Vitamin B12-Responsive Neonatal Megaloblastic Anemia and Homocystinuria With Associated Reduced Methionine Synthase Activity

By Lynnette J. Hallam, Margaret Sawyer, Arthur C.L. Clark, and Martin B. Van Der Weyden

We present findings on an infant with neonatal megaloblastic anemia, homocystinuria, and neurologic dysfunction that included developmental delay and tonic seizures. There was no methylmalonic aciduria. Cyanocobalamin therapy was accompanied by complete hematologic and neurologic recovery, diminished homocystine excretion, and subsequently normal neurologic development. Cultured fibroblasts and lymphoblasts showed a reduced methionine synthase activity and a growth requirement for methionine. Cobalamin incorporation by the patient's lymphoblasts was normal, but the proportion of cellular methylcobalamin in the patient's lymphoblasts and fibroblasts was markedly reduced and that of adenosylcobalamin normal. The reduced methionine synthase activity was independent of assay reducing (thiol) conditions, but normal levels of activity accompanied culture of the patient's lymphoblasts in medium with markedly increased cobalamin concentration. The characteristics of the reduced methionine synthase of our patient differ significantly from that of the previously described infant with cobalamin E disease and suggest that genetic heterogeneity may characterize this mutation.

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CASE REPORT

The patient, a Caucasian male infant, was referred at the age of 10 weeks for investigation of anemia, hypotonia, and developmental regression. He was the first born of unrelated parents who had had no fetal losses. He was born at 38 weeks gestation, birth weight 3,180 g, and was in good condition at birth. His parents stated that he was never a very responsive baby but fed successfully from the breast and smiled and responded to his parents at about six weeks. At that time he had a febrile illness with diarrhea and vomiting lasting about one week, after which he became unresponsive and unsmiling. On admission to the hospital at 10 weeks he was a pale, floppy, unresponsive infant, height 59 cm, weight 4,730 g, and head circumference 39.3 cm. The liver was palpable 3 cm below the costal margin, the spleen not palpable, and lymph nodes not enlarged. Despite his generalized hypotonia, deep tendon reflexes were present and symmetrical, and no other neurologic abnormalities were noted. However, during the subsequent three weeks he developed truncal spasticity with head retraction and spine extension when disturbed, and on the fifth hospital day he had a generalized seizure. Three further seizures followed until he was given phenobarbitone, after which these ceased. Investigations on admission revealed anemia with hemoglobin 7.7 g/dL, hematocrit 21%, MCV 101 fl, MCH 35.5 pg, MCHC 53.2 g/dL, reticulocytes 3.5%, obvious anisoctyosis, macroctytosis and poikilocytosis. The WBC was 6.8 x 10^9/L, neutrophils 27% with hypersegmentation, lymphocytes 71%, eosinophils 2%. Platelets were 719 x 10^9/L. Bone marrow was markedly cellular and megaloblastic. High voltage electrophoresis of the urine showed greatly increased homocysteine and increased serine, alanine, leucine, and ethanolamine. Methylmalonic acid was not detected, and gas liquid chromatography showed a normal organic acid pattern. Plasma amino acid analysis was not performed before treatment. Two weeks after the first dose of vitamin B12, no homocystine was detected in the plasma while methionine was 4 zmol/L (n, 5 to 34). Red cell folate was 1,280 nmol/L of packed cells (n, 230 to 2,200), serum folate 41 nmol/L, and B12 1 17 pmol/L (n, 160 to 500). Liver and renal function tests were normal. Cerebral ultrasound examination showed no abnormality, and an EEG showed medium voltage
with no consistent asymmetry, focal, or epileptiform features. His mother's serum folate and B12 were normal, and she had no antibodies to intrinsic factor. Transcobalamin II deficiency was excluded by normal findings for patient's serum mediated 57Co-cyanocobalamin uptake by normal lymphoblasts and serum 57Co-cyanocobalamin elution pattern with gel chromatography. One week after admission treatment was begun with both folate and B12, the latter as 1.000 µg of cyanocobalamin intramuscularly (IM) daily. Reticulocytes peaked at 23% on the fifth day, and there was progressive rise in hemoglobin and return toward normal of red cell changes. On treatment his general health improved, his muscle tone returned to normal, and there were no further seizures. Homocystine was no longer detected in his urine. Anticoagulants were discontinued at six months, and B12 was reduced to 1.000 µg twice a week at 12 months. At 2 years folic acid was discontinued. Developmental assessment at 3 years is within normal limits in all modalities. A female sibling was born 2 years later. She was physically and hematologically normal at birth. At 6 weeks she had a Hb 12.7 g/dL, MCV 100 fl, with normal red cell morphology. She made good progress until her sudden cot death at 9 weeks. Post mortem examination failed to reveal the cause of death. Attempts to establish Epstein-Barr virus (EBV)-transformed lymphoblasts from this individual were not successful.

