Alteration of NADH-diaphorase and cytochrome $b_5$ reductase activities of erythrocytes, platelets, and leucocytes in hereditary methaemoglobinaemia with and without mental retardation

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SUMMARY NADH-diaphorase and cytochrome $b_5$ reductase activities of platelets and leucocytes, as well as erythrocytes, were found to be deficient in a patient with hereditary methaemglobinaemia associated with moderate mental retardation and non-progressive neurological disturbance, in which hyperactive reflexes and involuntary movements were notable. In another methaemoglobinaemic patient with no mental or neurological abnormalities, these enzyme activities were defective in erythrocytes but normal in platelets and leucocytes. The first case was a generalised cytochrome $b_5$ reductase deficiency with non-progressive encephalopathy. It is suggested that the detection of cytochrome $b_5$ reductase activity in platelets, in addition to that in leucocytes, is useful for the assessment of a generalised enzyme defect. Genetic involvement of the present cases is discussed in association with the diaphorase gene loci in humans.

Hereditary methaemoglobinaemia is a recessive inborn error of metabolism which causes an increased methaemoglobin concentration in erythrocytes as a result of a deficiency in the ability to reduce methaemoglobin to haemoglobin. Scott demonstrated in erythrocytes of Alaskan Eskimos and Indians that the patients were deficient in red cell NADH-diaphorase activity. Recently, reports have indicated that the major pathway of methaemoglobin reduction is associated with cytochrome $b_5$ reductase and cytochrome $b_5$, and hereditary methaemoglobinaemia has been attributed to the deficiency of cytochrome $b_5$ reductase in erythrocytes. The sole clinical manifestation of this disease has been regarded as cyanosis. However, there have been several reports of methaemoglobinaemia associated with mental deficiency and neurological abnormalities. Kaplan and his associates established the relationship between a severe neurological syndrome and a generalised enzyme deficiency in a number of cases. They divided recessive hereditary methaemoglobinaemia into two types: type I (erythrocyte type) in which the cytochrome $b_5$ reductase deficiency is restricted to red cells and cyanosis is the sole symptom, and type II (generalised type) in which the enzyme defect is present in all tissues, and the methaemoglobinaemia is associated with a progressive encephalopathy causing death within a few years of birth. However, Arnold et al refer to a family with methaemoglobinaemia in which enzyme deficiency was found in both erythrocytes and leucocytes, but without mental and neurological symptoms. To discover the relationship between encephalopathy and the enzyme defect in the tissues, we determined the NADH-diaphorase and cytochrome $b_5$ reductase activities in the platelets and leucocytes of two methaemoglobinemic patients, one with and one without mental retardation. The patient who had a less severe form of neurological syndrome showed the enzyme defect in leucocytes and platelets.
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Materials and methods

NADH was obtained from Boeringer, Mannheim, West Germany. Dextran D-520 was the product of Sigma Chemical Co, St Louis, Missouri, USA. Cytochrome b₅ was prepared by the method of Passon et al.¹⁴

Subjects and collection of blood

Normal blood samples were obtained from healthy volunteer students and university officials. Blood specimens obtained from normal subjects and methaemoglobinaemic patients were anticoagulated with EDTA, K₂ (2·7 mg/ml of blood).

Collection of platelets and leucocytes

Platelets were collected from the platelet-rich supernatant which was obtained by centrifugation of the blood at 120 g for 10 minutes at room temperature. All subsequent procedures were performed at 4°C to 10°C in plastic equipment. Leucocytes were collected according to the modified method of Böyum.¹⁵ The bottom fraction containing erythrocytes and leucocytes was washed with saline, and fractionated by centrifugation at 600 g for 10 minutes in an equal volume of 2 g/100 ml dextran D-520 in saline. Leucocytes in the supernatant were collected and washed twice with saline. The leucocyte fraction was treated with 0·28 g/100 ml NaCl to haemolyse contaminated erythrocytes.

Preparation of enzyme extracts

The pellets of platelets and leucocytes were treated by repeated freezing and thawing in 3 vol 0·1 mol/l Tris-HCl buffer, pH 7·4, and homogenised in a Teflon homogeniser. The homogenates were centrifuged for 10 minutes at 600 g and the supernatant was used for the enzyme assay. Protein was determined according to the method of Lowry et al.¹⁶ using bovine serum albumin as a standard.

Assay procedures

Preparation of haemolsate and assay of NADH-diaphorase activity was performed essentially according to the method of Scott.¹ The assay system contained 50 μmol Tris-HCl, pH 7·4, 0·2 μmol 2,6-dichlorophenolindophenol, 1 μmol EDTA, 0·4 μmol NADH, and haemolsate or enzyme extract in a total volume of 1·5 ml. The reaction was started by the addition of NADH. The decrease in absorbance at 600 nm owing to reduction of the dye was measured with a Hitachi model 124 recording spectrophotometer at 23°C. A millimolar extinction coefficient of 20·1 for the oxidised 2,6-dichlorophenolindophenol was used in the calculation.

