Methylmalonyl-CoA Mutase Activity of Leukocytes in Variants and Heterozygotes of Methylmalonic Acidemia

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Methylmalonic acidemia is a rare inherited disorder. Clinically it is characterized by metabolic acidosis, vomiting, lethargy, muscular hypotonia, and failure to thrive (Oberholzer et al. 1967; Lindblad et al. 1968; Morrow et al. 1969a). Biochemically the sites of the metabolic block have been reported to be located at one of following four steps; 1) methylmalonyl-CoA mutase (Morrow et al. 1969b), 2) methylmalonyl-CoA racemase (Kang et al. 1972), 3) formation of 5′-deoxyadenosylocobalamin (DBCC) (Rosenberg et al. 1968, 1969), and 4) formation of both the methylcobalamin and DBCC (Mudd et al. 1969).

In the first case of methylmalonic acidemia, Oberholzer et al. (1967) demonstrated a possible defect in the methylmalonyl-CoA mutase activity of leukocytes on the basis that there was a marked deficiency in the conversion of propionyl-CoA to succinic acid when the white cell homogenates were incubated with propionyl-CoA and 14C-labeled sodium carbonate.

Rosenberg et al. (1968), and Marrow and Barness (1969) reported a defective formation of 14CO₂ from propionate-3-14C by leukocytes from a patient with methylmalonic acidemia.

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In 1972 Goodey and Gompertz investigated the mutase assay using leukocytes from patients with vitamin B$_{12}$ responsive or unresponsive type of methylmalonic acidemia and reported that the mutase activity of leukocytes was not detected in normal subjects as well as in the patients when DBCC was not added to the assay media.

In 1976 Giorgio et al. made an investigation on the mutase activity of leukocytes from normal subjects and patients with methylmalonic acidemia, and reported that there was no increase in the enzyme activity over a basal activity in leukocytes from normal persons even when DBCC was added to the assay media.

A defective production of $^{14}$CO$_2$ from propionate-$3^{-14}$C by platelets was also observed in a patient with methylmalonic acidemia by Hsia et al. (1970).

In 1969 Morrow et al. (1969b) measured the methylmalonyl-CoA mutase activity using liver biopsy specimens from patients with vitamin B$_{12}$ responsive or unresponsive type of methylmalonic acidemia, and reported that in the case with vitamin B$_{12}$ responsive type the mutase activity was restored to the normal when DBCC was added to the assay media.

In 1969 Rosenberg et al. demonstrated that there was a defect in conversion of DL-$[3H$-methyl$]$methylmalonyl-CoA to succinyl-CoA in fibroblasts from patients with methylmalonic acidemia.

The present paper of ours reports an assay method for the mutase activity of leukocytes obtained from 3 ml of blood and its clinical usefulness for the diagnosis of vitamin B$_{12}$ responsive and unresponsive types of methylmalonic acidemia and for the detection of heterozygotes with vitamin B$_{12}$ unresponsive type of this disorder.

**METHODS**

Assay of methylmalonyl-CoA mutase of leukocytes

Leukocytes were prepared from 3 ml of fresh blood in the same way as described by Rosenberg et al. (1968). The leukocyte pellet was so suspended in 250 µl of isotonic saline that 10$^7$ cells were contained. The suspension was subjected to rapid, alternating freeze-thawing twice in a dry ice-bath. Protein content was measured by the Lowry et al.'s method (1951). This cell lysate was used for the enzyme assay. All the procedures were done in the dark.

The enzyme assay was carried out according to the Morrow et al.'s method (1969b), and measurement of $^{14}$C-succinate was performed according to the Cardinale et al.'s method (1969).

The incubation mixture with a final volume of 0.1 ml contained: 10 µmoles of Tris-HCl buffer (pH 7.4), 7.4 nmoles of DL-$[14$C-methyl$]$methylmalonyl-CoA (specific activity 5.367 mCi/m mole, New England Nuclear, USA) and the leukocyte lysate containing 0.1-0.3 mg of protein. DBCC (Yamanouchi Pharm. Co., Ltd., Tokyo, Japan) was added in a dose of 20 µg as indicated. Incubation was done at 37°C for 30 min in the dark, then the reaction was terminated by addition of cold distilled water to bring the volume to 1.5 ml, and the test tubes were placed in a boiling water bath for 4 min to hydrolyze succinyl-CoA formed. The mixture in the tube was centrifuged and the supernatant was transferred into another test tube, to which 400 mg of NaCl, 0.3 mg of succinic acid, 0.3 mg of methylmalonic acid, and two drops of 10 N H$_2$SO$_4$ were then added. The mixture was subjected to extraction with 4 ml of diethylether. The extraction was repeated 5 times. The com-
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Combined ether extracts were concentrated in a rotary evaporator, then subjected to chromatography using the Toyo-roshi No. 50, and 4N formic acid-isooamylalcohol as a developer. After being dried, spots of succinic acid stained with bromcresol green, were cut out and counted in a liquid scintillation counter (Intertechnique, Model SL. 30).