MATERIALS AND METHODS

Cell lines. Human skin fibroblasts from the patient and normal individuals were routinely grown in Eagle's minimum essential medium (CS Laboratories, Parkville, Australia) with 10% fetal bovine serum (FBS Flow Laboratories, Australia). EBV-transformed lymphoblast cell lines were established from the patient, mother, father, and normal individuals as described previously. All lymphoblast cell lines were maintained in exponential growth in RPMI 1640 medium (Flow Laboratories) supplemented with 10% FBS. The concentration of methionine under normal culture conditions is 0.1 mmol/L (15 mg/L) and vitamin B12 3.7 nmol/L (5 µg/L). In experiments with modifications of concentration of vitamin B12, folate, or methionine in the culture medium, cells were grown in a modified RPMI 1640 medium (K-C Laboratories, Kansas City) with the appropriate vitamin or amino acid added at the indicated concentration. Fibroblast and lymphoblast growth studies were initiated at a cell density of 2 × 10^6/mL and 5 × 10^6/mL respectively with the indicated culture medium conditions, and cell counts were performed as previously described at 96 hours using trypan blue exclusion as the index of viability.

Fibroblasts were harvested by treatment with 0.2% trypsin (Flow Laboratories) for one minute at 37°C and subsequent inactivation with FBS.

Enzyme assays. Lymphoblasts or confluent fibroblasts were harvested as described, centrifuged at 1,000 g for 10 minutes at 4°C, and washed thrice with phosphate-buffered saline (PBS). Extracts of cells in appropriate buffers, usually 10 mmol/L Tris HCL pH 7.4. Hemoglobin was determined by the method of Lowry et al. Hematologically normal at birth. At 6 weeks she had a Hb 12.7 g/dL, MCV 100 fl, with normal red cell morphology. She made good progress until her sudden cot death at 9 weeks. Post mortem examination failed to reveal the cause of death. Attempts to establish Epstein-Barr virus (EBV)-transformed lymphoblasts from this individual were not successful.

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without added cobalamin (Table 2). Both holoenzyme and total enzyme activities were reduced in patient’s fibroblasts and lymphoblasts. This reduced methionine synthase activity was evident in patient’s lymphoblasts in culture at all intervals up to 96 hours. Assay of methionine synthase of patient and control lymphoblasts under anaerobic conditions showed no significant difference to that under aerobic conditions. Methionine synthase of control lymphoblasts were 2.2 and 0.9 (holoenzyme) nmol/hour/mg protein (aerobic) and 2.3 and 0.6 (anaerobic). Corresponding values for patient lymphoblasts were 0.36 and 0.21 (holoenzyme) nmol/hour/mg protein (aerobic) and 0.40 and 0.30 nmol/hour/mg protein (anaerobic). In data not shown, mixing control and patient lymphoblast extracts yielded the expected levels of methionine synthase activity, excluding the possibility of inhibition or, alternatively, activation of patient’s reduced methionine synthase activity by constituents of control extracts.

