CASE REPORT

Megaloblastic Anemia and Immune Abnormalities in a Patient with Methionine Synthase Deficiency

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ABSTRACT. Zittoun, J., Fischer, A., Marquet, J., Pérignon, J. L., Lagrue, A. and Griscelli, C. (Laboratoire d'Hématologie, Hôpital Henri Mondor, Créteil, Unité d'Hématologie et d'Immunologie Infantile and Département de Biochimie, Hôpital des Enfants Malades, Paris, France). Megaloblastic anemia and immune abnormalities in a patient with methionine synthase deficiency. Acta Paediatr Scand 76: 991, 1987.

We report a case of methionine synthase deficiency associated with cellular immune deficiency discovered in a 14-year-old boy. Principal findings were: developmental delay, recurrent upper and lower respiratory tract infections, megaloblastic anemia, discovered at 3 months of age, unresponsive to cyanocobalamin and poorly responsive to folinic acid. Biochemical studies showed: an abnormal deoxyuridine suppression test despite normal serum folate, cobalamin and transcobalamin levels; a normal intracellular uptake of these two coenzymes; and an absolute requirement of methionine for fibroblast growth, suggestive of defective methionine synthesis. An absence of methionine synthase activity in the patient's bone marrow and a profound depression of this activity in lymphocytes and liver were found. Hypergammaglobulinemia with variable lymphopenia, depressed lymphocyte transformation after lectin or recall-antigen stimulation, defective delayed-type hypersensitivity and decreased natural killer activity were noted as well. The patient died at the age of 14. Key words: methionine synthase deficiency, megaloblastic anemia, immune abnormalities.

Methionine synthase (5-methyltetrahydrofolate-homocysteine methyltransferase, EC 2.1.1.13) is the main enzyme involved in the biosynthesis of methionine. It catalyzes the transfer of a methyl group from 5-methyltetrahydrofolate to homocysteine using methyl-cobalamin as a coenzyme.

The impairment of methionine synthase can have various causes: cobalamin deficiency, a congenital defect in cobalamin metabolism (such as defective intracellular methylcobalamin synthesis or retention) (1, 2), a congenital deficiency of methionine synthase activity (3), and the inhibition of this enzyme by nitrous oxide (4). Each of these abnormalities in man leads ultimately to the impairment of DNA synthesis responsible for the megaloblastic anemia.

Congenital deficiency of methionine synthase is a very rare defect, and has been reported by Arakawa et al. (3) in a child suffering from megaloblastic anemia and severe mental retardation.

We report in the present paper a new case of methionine synthase deficiency in a young boy presenting with megaloblastic anemia associated with cellular immune defects which could possibly be related to the enzyme deficiency.

CASE REPORT

L. B., born in 1969, was the first child of unrelated healthy caucasian parents. At birth, he was described as normal. At the age of 3 months, he was admitted for anemia and severe infections. Physical findings included growth retardation, facial dysmorphism, hepatosplenomegaly, hypotonia and delayed neurological development. He learned to walk at the age of 27 months and was never able to learn to read and...
write. The D. O. score (Brune Lezine test) ranked about 55 on multiple occasions between the ages of 2 and 12. From the age of 3 months, he was subject to multiple infections: severe staphylococcal septicemia, pneumonia, pneumococcal meningitis, multiple upper respiratory tract infections over a 10-year period (no germ identified), and a coronavirus-positive diarrhea. The child died at the age of 14 of measles interstitial pneumonitis.

Throughout his life, hemoglobin levels averaged 70 g/l with a MCV of about 110 fl and a reticulocyte count of 20.10^7/l; white blood cell and platelet counts were within normal limits. Peripheral blood smears showed macrocytosis, polychromatophilic erythrocytes, Howell-Jolly bodies and hypersegmented neutrophils. Repeated bone marrow aspirates from the age of 3 months consistently demonstrated megaloblastic erythroblasts and giant metamyelocytes. At the age of 3 months, he received one injection of cyanocobalamin and a trial of folinic acid, both without effect. Folic acid was later administered between the ages of 8 and 20 months with only mild and transient improvement. At 2 years of age, hypergammaglobulinemia with 2 oligoclonal peaks (IgG K and IgG 2) was noted.