The cytochrome b₅ reductase activity was assayed by the method of Omura and Takesue¹⁷ with some modifications. The assay mixture contained 20 μmol phosphate buffer, pH 7·0, 1 μmol EDTA, 4 nmol cytochrome b₅, 0·4 μmol NADH, and haemolsate or enzyme extract in a total volume of 1·5 ml. The reaction procedure was the same as that in the NADH-diaphorase assay. A millimolar extinction coefficient of 100 at 424 nm was used for the difference between the oxidised and reduced form of cytochrome b₅.

Electrophoresis

Starch gel electrophoresis was performed as described by Kaplan and Beutler,¹⁸ and acrylamide disc gel electrophoresis was carried out according to Kitao et al.⁴

Patients

Case 1

The proband, a 13-year-old Japanese boy was described by Nishina et al.¹⁹ when he was 2 years 4 months old. The noticeable abnormal features of his early life were congenital cyanosis and retardation of mental and physical development. He was born after an uncomplicated 36-week pregnancy, weighing 2050 g. Cyanosis was noted at birth, and because of low body weight and cyanosis, he was in an incubator for 2 months. At the time of first description he had 3·4 g methaemoglobin per 100 ml haemoglobin (24% of total). Treatment with methylene-blue (40 mg/day) was effective and cyanosis faded but it reappeared when medication was stopped. Methaemoglobin was decreased to 10% by the administration of ascorbic acid (1000 mg/day). Neurological disturbances were first noted at 6 months of age by the instability of his head. Mental development continued to be moderately retarded. Developmental assessment and intelligence tests were carried out four times between the ages of 5 and 13. IQ was estimated at between 25 and 35, with no significant change over this period. On examination the lips were slightly violet. He was friendly and tried to co-operate with the tester but was restless and unable to concentrate. He was a slender boy weighing 34 kg and measuring 141 cm, moderately retarded in development, especially in weight. On neurological testing moderate choreoathetoid movements throughout the body were observed, resulting in facial grimacing and disturbance of speech and of gait, which appeared bizarre and theatrical. Co-ordination, diadochokinesia, finger to nose test, and finger to finger test were clumsy. The involuntary movements have not progressed for several years. Among other neurological findings, the convergence reflex was poor and double vision was noted, but no strabismus was
found. The bilateral deep tendon reflexes were symmetrically hyperactive and there were no extensor planter responses. Slight scoliosis and torticollis were observed. No sign of paralysis was found. An EEG and a computerised tomogram showed normal findings.

The pedigree from Nishina et al\textsuperscript{19} is outlined in the figure. The proband’s parents are first cousins. No other members of his family were known to have unexplained cyanosis or mental retardation. However, examination of NADH-diaphorase activities of nine members of the family showed the enzyme activities of both the proband’s parents (III.3 and III.7) to be approximately half of normal. Both the paternal and maternal grandmothers were heterozygotes (II.2 and II.4). Of two sibs of the father, the sister was heterozygous (II.2). The mother has five sibs: one sister (III.8) was heterozygous and one brother and another sister were normal (III.4 and III.6). The patient has a brother (IV.1) who was heterozygous.

**CASE 2**

This patient was chosen as an example of hereditary methaemoglobinaemia without mental retardation. A 31-year-old Japanese male was first examined at Fujita Gakuen University Hospital at the age of 28 because of persistent cyanosis.\textsuperscript{20} Methaemoglobin content was 16% of total haemoglobin and the haemolysate showed maximum absorbance at 630 nm (pH 6.3). Starch block electrophoresis revealed normal haemoglobin A. NADH-diaphorase activity in erythrocytes was 35% of normal. Cyanosis of the lips, oral mucosa, and nail beds was noted on examination. The heart and lungs were clinically normal. Apart from the cyanosis, physical examination including neurological evaluation revealed no abnormalities. No mental retardation was noted. The patient’s parents were first cousins and his father had died of stomach cancer. Of six relatives, his elder sister was cyanotic and enzyme defect was demonstrated. Two (one male, one female) of four cousins, whose parents were consanguineous, were cyanotic.