Effect of alteration of assay conditions on methionine synthase of patient’s cells. Rosenblatt et al. have demonstrated that methionine synthase in the previously described patient with megaloblastic anemia and homocystinuria was normal at conventional thiol concentrations. The effect of varying assay concentrations of dithiothreitol (DTT 0.2 to 25 mmol/L) and B mercaptoethanol, (7.5 to 200 mmol/L) were determined with lymphblast extracts of the current patient. In both instances at all concentrations of thiol assayed the patient’s methionine synthase activities (Figs 1 and 2) were significantly lower than those of control lymphoblasts. For both patient and control lymphoblast extracts, methionine synthase activity at the maximal concentration of DTT (25 mmol/L) was approximately four times that observed with B mercaptoethanol (200 mmol/L), suggesting that the reducing conditions produced by these thiol reagents are neither equivalent nor a unique interaction of DTT with methionine synthase.

Methionine synthase of patient and control fibroblasts were also assayed with varying concentrations of B mercaptoethanol: 5, 75 and 200 mmol/L. With these thiol concentrations, control fibroblast methionine synthase activities were 0.17, 1.45, and 2.5 nmol/hour/mg protein. Corresponding values for patient fibroblast methionine synthase were 0.04, 0.39, and 0.8 nmol/hour/mg protein. In data not shown, increasing the assay concentration of methylcobalamin (up to 50-fold), S-adenosyl methionine (up to fivefold), and 5 methylTHF (up to fivefold) did not significantly alter the reduced methionine synthase activity exhibited by patient cell extracts. In addition, substitution of methyl cobalamin by hydroxocobalamin or cyanocobalamin in the assay system produced no significant differences.

Effect of varying culture concentration of cobalamin on methionine synthase activity. The methionine synthase activities for control and patient lymphoblasts cultured with either varying cobalamin compounds or concentration are shown in Table 3. Control lymphoblasts cultured with increasing concentration of cyanocobalamin (0.5 μg/L, 0.375 mmol/L to 500 μg/L, 375 nmol/L) exhibited a fivefold increase in methionine synthase, and the enzyme levels with hydroxocbl or cyanoCbl at the higher cobalamin culture concentration used were comparable (Table 3). Patient’s lymphoblasts cultured under identical conditions showed a twofold increase in methionine synthase activity, and this activity level was now in the normal range.

Accumulation and distribution of labeled cobalamin and effect of methionine culture deprivation on cell growth. The accumulation of label from 57Co-cyanocobalamin in patient and control lymphoblasts after two hours or 24 hours
in culture was comparable: at two hours patient 3.2 x 10^6 cpm, control 2.4 x 10^6 cpm/10^6 cells; at 24 hours patient 19 x 10^6 cpm, control 17 x 10^6 cpm/10^6 cells. The distribution of methylICbl and adenosylICbl in human lymphoblasts showed that in contrast for fibroblasts the major cellular cobalamin is adenosylCbl, but the concentration of methylCbl in patient's lymphoblasts and fibroblasts was markedly reduced (Table 4). The patient's cells exhibited reduced growth rates when cultured in methionine-replete medium or when methionine was replaced by homocysteine. When 2 x 10^5/mL control and patient fibroblasts were plated in complete medium, growth at 96 hours for control cells was 9 ± 0.3 x 10^6 (SEM)/mL and for the patient's cells 5 ± 0.2 x 10^6/mL. When homocysteine (0.2 mmol/L) was substituted for methionine in the media, growth at 96 hours was 8 ± 0.5 x 10^6/mL for control fibroblasts and 3 ± 0.2 x 10^6/mL for the patient fibroblasts. Lymphoblasts were also studied. Both control and patient lymphoblasts cultures were initiated at a cell density of 0.5 x 10^6 cells/mL. Growth at 96 hours in methionine replete medium was 3.2 ± 0.3 x 10^6 (SEM)/mL for the control lymphoblasts and 2.4 ± 0.1 x 10^6/mL for patient's lymphoblasts. Both the control and patient lymphoblasts showed a preferential growth requirement for methionine with the patient's lymphoblasts exhibiting no increase in cell numbers in the absence of methionine. When homocysteine was substituted for methionine, control lymphoblasts' density at 96 hours had increased to 0.9 ± 0.1 x 10^6 (SEM)/mL, whereas the patient's lymphoblast density at 96 hours was 0.4 ± 0.1 x 10^6/mL.