A younger brother born in 1975 also suffered from hypotrophy, recurrent infections and megaloblastic anemia. He died at the age of 2 1/2 years of a pneumonitis and gastrointestinal infection. No investigation was made.

Methods. Serum and erythrocyte cobalamin were assayed microbiologically with *L. leichmannii*, serum folate with *L. casei* and *P. cerevisiae* and total red blood cell folate with *L. casei*. Monoglutamate forms of red blood cell folate were measured using the method reported by Chanarin et al. (5). Serum unsaturated B12 binding capacity was determined by saturating 0.5 ml of serum with an excess of [^57Co]-cyanocobalamin (Amersham, specific activity 300 µCi/µg) and removing the unbound [^57Co]-cyano- cobalamin by dialysis (6). Fractionation of serum transcobalamins (R binders and transcobalamin II) was done by gel filtration on a 2.5/70 cm column of Sephadex G200 (6).

Uptake of 5-[^14C]methyl-tetrahydrofolic acid (Amersham, specific activity 45 mCi/mmol) and [^57Co]-cyanocobalamin (300 µCi/µg) was tested on bone marrow cells of the patient and on bone marrow cells from a normal control. The cells obtained after sedimentation of the heparinised aspirate on dextran were washed twice with Hank's balanced salt solution (HBSS) pH 7.3. 1.10^6 cells resuspended in HBSS were incubated at 37°C for 3 hours with 10% of the patient's serum or normal AB serum and either 5 µCi of 5-[^14C]methyl-tetrahydrofolic acid or 0.1 µCi of [^57Co]-cyanocobalamin. After 3 hours, the reaction was terminated with cold saline. The cells were then centrifuged and washed twice in saline. Their radioactivity was then counted.

The dU suppression test was carried out on bone marrow cells as described previously (7). The test is based on the suppression by cold deoxyuridine (dU) of the incorporation of [^3H]-thymidine ([^3H]dR) into DNA in cell culture. A deficiency of folate or cobalamin or a disturbance in their metabolism leads to the impaired methylation of deoxyuridine to thymidylate acid. Such impairment results in the increased incorporation of [^3H]-thymidine into DNA. Attempts at correcting the dU test were performed by addition of various folate and cobalamin derivatives (folinic acid, 5-methyltetrahydrofolate (5-methylTHF), 5-formylTHF, methylcobalamin (methylCbl), or 5-methylTHF and methylCbl). dU suppression was calculated as per cent residual [^3H]dR incorporated into DNA in the presence of dU alone or with derivatives as compared to the incorporation in the absence of dU. Normally residual incorporation is less than 9%. Because of the sensitivity of some folate and cobalamin derivatives to light, all incubations were done in the dark.

Plasma and urinary aminoacids were analyzed by cation exchange chromatography on a Technicon TSM apparatus. The limit of detection for homocysteine was 1 µmol. Methylmalonic acid was assayed by the method reported by Kesner et al. (8). Red cell adenosine deaminase, purine nucleoside phosphorylase, hypoxanthine phosphoribosyltransferase and orotate phosphoribosyltransferase were assayed as previously described (9). Dihydrofolate reductase activity was assayed in the patient's lymphocytes established in continuous cell line according to the method described by Rothenberg et al. (10). 5,10-methylene tetrahydrofolate reductase activity in lymphocytes was determined as described by Kutzbach & Stockstad (11). Methionine synthase activity was measured in lymphocytes, bone marrow cells and liver according to the method described by Peytremann et al. (12). The patient's liver samples were collected some hours before death and stored immediately at −80°C. Five control liver samples were obtained from patients without hepatic disease. The duration and conditions of storage were similar for the patient's and control's samples and the hepatic enzyme activities were measured simultaneously. Before the assay, liver specimens (150 to 250 mg) were homogenized in 1 ml PBS 0.01 M pH 7.4 containing 5 µmol 2-mercaptoethanol. The homogenates were then dialyzed against the same buffer for 24 hours. The reaction mixture contained (in a total volume of 500 µl) 0.25 mmol/l of 5-[^14C]methyltetrahydrofolic acid, 0.1 mmol/l of S-adenosylmethionine, 1.25 mmol/l of 1-homocysteine, 0.1 mmol/l of methylcobalamin, 200 mmol/l of 2 mercaptoethanol, 50 mmol/l of PBS pH 7.4 and 140 µ of enzyme solution adjusted to contain between 0.7 mg and 1 mg of protein. The reaction was performed in the dark and under nitrogen.
Culture of the patient's fibroblasts was attempted in order to assay the activities of various enzymes, in particular that of methionine synthase. On two occasions, fibroblasts from the patient's skin biopsy failed to grow when cultured in Eagle's minimal essential medium (MEM) which contained essential and non-essential aminoacids and to which was added 20% dialyzed fetal calf serum. On a third occasion, after supplementation of the culture medium with methionine (15 mg/l), hypoxanthine (10 mg/l) and thymidine (10 mg/l), cells began to grow but did so more slowly than cells from two normal controls. In addition, these cells were morphologically abnormal (giant cells) and died before they had grown sufficiently to allow enzyme assay.