**Results**

The NADH-diaphorase activities in erythrocytes of both case 1 and case 2 were 30 to 40% of those of normal subjects, as shown in table 1. The cytochrome $b_5$ reductase activities were essentially zero, which indicated that the enzyme was defective in the erythrocytes of both the patients. The methaemoglobin content of case 1 was 24% and that of case 2 26·3% of total haemoglobin. The amount of methaemoglobin was decreased by treatment with ascorbic acid (500 to 1000 mg/day) to 13% and 16%, respectively. No biochemical differences in the erythrocytes were shown between the patients with and without mental and neurological disturbances.

**TABLE 1**  \textit{NADH-diaphorase and cytochrome $b_5$ reductase activities in erythrocytes from methaemoglobinaemic patients and normal subjects}

<table>
<thead>
<tr>
<th>Enzyme activities (nmol/min/mg Hb)</th>
<th>Case 1</th>
<th>Case 2</th>
<th>Normal*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH-diaphorase</td>
<td>0·14</td>
<td>0·19</td>
<td>0·45±0·19</td>
</tr>
<tr>
<td>Cytochrome $b_5$ reductase</td>
<td>0</td>
<td>0</td>
<td>0·41±0·12</td>
</tr>
</tbody>
</table>

*Mean ± SD of 14 experiments.

**TABLE 2**  \textit{NADH-diaphorase and cytochrome $b_5$ reductase activities in platelets and leucocytes from methaemoglobinaemic patients and normal subjects}

<table>
<thead>
<tr>
<th>Enzyme activities (nmol/min/mg protein)</th>
<th>Case 1</th>
<th>Case 2</th>
<th>Normal*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH-diaphorase activity</td>
<td>1·99</td>
<td>19·6</td>
<td>23·5±4·1</td>
</tr>
<tr>
<td>Leucocytes</td>
<td>5·03</td>
<td>35·1</td>
<td>39·8±8·7</td>
</tr>
<tr>
<td>Cytochrome $b_5$ reductase activity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelets</td>
<td>0·85</td>
<td>7·2</td>
<td>7·6±1·4</td>
</tr>
<tr>
<td>Leucocytes</td>
<td>0·87</td>
<td>8·7</td>
<td>10·3±2·3</td>
</tr>
</tbody>
</table>

*Mean ± SD of 6 experiments.
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order to see whether any electrophoretic variant was detectable, haemolysates from normal blood and from the patients were freed from haemoglobin by treatment with CM cellulose chromatography according to Kitao et al.4 and concentrated by ultrafiltration. The haemoglobin-free extracts from normal blood showed one band of NADH-diaphorase and those from both the patients revealed a faint band showing the same mobility as normal diaphorase, both on starch gel and acrylamide gel electrophoresis. The NADH-diaphorase and cytochrome $b_5$ reductase activities in platelets and leucocytes were determined (table 2). While NADH-diaphorase activities for case 1 decreased to approximately 8 and 13% of normal, respectively, those of case 2 were within normal limits, as shown in table 2. These results were further confirmed by the determination of cytochrome $b_5$ reductase activity. The enzyme activity in the platelets and leucocytes of the patient with mental retardation was diminished to about 10% of normal. The activities in the cells of case 2 were within normal limits. These results indicate that the use of platelets, as well as leucocytes, can be a satisfactory means of determining the deficiency of cytochrome $b_5$ reductase in non-erythroid cells.

Discussion

The NADH-diaphorase activity in erythrocytes of both patients was high (table 1) in comparison with the levels reported by Kaplan et al.5–12 While Scott’s method, used in this report, employs 2,6-dichlorophenolindophenol as an electron acceptor,1 Kaplan measured ferrihaemoglobin reductase activity by the method of Hegesh et al.21 Many enzymes are known to act as diaphorases and the specificity of diaphorase for dichlorophenolindophenol might be different from that for ferrihaemoglobin reductase. NADH-cytochrome $b_5$ reductase is considered to be the major enzyme involved in the reduction of methaemoglobin in vivo5 and the defect of this enzyme resulted in hereditary methaemoglobinemia.4 The activity of this enzyme was determined using cytochrome $b_5$ as an electron acceptor, and it was virtually undetectable in the erythrocytes of both the patients (table 1).

Eletrophoresis of haemolysates from both the patients revealed a weak diaphorase band when concentrated and its mobility was the same as the usual major NADH-diaphorase. As cytochrome $b_5$ reductase was undetectable in red cells from these patients, the residual reductase may be diaphorase I as defined by DEAE cellulose chromatography, which is not defective in hereditary methaemoglobininaemia.4 Diaphorase I and II (cytochrome $b_5$ reductase) showed the same electrophoretic mobility on starch gel and disc gel electrophoresis. In contrast, residual activity of cytochrome $b_5$ reductase was demonstrated in platelets and leucocytes of both the patients. It would be of value to study the enzymatic and electrophoretic characteristics of cytochrome $b_5$ reductase of the patients, but the enzyme is insoluble and obtaining a large enough sample is difficult.

