Hereditary Methemoglobinemia due to Cytochrome b\textsubscript{5} Reductase Deficiency in Blood Cells Without Associated Neurologic and Mental Disorders

By Kiyoh Tanishima, Kazuo Tanimoto, Akio Tomoda, Kazuhiro Mawatari, Shigeru Matsukawa, Yoshimasa Yoneyama, Hitoshi Ohkuwa, and Eisuke Takazakura

Following the observation of two fraternal patients without neurologic symptoms, but with hereditary methemoglobinemia due to cytochrome b\textsubscript{5} reductase deficiency in erythrocytic and nonerythrocytic cells, a familial study of their paternal and maternal relatives was undertaken. Ferrihemoglobin reductase activities in erythrocytes from the two patients were found to be impaired, and cytochrome b\textsubscript{5} reductase activities in platelets and leukocytes were essentially absent. Any deficiencies of the enzyme activities seemed not to be found in nonhematopoietic cells. The enzyme activities in blood cells derived from the parents and some of their paternal and maternal family members showed levels intermediate between those of the patients and those of the normal control, which seemed to be heterozygous. The present cases did not belong to either the classic erythrocytic or the generalized type, and their enzyme deficiency was found rather to be restricted to their blood cells and not associated with neurologic and mental disorders. A necessity of diagnosis by tissues other than blood cells is discussed for a severe form of generalized-type hereditary methemoglobinemia with associated neurologic and mental disorders.

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METHEMOGLOBIN REDUCTASE DEFICIENCY

is an autosomal recessive trait with homozygotes completely lacking cytochrome b\textsubscript{5} reductase activity (NADH: ferricytochrome b\textsubscript{5} oxidoreductase, EC 1.6.2.2.); the disorder has also been described as methemoglobin reductase, NADH-diaphorase, or ferrihemoglobin reductase deficiency. Since Gibson and Scott and Griffith found hereditary deficiencies of the enzyme catalyzing reduction of methemoglobin in erythrocytes, a number of cases have been reported in several countries.

Among the reported cases, two types of the disease have been described:\textsuperscript{4} Type I is a benign form, in which methylene blue and sometimes ascorbic acid or riboflavin are used to control methemoglobinemia, but methylene blue is far more effective than either ascorbic acid or riboflavin. Most patients with this disease belong to this type, and the enzyme defect is restricted to erythrocytes. Type II is a severe form in which methemoglobinemia is associated with a progressive encephalopathy. This type is relatively uncommon and characterized by a generalized deficiency of cytochrome b\textsubscript{5} reductase in erythrocytes, leukocytes, and platelets as well as other tissues. In 1978, however, Arnold et al.\textsuperscript{5} reported another type of deficiency. This type III form was also due to cytochrome b\textsubscript{5} reductase deficiency in erythrocytes and leukocytes, but here methemoglobinemia was not associated with neurologic symptoms. They reported the case in the 17th Congress of the International Society of Hematology, but the paper has not been published, and the details are unknown. The present report describes a Japanese family with this type III cytochrome b\textsubscript{5} reductase deficiency.

MATERIALS AND METHODS

Heparinized venous blood specimens were obtained from two fraternal patients, their immediate relatives, and 11 healthy volunteers (one male and ten females, aged 19 to 20). Platelet-rich plasma was prepared by centrifugation at 120 g for ten minutes at room temperature. Subsequent collections of platelets, separation of leukocytes, and extraction of the enzymes were carried out according to the method previously reported, except in the use of Triton X-100 (Wako Pure Chemical Industries Ltd, Tokyo, Japan) (final concentration, 0.1%) as a detergent.

Diaphorase activity and cytochrome b\textsubscript{5} reductase activity were each assayed by the method of Scott\textsuperscript{2} and that of Kitao et al\textsuperscript{11} using 2,6-dichlorophenol-indophenol and cytochrome b\textsubscript{5} prepared from rabbit liver as substrate, respectively. Ferrihemoglobin reductase activity was measured by the method of Heglath et al.\textsuperscript{12} using a semiphysiologic electron acceptor, ferrocyanide–methemoglobin complex. Lactate dehydrogenase activity was measured using commercial reagents (LDH-UV Test, Wako Pure Chemical Industries Ltd, Tokyo, Japan) according to the manufacturer's instructions. Assay temperature was 30 °C during the assessment of all enzyme activity measurements using a Cary 17 D spectrophotometer (Varian Instrument Div, Calif). Glucose-6-phosphate dehydrogenase activity was measured by the method of Noble and Tanaka.\textsuperscript{13}

Hemoglobin contents were determined using commercial reagents (Hemoglobin Test, Wako), according to the method recommended by the International Committee for Standardization in Hematology. Protein concentration was determined by the method of Lowry et al.\textsuperscript{14} using bovine serum albumin as a standard.