Boiled leukocyte lysate was subjected to the same procedures as described above as a blank.

Preliminary studies indicated that under these assay conditions succinate formation proceeded linearly up to 40 min and was proportional to the protein amounts in the range of 0.08 to 0.40 mg.

The methylmalonyl-CoA mutase activity of leukocytes was expressed as nmoles of $^{14}$C-succinate formed per mg protein per hr.

Methylmalonic acid in 24 hr urine specimens was estimated by the Frenkel and Kitchen's method (1975).

Case Report

Case 1. H.T., a 6-month-old boy was referred to the University Hospital because of frequent episodes of lethargy and muscular hypotonia.

The patient was born to healthy but consanguineous parents after an uneventful pregnancy and delivery, weighing 2,850 g. There were two healthy siblings in his family. He had been well until the age of 4 months, when vomiting, lethargy and dehydration developed after an acute upper respiratory infection. At that time a positive reaction to urinary acetone and large amounts of methylmalonic acid in urine (1,390 mg/24 hr) were found. Clinical symptoms and signs were improved by an intravenous fluid therapy including sodium bicarbonate and by feeding on a diet low in protein (approximately 2 g/kg/day).

On admission (when 6 months old) physical examination revealed a floppy and pale infant with apathetic facies. His body weight was 6,450 g (normal 7,300 g), height 62 cm (normal 65.2 cm), circumference of the head 43.5 cm (normal 43.1 cm), and that of the chest 39.0 cm (normal 42.0 cm). The liver was palpable four finger breadths below the right costal margin. He was markedly hypotonic, but other neurologic abnormalities were not found.

Laboratory examination revealed the serum level of vitamin B$_{12}$ of 438 pg/ml. The hemoglobin was 9.7 g/100 ml, the red blood cell count $3.17 \times 10^{6}$/mm$^3$, the hematocrit 26%, the white blood cell count 10,900 mm$^3$, and thrombocyte count $22 \times 10^{4}$/mm$^3$. No megaloblasts were found in bone marrow smears. Serum electrolyte concentrations were sodium 140, potassium 5.8, and chloride 96 mEq/liter. The plasma bicarbonate content was 16.3 mEq/liter. Fasting blood sugar was 78 mg/100 ml. Methylmalonic acid concentrations were 879 mg in 24 hr urine, 113 µg/ml in serum, and 82.1 µg/ml in cerebrospinal fluid. There was no changes in urinary methymalonic acid after intramuscular injections of vitamin B$_{12}$, 1 mg daily, for successive five days. Serum glycine level was 9.5 mg/100 ml (normal range from 0.77 to 2.90 mg/100 ml), and methionine 0.34 mg/100 ml (normal range from 0.02 to 0.46 mg/100 ml). Ophthalmologic examination revealed no abnormalities. Routine urinalysis was negative and the cyanide-nitroprusside test was negative.
Case 2. H.A., a 4-month-old girl was admitted to the University Hospital because of frequent episodes of vomiting, anorexia and metabolic acidosis since 3 months of life. The patient was born to healthy, but consanguineous parents after an uneventful pregnancy and delivery, weighing 2,800 g. Her elder brother was healthy.

Episodes of metabolic acidosis with anorexia, vomiting and lethargy developed again at the age of 3 and 1/2 months, with recovery by an intravenous fluid therapy including sodium bicarbonate. During the acidotic episodes, hyperammonemia and neutropenia were observed.

On admission, when 4 months old, physical examination revealed a floppy female infant. The body weight was 6.0 kg (normal 6.4 kg), length 58.4 cm (normal 61.3 cm), circumference of the head 39.6 cm (normal 41.6 cm). The liver was palpable one finger breadth below the right costal margin, but the spleen not. There was generalized muscular hypotonia, but no other neurological abnormalities.

