GST) (16.1%, 29.3%, and 9.3%, respectively) as well. Slightly decreased GST activity (60.5% to 78.9%) was also found in the examined family members except for the mother of case 2. The other RBC enzyme activities were normal or moderately increased because of reticulocytosis. The levels of 5-oxoproline in the patients' plasma and urine were within normal limits (Table 1).

DISCUSSION

Two different enzymatic deficiencies have so far been reported to cause marked depletion of RBC GSH. In the present study, we found that cases 1 and 2 had RBC GC-S deficiency and case 3 had GSH-S deficiency. Deficiency of GC-S has previously been found only in three patients from two unrelated families. Our patients are the fourth and the fifth documented cases of this disorder. Table 2 compares the clinical and biochemical characteristics of our patients and three earlier cases. Although there are some differences in the degree of GSH levels and residual GC-S activities in RBCs, hematologic manifestations of these cases are almost identical, ie, mild or well-compensated chronic nonspherocytic hemolytic anemia. The patients of the first GC-S–deficient family were reported to have spinocerebellar degeneration of late onset in addition to hemolytic anemia, whereas no neurologic deficit was noted in the second family. We also could not find any neurologic deficit in the probands and the other members in the present families. It should be noted that the spinocerebellar degeneration found in two siblings in the first family was late onset and neither of the patients had shown neurologic abnormalities until age 29. Considering the relatively young ages of our patients, it might still be possible that they will develop neurologic manifestations later. The differences in the RBC GC-S activities between our two patients are assumed to be

Table 1. Glutathione-Related Enzyme Activities and GSH Content in RBCs, and Plasma and Urine 5-Oxoproline Levels

<table>
<thead>
<tr>
<th></th>
<th>Normal Range</th>
<th>Case 1</th>
<th>Case 2</th>
<th>Case 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Proband</td>
<td>Mother</td>
<td>Proband</td>
</tr>
<tr>
<td>Glutathione reductase</td>
<td>6.19-9.20</td>
<td>6.18</td>
<td>ND</td>
<td>8.77</td>
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<tr>
<td>Glutathione peroxidase</td>
<td>17.4-26.4</td>
<td>29.3</td>
<td>ND</td>
<td>36</td>
</tr>
<tr>
<td>GST</td>
<td>5.45-6.85</td>
<td>0.99</td>
<td>3.72</td>
<td>1.80</td>
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<td>GC-S</td>
<td>0.82-9.08</td>
<td>0.05</td>
<td>0.50</td>
<td>0.23</td>
</tr>
<tr>
<td>GSH-S</td>
<td>0.43-0.55</td>
<td>0.57</td>
<td>0.48</td>
<td>0.52</td>
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<tr>
<td>GSH</td>
<td>54.37-73.4</td>
<td>2.8</td>
<td>22.8</td>
<td>8.4</td>
</tr>
<tr>
<td>Plasma 5-oxoproline</td>
<td>310-490</td>
<td>330</td>
<td>400</td>
<td>410</td>
</tr>
<tr>
<td>Urine 5-oxoproline</td>
<td>760-1,250</td>
<td>900</td>
<td>ND</td>
<td>870</td>
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</table>
caused by a diversity of mutant enzymes, which is common in other erythroenzymopathies. Deficiency of GC-S in our families seems to be inherited as an autosomal recessive trait as was reported in the previous cases.

Case 3 is the first reported Japanese patient with GSH-S deficiency. Because hemolytic anemia was the only symptom and there was no accumulation of 5-oxoproline in plasma or urine, our patient’s GSH-S deficiency apparently belongs to the RBC type. An RBC type of GSH-S deficiency has been reported in 12 patients from eight families. These patients showed mild, and often fully compensated, nonspherocytic hemolytic anemia as their only clinical manifestation. Although RBCs are capable of producing 5-oxoproline from accumulated γ-glutamylcysteine, patients with an RBC type deficiency including ours did not present with 5-oxoprolinuria, with the exception of two cases with mild 5-oxoprolinuria. This might be due to a low contribution of RBCs to an overall tissue production of 5-oxoproline.

It should be noted that the proband’s father also showed persistent nonspherocytic hemolytic anemia since his childhood. Because GSH-S deficiency is considered to be inherited as an autosomal recessive trait, the father may be deficient in both of his GSH-S alleles or he may have another precipitating factor for hemolysis in addition to the heterozygous GSH-S deficiency. Unfortunately, we had no opportunity to examine his blood sample and the precise cause of his hemolytic anemia remains unknown.

The decreased activity of GST in RBCs found in our patients has previously been reported in several GSH deficiency cases. This reduced activity of GST is considered to be an epiphenomenon associated with the decreased level of GSH a potent stabilizer of GST, rather than the primary cause of GSH deficiency.

ACKNOWLEDGMENT

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REFERENCES

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Table 2. Summary of the Five Reported Patients With γ-Glutamylcysteine Synthetase Deficiency

<table>
<thead>
<tr>
<th>Families</th>
<th>Age (yr)</th>
<th>Sex of Patients</th>
<th>Ethnic Groups</th>
<th>Hematocrit (% )</th>
<th>Reticulocytes (%)</th>
<th>GSH (%)</th>
<th>GC-S Activity (%)</th>
<th>GSH in Carriers (%)</th>
<th>GC-S Activity in Carriers (%)</th>
<th>CNSHA</th>
<th>Neurologic Disorders</th>
<th>Reference</th>
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<tbody>
<tr>
<td>1-1</td>
<td>38</td>
<td>M</td>
<td>German</td>
<td>39</td>
<td>8.6</td>
<td>3.1</td>
<td>13.3</td>
<td>Normal</td>
<td>47-69</td>
<td>+</td>
<td>+</td>
<td>Beutler et al 3</td>
</tr>
<tr>
<td>1-2*</td>
<td>36</td>
<td>F</td>
<td>German</td>
<td>36</td>
<td>10.3</td>
<td>2.0</td>
<td>8.8</td>
<td>Normal</td>
<td>47-69</td>
<td>+</td>
<td>+</td>
<td>Beutler et al 3</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td>F</td>
<td>Polish</td>
<td>39</td>
<td>11.5</td>
<td>10.6</td>
<td>6.4</td>
<td>Normal</td>
<td>50-59</td>
<td>+</td>
<td>Beutler et al 3</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>M</td>
<td>Japanese</td>
<td>33</td>
<td>6.8</td>
<td>4.4</td>
<td>5.6</td>
<td>36</td>
<td>50-59</td>
<td>+</td>
<td>Present case</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>17</td>
<td>F</td>
<td>Japanese</td>
<td>31</td>
<td>6.6</td>
<td>13.1</td>
<td>23.3</td>
<td>Normal</td>
<td>49-76</td>
<td>+</td>
<td>Present case</td>
<td></td>
</tr>
</tbody>
</table>

* Sister of 1-1.

Abbreviation: CNSHA, chronic nonspherocytic hemolytic anemia.
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