**DISCUSSION**

Inherited defects of cobalamin metabolism resulting in megaloblastic anemia and homocystinuria include the mutations of congenital methylmalonic aciduria-homocystinuria. On the basis of biochemical and genetic complementation studies, these disease states represent at least two different genetic entities termed CblC and CblD mutations, which both involve an assumed defect in Cob(II1)alamin reductase. This activity reduces the charge of the cobalt of cobalamin from +3 to +2, an event that normally occurs after cobalamin enters into the cell and precedes the formation of both adenosylCbl and methylCbl. Thus common to these mutations is the inability of affected cells to accumulate cobalamin as adenosylCbl and methylCbl and reduced hololenzyme activities of methylmalonylCoA mutase and methionine synthase. Clinical findings include methylmalonic aciduria, homocystinuria, neurologic disorders, and a variable incidence of megaloblastic anemia confined to individuals with CblC mutation. Cells of our patient (1) were not defective in accumulating cobalamin but exhibited decreased methyl Cbl but not adenosylCbl content, (2)

**Table 3. Methionine Synthase Activity of Patient's and Control Lymphoblasts Cultured Under Varying Cobalamin Conditions**

<table>
<thead>
<tr>
<th>Culture Conditions*</th>
<th>Added Cobalamin</th>
<th>HydroxyB12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of</td>
<td></td>
<td></td>
</tr>
<tr>
<td>nmol/L</td>
<td></td>
<td></td>
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<tr>
<td>Methionine synthase (nmol/hour/mg protein)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.8 (0.8)</td>
<td>8.1</td>
</tr>
<tr>
<td>Patient</td>
<td>0.8 (0.4)</td>
<td>1.7</td>
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<table>
<thead>
<tr>
<th>Culture conditions†</th>
<th>MethyTHF</th>
<th>HydroxO812</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>- HydroxO812</td>
</tr>
<tr>
<td>Methionine synthase (nmol/hr/mg protein)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4.8</td>
<td>2.1</td>
</tr>
<tr>
<td>Patient</td>
<td>1.5</td>
<td>0.6</td>
</tr>
</tbody>
</table>

*Lymphoblasts were cultured in standard RPMI 1640 supplemented with 10% fetal calf serum with varying concentrations of cyanocobalamin or hydroxocobalamin as indicated. Cells were harvested at 48 hours and assayed for methionine synthase activity as described in the text.
†Lymphoblasts were cultured in RPMI 1640 medium without or with hydroxocobalamin at concentration of 3.75 nmol/L and methyTHF 2 μmol/L, harvested at 48 hours, and assayed for methionine synthase as described in the text.
‡Figures in parenthesis are hololenzyme values.

**Table 4. Distribution of Methylcobalamin and Adenosylcobalamin in Lymphoblast and Fibroblast Extracts**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>5O3-B12</th>
<th>CN-B12</th>
<th>CH3-B12</th>
<th>Ado-B12</th>
<th>OH-B12</th>
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<tbody>
<tr>
<td>% of Total Cobalamin*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphoblast</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>3</td>
<td>2</td>
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<td>60</td>
<td>7</td>
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<tr>
<td>Patient</td>
<td>4</td>
<td>0</td>
<td>3</td>
<td>87</td>
<td>7</td>
</tr>
<tr>
<td>Fibroblast</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3</td>
<td>0</td>
<td>58</td>
<td>31</td>
<td>8</td>
</tr>
<tr>
<td>Patient</td>
<td>3</td>
<td>0</td>
<td>10</td>
<td>76</td>
<td>11</td>
</tr>
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</table>