Laboratory findings: The red cell count was 5.65×10^6/mm³, hemoglobin 12.2 g/100 ml, white cell count 4,450/mm³, and platelet count 38×10⁴/mm³. No megaloblasts were found in bone marrow smears. Results of urinalysis were: pH 6.0, acetone (−), protein (−), sugar (−), and sediments within normal limits. The cyanide-nitroprusside reaction was negative. The serum total protein was 6.1 g/100ml and its distribution normal. The plasma bicarbonate content ranged from 10.2 to 21 mEq/liter. Fasting blood sugar was 92 mg/100 ml. In serum urea nitrogen was 8 mg/100 ml, and uric acid 1.1 mg/100 ml. Serum glycine level was 10.9 mg/100 ml and methionine 0.24 mg/100 ml. Urinary excretion of methylmalonic acid was 1,054 mg/24 hr, and not decreased by intramuscular injections of cyanocobalamin, 1 mg per day, for successive five days.

Case 3. A.M., a boy, was born on March 15, 1976, after an uneventful pregnancy and delivery, weighing 3,150 g. He was a first child of healthy unrelated parents.

When one month old, he suffered from urinary tract infection with metabolic acidosis, which was treated accordingly with a complete recovery.

At the age of two months, he sucked poorly, then tachypnea, lethargy, involuntary movements of extremities, and generalized convulsions developed. At that time serum glycine was 7.37 mg/100 ml, the plasma bicarbonate content was 5.3 mEq/liter, and there were neutropenia and failure to thrive. Clinical conditions were improved by an intravenous fluid therapy including sodium bicarbonate. About one week after the recovery, urinary excretion of methylmalonic acid was found to be 24.9 mg/24 hr. The urinary cyanide-nitroprusside reaction was negative.

Even though urinary excretion of methylmalonic acid was less than that in previously reported cases with methylmalonic acidemia, he was then treated with a diet low in valine, isoleucine, threonine and methionine (specially prepared by Yukizirushi Co. Ltd., Tokyo) and oral administration of cyanocobalamin, 1 mg
daily. These therapy was continued for following 5 weeks, during which period the patient was free from vomiting, lethargy, and convulsions.

At the end of five weeks on the therapy with the low protein diet and an oral administration of vitamin B₁₂, only the vitamin B₁₂ administration was discontinued. Three months later, he refused food, and showed involuntary movements of the head, vomiting, acidosis and no interest to his surroundings. At this time urinary excretion of methylmalonic acid was found to be 136 mg/24 hr and serum vitamin B₁₂ 1,226 pg/ml. An intravenous fluid therapy including sodium bicarbonate resulted in clinical improvement. At this stage blood samples were obtained for assay of leukocyte mutase activity.

Urinary excretion of methylmalonic acid after an oral valine load in Case 3 was examined in the following way: Twenty four-hr urine was collected before and after an oral load with L-valine (100 mg per kg body weight) and assayed for methylmalonic acid, with results of 9.3 mg and 143 mg/24 hr, respectively. After intramuscular injections of cyanocobalamin, 1 mg per day for successive 10 days, the oral valine loading was done again. In this case, urinary methylmalonic acid was found to be 23.8 mg and 15.1 mg/24 hr before and after the valine load, respectively, indicating that there was practically no difference between the two tests.

**SPECIAL STUDIES**

*Methylmalonyl-CoA mutase activity of leukocytes* (Table 1)

The methylmalonyl-CoA mutase activities of leukocytes from normal subjects aged from 4 months to 40 years were assayed and the values of 0.50±0.11 (ranging from 0.24 to 0.61) and 6.24±1.41 (ranging from 4.82 to 10.10) were obtained in terms of the formation of nmoles ¹⁴C-succinate per mg protein per hr without or with the in vitro addition of DBCC in the assay medium, respectively.

The methylmalonyl-CoA mutase activities of leukocytes, when measured without the in vitro addition of DBCC, were found to be one tenth or less of the normal in Cases 1, 2 and 3.

When the enzyme assay was carried out with the in vitro addition of DBCC, the mutase activity was restored to be within the normal range in only Case 3, but not in Cases 1 and 2, indicating that Case 3 was of the vitamin B₁₂ responsive type of methylmalonic acidemia.