There have been several reports of hereditary methaemoglobininaemia accompanied by mental retardation and neurological disturbances which have ranged from mild to severe. Worster-Drought et al22 reported three methaemoglobinemic relatives with mild mental retardation. Both the cases of Dine23 and Lees and Jolly24 were young babies of less than 16 months who showed signs of poor development and moderate mental retardation. Fialkow et al8 reported three members with methaemoglobinemia in a family. Though two of them showed moderate mental retardation, a younger child of 3 years was mentally normal and showed only cyanosis. These workers suggested a complex aetiology based on the interaction with diaphorase deficiency of hypoxia, neonatal jaundice, and/or the presence of the genes predisposing to mental retardation. They also reviewed 58 biochemically proved cases of hereditary methaemoglobinemia in 35 families.8 Mental retardation was demonstrated in nine patients of five families, which is more frequent than would have been expected by chance. The patients which were reported by Jaffé et al25 and Heusden et al7 showed methaemoglobinemia and severe encephalopathy and died at a young age. The relationship between methaemoglobinemia and progressive neurological involvement was first clarified by Kaplan and his colleagues.8–12 The patient, whose cyanosis was associated with a progressive neuropathy, showed deficiency of NADH-cytochrome $b_5$ reductase in leucocytes and other tissues (type II),8 and the symptomatology of this type of disease has been established.12 In 24 cases of methaemoglobinemia, eight cases showed the enzyme defect in leucocytes and cyanosis was associated with the following common features: severe mental retardation, opisthotonus, microcephaly, and bilateral athetosis.12 Three patients with generalised enzyme defect were also reported by Arnold et al13 but these patients showed neither neurological symptoms nor mental retardation. Case 1 reported here showed mild and non-progressive mental and neurological disturbances. It might be possible to classify the generalised enzyme defect into three groups on the basis of severity of the neurological signs: (1) severe and progressive,
(2) mild and non-progressive, (3) cases without neurological signs. In the progressive case, the enzyme defect might be continuously affecting the metabolism (probably of fatty acids) and making the neurological symptoms worse until death occurs at a young age. However, in our non-progressive case the neurological and mental disturbances might have been established at some time in the fetal or newborn period and residual enzyme activity might compensate and prevent further chemical deterioration. Red cell cytochrome b₅ reductase activity is low in the newborn period.66 If cytochrome b₅ reductase in the erythrocytes and in the tissue cells is a common gene product, the tissue cell enzyme may also be low in the fetal or newborn periods. When the enzyme defect in tissue cells does not cause any disorder at any period because of sufficient residual activity or for some other unknown reasons, the proband might be neurologically normal, as Arnold et al13 observed.

Fisher et al27 reported that human diaphorase is controlled by at least three gene loci: DIA₁, DIA₂, and DIA₃. It is suggested that the 'red cell' and main 'tissue' forms of NADH-dependent diaphorase are coded for by the same gene locus, DIA₁. The NADPH-diaphorase and the 'sperm' diaphorase are controlled by DIA₂ and DIA₃, respectively. Recently, a fourth enzyme (diaphorase 4) which is dependent on FAD has been reported.28 The physiological role of these diaphorases, apart from the DIA₁ product, is not yet clear. The 'red cell' type diaphorase has been demonstrated in a variety of human tissues on the basis of immunological and electrophoretic studies,27 and it has been indicated that this enzyme corresponds to NADH-cytochrome b₅ reductase.27-30 If the 'tissue' and 'red cell' diaphorases are the products of the same gene locus,27 a possible explanation for the occurrence of type 1 and 2 methaemoglobinemia might be as follows. Type 1 methaemoglobinemia could result from a mutation producing an unstable enzyme leading to loss of enzyme activity in erythrocytes, while synthesis of enzyme protein might compensate in other tissues. In type 2, the mutation might cause underproduction of the enzyme or production of an inactive enzyme. Alternatively, 'red cell' type methaemoglobinemia might occur when the mutation causes impairment of the release of soluble enzyme from a microsomal precursor in the process of red cell maturation.

Cytochrome b₅ reductase plays a role in the reduction of methaemoglobin in mammalian erythrocytes. In tissue cells, however, the microsomal cytochrome b₅ reductase system has been thought to participate in fatty acid desaturation.31 Moreover, recent reports have suggested that the system may play a more extensive role in the metabolic pathways. These include eicosatrienoyl lecithin desaturation,32 cholesterol dehydrogenation, plasmalogen biosynthesis, and fatty acid elongation.33 Though the direct cause of mental and neurological disorder is not elucidated, impaired fatty acid metabolism is quite possible and this should be clarified in future studies.

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References
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