Immunological investigations were done as previously described (13). T lymphocytes were enumerated by E rosette formation. B lymphocytes by indirect immunofluorescence using a goat F(ab')2 antihuman IgG Fab labelled with fluorescein (13). Assays of lymphocyte proliferation to phytohemagglutinin (PHA) and to various antigens (tetanus toxoid, candida, allogeneic cells), and assays of natural killer (NK) activity were carried out in RPMI 1640 as previously described (14). Serum immunoglobulin levels (IgG, A, M, D) were measured by immunodiffusion. IgE levels were measured by radioimmunoassay. Serum antibody titers to tetanus toxoid and Toxoplasma gondii were determined by neutralization, titers to poliovirus by complement fixation, and titers to smooth muscle by immunofluorescence.

RESULTS

Cobalamin, transcobalamin and folate studies. Serum and erythrocyte cobalamin levels were normal as were serum and erythrocyte folate levels (Table 1). Levels of reduced forms of folate as assayed with P. cerevisiae were also in the normal range. In addition, the intracellular ratio of pteroylmonoglutamate to total pteroylglutamate was normal. Serum unsaturated B12 binding capacity was within normal limits (1805 ng/l): of the labelled vitamin B12 added to the serum, 60% was bound to transcobalamin I, 40% to R proteins. In addition, no difference between the patient's and controls' bone marrow cells was found in the uptake of 5-[14C]methyl-tetrahydrofolic acid and [57Co]-cyanocobalamin. This uptake was independent of the serum used as a source of transcobalamin I, whether this serum was the patient's or normal AB serum.

\(dU\) suppression test values. The \(dU\) suppression test was performed at various times as shown in Table 2. The first \(dU\) suppression test (1977) was mildly abnormal and improved only after the addition in vitro of 5-formyl-THF. In 1980, the \(dU\) test was frankly abnormal and improved only after the addition in vitro of 5-formyl-THF. The addition of either 5-

Table 1. Serum and erythrocyte cobalamin and folate levels

| Cobalamin and folate levels | 2.1977 | 5.1980\(^a\) | 9.1980 | 3.1982\(^b\) |
|-----------------------------|--------|-------------|--------|-------------|
| Serum cobalamin (ng/l)      | 350    | 3000        | 860    | 550         |
| (200–500)\(^c\)             |        |             |        |             |
| Erythrocyte cobalamin (ng/l)|        | 420         |        |             |
| (120–400)\(^c\)             |        |             |        |             |
| Serum folate (µg/l)         |        |             |        |             |
| \(L. \text{casei}\)         | 8      | 15.9        | 9.3    | 7.7         |
| (5–12)\(^c\)                |        |             |        |             |
| \(P. \text{cerevisiae}\)    | 0.9    | 0.5         |        |             |
| (0.4–1.2)\(^c\)             |        |             |        |             |
| Total erythrocyte folate (µg/l)| 305 | 535        | 395    |             |
| (>200)\(^c\)                |        |             |        |             |
| Erythrocyte pteroylmonoglutamate | 210 | 215        |        |             |

\(^a\) After treatment with hydroxocobalamin and folinic acid.

\(^b\) After treatment with folinic acid.

\(^c\) Normal values.
methyl-THF, methylCbl or both worsened the abnormality. Therapy with folinic acid and hydroxocobalamin or with folinic acid alone (3.1982) normalized the dU test.