Isoelectrofocusing of methemoglobin reductase and enzyme activity staining were both carried out according to the methods of Kaplan and Beutler\textsuperscript{15} and Yubisui and Takeshita\textsuperscript{16} with minor modifications. The erythrocytic hemolysates were applied to an Amphotile PAG plate (pH 3.5 to 9.5, LKB), and isoelectrofocusing electrophoresis was performed at 4 °C. Staining for the enzyme activity after electrophoresis was performed on NADH-diaphorase and on NADH-cytochrome b\textsubscript{5} reductase using staining solutions containing 0.1 mol/L Tris-HCl buffer (pH 8.0), 1 mmol/L NADH, 0.1 mmol/L 2,6-dichlorophenol-indophenol or 10 μ mol/L cytochrome b\textsubscript{5} purified from rabbit liver microsomes, and 1.2 mmol/L MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide).

CASE REPORT

Patient W.N., an 18-year-old Japanese male, was admitted to our hospital for further detailed investigation of cyanosis. At birth, he...
was noted to have moderate cyanosis without any other abnormalities and was diagnosed as having methemoglobinemia. His older brother was also confirmed to have cyanosis. At the present investigation, cyanosis of lips, nail beds, and cheeks was noted. No obvious cardiovascular or pulmonary cause for this clinical feature could be found. Hematologic investigations showed hemoglobin 15.6 g/dL, RBC 5.09 x 10^{12}/L, WBC 4.9 x 10^9/L, reticulocytes 3.2%, and methemoglobin 24.0%. Heinz bodies were negative. Patient M.N., aged 20, the older brother of W.N., was found to be healthy except for a gray cyanosis of his nail beds. At the request of his parents, a detailed clinical examination was performed. Hematologic investigations showed hemoglobin 15.3 g/dL, RBC 5.09 x 10^{12}/L, WBC 6.0 x 10^9/L, reticulocytes 5.3%, and methemoglobin 25.3% to 26.7%. Heinz bodies were negative. No cardiovascular or pulmonary causes could be found.

Although both patients had reticulocytosis, serum levels of bilirubin, lactate dehydrogenase, and haptoglobin were normal, and anemia, jaundice, and splenomegaly have not been observed. Unrecognized hemolysis or hidden bleeding may occur and be compensated, but we could not perform the examinations to find it. Mental and neurologic evaluations revealed no abnormalities. Both were educated to the senior high school level. Patient M.N. is a cook at a restaurant, and patient W.N. is his assistant. It is said that the maternal grandfather and the paternal grandmother of the patients were consanguineous, but it was not evident how the grandparents were related.

RESULTS

Methemoglobin reductase activities. The erythrocytic cytochrome b5 reductase activities (measured as ferrihemoglobin reductase by Hegesh et al13) of the two fraternal patients were undetectable in contrast to control values, and the enzyme activities of their parents' erythrocytes were intermediate between those of the patients and those of the control and were classified as heterozygous (Table 1). Most members of both the paternal and the maternal families showed intermediate values in erythrocytic methemoglobin reductase activity. Figure 1 depicts the results of this familial study.

The lymphocytic, granulocytic, and platelet cytochrome b5 reductase activities of the patients were also essentially absent. In addition, activities of the father and mother were lower compared with control values, whereas platelet and lymphocytic enzyme activities of all relatives whose erythrocytic enzyme activities showed intermediate values were essentially at the same intermediate levels as found in their erythrocytes (Table 2). The lymphocytic enzyme activity in some cases and the granulocytic enzyme activity in all cases could not be determined because of miscarriage of the cell preparations. Glucose-6-phosphate dehydrogenase activities in erythrocytes and lactate dehydrogenase activities in platelets and leukocytes were at almost equal levels in all blood specimens examined, as compared with normal controls.

Isoelectrofocusing of the erythrocytic hemolysate. Figure 2 shows the result of the methemoglobin reductase activity staining of the gels after isoelectrofocusing of the erythrocytic hemolysates from the patients and their parents. It is evident that the blood specimens of both fraternal patients lack the activity staining bands representing cytochrome b5 reductase. Regarding NADH-diaphorase, only the staining bands corresponding to cytochrome b5 reductase activity were defective in both patients.

DISCUSSION

Hereditary methemoglobinemia is due either to methemoglobin reductase (cytochrome b5 reductase) deficiency or to the presence of an abnormal hemoglobin, Hb-M. The present patients were confirmed not to have Hb-M by isoelectrofocusing electrophoresis on a PAG plate and by the absorption spectral analysis.

Results from measuring the enzyme activities in erythrocytes showed apparent autosomal recessive transmission; both fraternal patients were homozygous, and their parents and some other relatives seemed to be heterozygous. The homozygous patient, W.N., has been healthy during his growth and presented nothing other than a slate-gray cyanosis as an adolescent during a medical examination. Because no neurologic or mental abnormalities were found, he seemed to present with hereditary methemoglobinemia of the type I category, as is the case with his older brother.