The enzyme assay with the in vitro addition of DBCC revealed that the mutase activities of leukocytes from both parents of Cases 1 and 2 were approximately half the normal and that from the second elder brother of Case 1 was 26.4% of the normal value.

**DISCUSSION**

Clinical pictures of methylmalonic acidemia, including acidosis, vomiting, lethargy, muscular hypotonia and failure to thrive (Oberholzer et al. 1967; Lindblad et al. 1967; Morrow et al. 1969a) were observed in all the three cases of ours. But
it was noticed that in Case 3, the amount of methylmalonic acid excreted in urine (9.3-14.30 mg/24 hr) was less than those for the cases reported as methylmalonic acidemia except for the following five cases: Giorgio's two adult cases (1976) which were free from clinical findings except for those of diabetes mellitus and showed urinary methylmalonic acid of 115 mg or less/24 hr, and Garnica's three infants (1976) which showed intermittent acidosis and urinary excretion of methylmalonic acid of 145 mg or less/24 hr.

In our three cases with methylmalonic acidemia, Cases 1 and 2 showed no change in urinary excretion of methylmalonic acid after intramuscular injections of cyanocobalamin. However in Case 3, there was a marked decrease in urinary methylmalonic acid excretion following an oral load with L-valine when tested after pretreatment with intramuscular injections of cyanocobalamin, 1 mg per day for successive ten days.

The results of the assay for methylmalonyl-CoA mutase of leukocytes with the in vitro addition of DBCC revealed that methylmalonic acidemia in Cases 1 and 2 belonged to the B₁₂ unresponsive type and that in Case 3 to the B₁₂ responsive type. These results of ours were consistent with those of Morrow et al. (1969b), who showed that the methylmalonyl-CoA mutase activity of the liver from patients with the vitamin B₁₂ responsive methylmalonic acidemia was restored to the normal when the enzyme assay was done with the in vitro addition of DBCC.
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The similar tendency with respect to the enzyme activity was reported by Rosenberg et al. (1969) in fibroblasts cultured from the vitamin B\textsubscript{12} sensitive methylmalonic acidemia.

The failure to find a significant formation of 14C-succinate with DL-14C-methylmalonyl-CoA as substrate in Cases 1 and 2 implicated the methylmalonyl-CoA mutase activity as being at least one site of the defect, though the possibility that an impaired racemase activity might coexist was not thoroughly excluded.

The mutase activities of leukocytes from both parents of Cases 1 and 2 were found to be half the normal when the enzyme assay was carried out with the in vitro addition of DBCC. Moreover, there was no overlap in the mutase activity of leukocytes between obligate heterozygotes and normal subjects. The mode of transmittance was reported as an autosomal recessive for both the apoenzyme defect and the vitamin B\textsubscript{12} responsive variant (Morrow et al. 1969a). Thus the assay technique for the mutase activity of leukocytes described here could be of value for the detection of heterozygotes with the vitamin B\textsubscript{12} unresponsive type. The second elder brother of Case 1, who was clinically normal, showed a mutase activity 26.4% of the normal, suggesting that he was also a heterozygote.

Morrow (1974) described, however, that he failed to make differentiation between heterozygotes of methylmalonic acidemia and controls by the mutase assay of leukocytes or fibroblast. Discrepancy between Morrow’s results and ours remains unexplained because detailed procedures of assay for the mutase activity were not described in the Morrow’s report.

In our assay for the mutase activity, we employed 0.074 mM DL-methylmalonyl-CoA as the substrate and therefore the concentration of L-methylmalonyl-CoA should be considerably lower than the Km value which had been reported to be 0.24 mM for the mutase from sheep liver (Cannata et al. 1965). Consequently, the rate of formation of 14C-succinate from 14C-racemic methylmalonyl-CoA under our assay conditions would be influenced considerably by the racemase activity in the enzyme preparation used. In other words, it is expected that when our method of assay of the mutase activity was applied to leukocytes from patients with racemase defect, the mutase activity would appear to be less than the normal. These situations raise the question that both the parents of Case 1 and 2 might have been deficient in the racemase activity, for, as shown in Table 1, the “mutase” activity of leukocytes from both parents from Case 1 and 2 were found to be half the normal when DBCC was added to the assay medium. However, this does not appear to be the case because urinary excretion of methylmalonic acid in both the parents was found to be within normal limits.

References


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