**Aminoacid and methylmalonic acid studies.** Plasma aminoacid analysis twice showed decreased methionine levels of 6.7 μmol/l and 15 μmol/l (normal = 28±6). The cyanide-nitroprusside test was negative. Urinary excretion of aminoacids and organic acids was normal and in particular, there were neither homocystinuria nor methylmalonilaciduria.

**Investigations of purine and pyrimidine metabolism.** Table 3 shows that the activity of the various enzymes involved in these pathways were found to be normal or increased, a common finding in megaloblastic anemias (unpublished data); in particular there was no decrease of orotase phosphoribosyltransferase and hypoxanthine phosphoribosyltransferase. No urinary excretion of orotic acid was noted on different occasions.

**Enzyme activities of folate and cobalamin metabolism.** Dihydrofolate reductase activity (0.58 pmol/min/mg protein) was within the normal limits (0.7±0.35 pmol/min/mg protein), 5,10-methylene tetrahydrofolate reductase activity was normal (7.2 nmol/h/mg protein) and comparable to that of controls (5.3±1.46 nmol/h/mg protein). Methionine synthase activity was found to be frankly abnormal (Table 4). It was undetectable in the patient's bone marrow cells in comparison to an activity in controls ranging from 2.44 to 6.7 nmol/h/10⁶ immature cells. Values for methionine synthase activity in lymphocytes (0.22 nmol/h/mg protein) and in liver (0.13 nmol/h/mg protein) were less than one quarter of those found in control samples and were in the same range as those found in control samples measured without methylcobalamin.

**Immunological findings.** From 1980, the absolute lymphocyte count was low. T lymphocyte proliferation induced by lectins and recall-antigens (PHA, tetanus toxoid, poliovirus I, II, III, candida antigen, allogeneic cells) was found initially to be low and later, a month before the patient's death, to be absent. The delayed hypersensitivity response at 48 hours, as meas-

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### Table 2. Deoxyuridine (dU) suppression test values

| Additive                  | 2.1977 | 5.1980* | 9.1980 | 3.1982* |
|---------------------------|--------|---------|--------|---------|
| dU                        | 13     | 2.7     | 29     | 4.1     |
| dU + folic acid           | 11     | 2.7     | 27.6   | 3.2     |
| dU + 5 formyl THF         | 7      | 2.8     | 8.8    | 3.3     |
| dU + 5 methyl THF         | 11     | 2.5     | 30.7   | 3.6     |
| dU + hydroxocobalamin     | 11     |         | 32     |         |
| dU + methylcobalamin      | 11     | 3       | 48     |         |
| dU + methylcobalamin + 5-methyl THF | 48 |        |       |         |

* After treatment with hydroxocobalamin and folinic acid.
* After treatment with folinic acid.

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### Table 3. Enzyme activities of purine and pyrimidine pathways

| Activities assayed on red blood cells (nmol/min/ml red blood cells) | Patient | Normal controls (mean±1 SD) |
|---------------------------------------------------------------------|---------|-----------------------------|
| Adenosine deaminase                                                 | 773     | 494±60                      |
| Purine nucleoside phosphorylase                                     | 49 900  | 47 500±6 200                |
| Hypoxanthine phosphoribosyltransferase                              | 734     | 555±110                     |
| Orotate phosphoribosyltransferase                                   | 4.2     | 1.35±0.5                    |
ured by skin testing with the same antigens, was also diminished. Similarly, the NK activity, present initially, disappeared (Table 5). An important hypergammaglobulinemia, due mainly to the presence of high levels of oligoclonal IgG K and IgG λ (38 to 67 g/l) was observed from the age of one (Table 5). A normal antibody response to recall-antigens was observed while smooth muscle antibodies were detected.

DISCUSSION

Congenital megaloblastic anemias are usually related to defects in purine or pyrimidine biosynthesis (15, 16) or to inborn errors of folate and cobalamin metabolism. In our patient, an impairment of purine and pyrimidine synthesis was unlikely because of the absence of oroticicaciduria and a normal red cell specific activity of the enzymes involved in these pathways.