*Results expressed as % of total cobalamin in cell extracts following growth for 72 hours in RPMI 1640 medium containing 57Co-cyanocobalamin (0.1 ng/mL). Values given are means of two separate determinations in duplicate.
utilized the methyl group of methylTHF less effectively than normal cells, and (3) incorporated the radio label from 14C propionate into amino acid at normal rates. In addition, our patient exhibited megaloblastic anemia with homocystinuria but not methylmalonic aciduria. These findings exclude CbIC or CbID mutations and are identical with those reported for the patient with CbIE disease. At variance are the differing characteristics of methionine synthase. Specifically, the reduced methionine synthase activities of our patient’s lymphoblasts and fibroblasts were not normalized with optimal assay reducing conditions, either with DTT or 0-mercaptopethanol. In addition, increasing substrate concentrations of methylTHF, methylCbl, or 5-adenosylmethionine had no effect on the reduced activity. Although these findings suggest that our patient with altered methionine synthase activity may have a defect at the same locus as that of the previously described patient with congenital homocystinuria and abnormal methionine synthase activity, only genetic complementation studies will resolve this question. That this altered methionine synthase is of significance for intact cells is suggested by the reduced methylCbl content of the patient’s lymphoblasts and fibroblasts and an impaired growth rate of patient fibroblasts and lymphoblasts when methionine was substituted in culture medium by homocysteine. The mechanisms involved in methionine-dependent cell growth are controversial and evolving. In the absence of extracellular methionine, this amino acid in cells is generated in human cells from either homocysteine by methionine synthase or by a series of reactions from the methylthio-containing portion of methylthioadenosine, a byproduct of polyamine metabolism. Methionine for cell growth is potentially a summation of the contributions of these two pathways. The differing growth capacity of our patient’s cells in culture when methionine has been replaced by homocysteine reflects in part the reduced methionine synthase activity of these cells and the relative contribution of this activity to the cellular methionine pool. Changing culture cobalamin conditions produced differing effects for the patient’s and control lymphoblasts’ methionine synthase activity. An increase in the concentration of cobalamin added to the culture medium was accompanied by a fivefold increase in this activity of control lymphoblasts compared to a twofold change for patient’s cells cultured under identical conditions. Limiting cobalamin in the culture medium was accompanied by greater degrees of reduction of patient’s activity, and it is of some interest that our patient’s serum B12 on presentation was suboptimal. Although the magnitude of increase of patient’s methionine synthase with increased cobalamin in the culture medium was less than that exhibited by control lymphoblasts, the absolute level of activity achieved by the patient was of the same order as in normal cells grown under standard conditions for cobalamin. This finding may have in vivo significance since both the homocystinuria and megaloblastic anemia responded completely to large doses of cyanocobalamin, and it is feasible that this is mediated by augmentation of the reduced activity in vivo. The molecular basis for cobalamin-related changes in methionine synthase of culture cells is not entirely clear. It is independent of protein synthesis and probably reflects stabilization of enzyme by cobalamin with decreased enzyme turnover. If this is so, the differing pattern of response of the patient’s methionine synthase to cobalamin concentration in culture suggests a fundamental difference for mutant activity. Only purification and characterization of the enzyme will satisfy this hypothesis. As the two described patients with neonatal megaloblastic anemia, homocystinuria, and altered methionine synthase activity exhibited neurologic features, including seizures and developmental delay, the impact of altered cobalamin metabolism on the developing neurologic system deserves some comment. Whether in hereditary disorders such as transcobalamin II deficiency, CbIC, and now CbIE mutation or the acquired defects of vitamin B12 in breast-fed infants of vegan or pernicious anemia mothers, the accompanying neurologic syndromes are characterized by their diversity if one sets aside the background of failure to thrive, listlessness, and irritability. Possibly the specific neurologic syndromes depend primarily on the developmental stage of the infant at the onset of vitamin deficiency. Whatever the manifestations, failure to diagnose the specific defect and to institute appropriate therapy can have dire consequences for the infant’s ultimate neurologic status, whereas early recognition is associated with a favorable outcome as illustrated in our patient.

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REFERENCES

7. Lowry OH, Rosebrough WJ, Farr AL, Randall RJ: Protein
measurement with the folin phenol reagent. J Biol Chem 193:265, 1951
Vitamin B12-responsive neonatal megaloblastic anemia and homocystinuria with associated reduced methionine synthase activity

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