It is well documented that most cases of hereditary methemoglobinemia belong to type I, a benign form, but a small number of cases belong to type II, a severe form. Recently, therefore, the estimation of enzyme activity in

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#### Table 1. Methemoglobin Reductase Activities in Erythrocytic Hemolysates From Two Fraternal Patients With Hereditary Methemoglobinemia and Their Familial Relatives

<table>
<thead>
<tr>
<th></th>
<th>Ferrihemoglobin Reductase Activity</th>
<th>Diaphorase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Father</td>
<td>1.6</td>
<td>1.4</td>
</tr>
<tr>
<td>Mother</td>
<td>2.1</td>
<td>1.5</td>
</tr>
<tr>
<td>Patient M.N.</td>
<td>0.0</td>
<td>0.7</td>
</tr>
<tr>
<td>Patient W.N.</td>
<td>0.0</td>
<td>0.8</td>
</tr>
<tr>
<td>Paternal family</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grandmother</td>
<td>1.1</td>
<td>0.4</td>
</tr>
<tr>
<td>Uncle</td>
<td>2.0</td>
<td>0.9</td>
</tr>
<tr>
<td>Maternal family</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grandfather</td>
<td>2.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Grandmother</td>
<td>4.1</td>
<td>2.6</td>
</tr>
<tr>
<td>Uncle</td>
<td>1.9</td>
<td>1.3</td>
</tr>
<tr>
<td>Aunt 1</td>
<td>4.5</td>
<td>2.0</td>
</tr>
<tr>
<td>Aunt 2</td>
<td>2.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Normal control</td>
<td>3.0</td>
<td>2.9</td>
</tr>
<tr>
<td>Normal range</td>
<td>4.1 ± 1.1</td>
<td>2.9 ± 0.8</td>
</tr>
<tr>
<td>(sample size)</td>
<td>(14)</td>
<td>(14)</td>
</tr>
</tbody>
</table>

Enzyme activity is given in nmol/min/mg hemoglobin.
leukocytes or platelets has been considered important in rapid diagnostic disease typing\textsuperscript{37} because the enzyme deficiency associated with type II also accompanies progressive neurologic and mental disorders. This deficiency appears not only in erythrocytes, but also in leukocytes and platelets. Vives-Corrons et al\textsuperscript{19} also reported that leukocytes were found to be enzyme deficient in several cases of methemoglobinemia with neurologic involvement, in contrast to patients with the benign type of congenital methemoglobinemia in which only erythrocytes are found to be defective in enzyme activity.

The present patients were apparently defective in enzyme activities both in erythrocytic and in nonerythrocytic cells, but this deficiency was not associated with any mental or neurologic disorders. Concerning nonhematopoietic cells, deficiencies of the enzyme activities seemed not to be found from the study of hair root and buccal cells by means of agar plate staining of the enzyme activities (the plate included the substrate mixture for activity measurement in the text, at the equal final concentrations. Controls included the same mixture without substrate cytochrome b$_5$). They were thus relegated to the type III category of Arnold et al.\textsuperscript{8}

This fact presents important problems because determination of the enzymatic activities in leukocytes or platelets is insufficient to estimate the generalized deficiency of the enzyme in hereditary methemoglobinemia, although it has been considered a valuable diagnostic indicator for the generalized type.\textsuperscript{9,17-20} Type III, an erythrocytic and non-erythrocytic cell type, is probably different from the two other types; in this third type, the enzyme deficiency seems to be restricted to erythrocytes, platelets, and leukocytes and is not found in other tissues, such as brain and muscle. From the results of Tables 1 and 2, this third form of cytochrome b$_5$ reductase deficiency appears to be an autosomal recessive, although Arnold et al\textsuperscript{8} reported that they found a pronounced deficiency of the enzyme in the father's and the two affected sons' leukocytes, whereas the enzyme activity was normal in the mother's leukocytes.

The reason the enzyme deficiency is restricted to erythrocytic cells, or to erythrocytic and nonerythrocytic cells is unclear. Kaplan et al\textsuperscript{21} suggested that the enzyme deficiency was restricted to the erythrocyte-soluble cytochrome b$_5$ reductase in the type I deficiency, whereas in the type II form, the enzyme defect was generalized to all tissues, involving both soluble and the microsomal forms of cytochrome b$_5$ reductase. Different mutations occurring at the same locus may account for this heterogeneity. However, if the microsomal forms of cytochrome b$_5$ reductase are identical in leukocytes, platelets, nervous tissues, and other tissues, the enzyme deficiency might be found in all tissues, including the brain and the nervous systems. The present patients, however, do not display any mental or neurologic symptoms. It has recently been considered that the mental and neurologic disorder observed in type II deficiency may be due to a defective metabolism of lipids in the nervous tissue, resulting from a defect in the desaturation of fatty acids by the cytochrome b$_5$ system.\textsuperscript{18,22-24} The present patients thus
may not be relegated to the type II category and their nervous systems appear to be normal.

From a practical point of view, it is important to establish the homozygous carriers in this inheritance who possibly exhibit a generalized deficiency of the enzyme from the infantile stage. Junien et al. reported on a prenatal diagnosis of congenital enzymopenic methemoglobinemia with mental retardation by analyzing the amniotic cells of two fetuses at risk for generalized cytochrome b5 reductase deficiency. Prospective studies of such prenatal diagnosis are expected to prevent neurologic or mental damage in carriers at risk for generalized cytochrome b5 reductase deficiency.

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