Folate and cobalamin deficiencies were also excluded. Serum levels of unsaturated transcobalamins were in the normal range, especially those of transcobalamin II. A deficiency of transcobalamin II could be invoked to explain a megaloblastic anemia appearing in the first weeks of life (17). Functionally abnormal transcobalamin II (18, 19) was excluded by the normal uptake of $^{137}$Co-cyanocobalamin in bone marrow cells, independent of the serum used as a source of transcobalamin II, normal or patient's serum. A possible defect of cellular fo-

| Lymphocytes (nmol/h/mg protein) | Bone marrow cells (nmol/h/10^9 immature cells) | Liver (nmol/h/mg protein) |
|---------------------------------|-----------------------------------------------|--------------------------|
| Patient                         | Controls (6) \(^a\)                             | Controls (5) \(^a\)      |
| 0.22                            | 2.32±1.42 \(^a\)                               | 0.13                     |
| (0.9–5.26)                      | (4.2±1.5)                                      | (0.82±0.25)              |
| Control (20) \(^a\)             |                                               | 0.13                     |
| \(^a\) Number of controls.     |                                               | 0.82±0.25                |
| \(^b\) Mean ± SD.              |                                               | (0.5–1.12)               |
| \(^c\) Range.                  |                                               |                          |
| \(^d\) The enzyme assay was performed on two different samples of bone marrow cells.

Table 5. Immunological investigations

| ND = not done |
|---------------|
| 1980 \(^a\)  | 1982 \(^a\)  | Normal range                       |
| Lymphocytes (mm^3) | 250–4 000 | 500–1 000 | 1 500–3 000 |
| T Lymphocytes (%) (E. Rosettes) | 70–85 | 54 | 60–80 |
| NK activity (%)          | 70 | 1(±1FNγ) \(^c\) | 30–55 |
| B Lymphocytes (%)        | 3–15 | 29 | 2–10 |
| Serum IgG (g/l)          | 38–65 \(^d\) | 34 \(^d\) | 7–12 |
| IgA                      | 7.6–8.4 | 5.5 | 1.5–2.5 |
| IgM                      | 0.9–1.7 | 1.0 | 0.6–1.5 |
| IgD                      | 0.24–0.32 | 0.28 | 0.1 |
| IgE (IU/ml)              | 20–170 | ND | 5–50 |

\(^a\) The patient was uninfected in 1980 at time of immunological investigations.

\(^b\) Immunological investigations performed one month prior to death. At this time, the child was not infected by measles virus that eventually led to death.

\(^c\) Addition of 700 units of interferon γ during the cytolytic test.

\(^d\) Immuno-electrophoresis studies showed several IgG components.
late uptake similar to that reported by Branda et al. (20) in a patient with megaloblastic anemia was also ruled out. In L. B., the uptake of 5-[14C]methyltetrahydrofolinic acid by PHA-stimulated lymphocytes and bone marrow cells was normal.

A subnormal dU suppression test suggestive of impaired thymidylate biosynthesis prompted us to investigate the possibility of an intracellular derangement of folate or cobalamin metabolism. The dU test was normalized in vitro by folinic acid. Moreover, on some occasions, injection of folinic acid resulted in an increased Hb level but did not improve the macrocytosis or the megaloblastosis.

The possibility of dihydrofolate reductase deficiency was considered. Tauro et al. (21) reported cases in whom the abnormality was associated with a megaloblastic anemia that responded to folinic acid and in whom the abnormal dU suppression test was corrected by the addition of folinic acid. In our patient, the activity of this enzyme was normal as well as that of 5,10-methylene tetrahydrofolate reductase.

A defect in methionine synthesis remained the most likely etiology of the megaloblastic anemia, given the low serum levels of methionine and the inability of fibroblasts to grow in usual culture medium, an inability corrected by the addition of methionine. No methionine synthase activity was detected in bone marrow cells and this activity was profoundly depressed in lymphocytes and in liver. The enzyme activity was not restored, even partially, when methylcobalamin was used as coenzyme in cell extracts. It is likely that the patient's younger brother in whom a megaloblastic anemia was discovered during the neonatal period and who died of severe infections when he was 2 1/2 years old also suffered from the same abnormality.

The present case is similar in some respects to that reported by Arakawa et al. (3) in which a 6-month-old girl presented with megaloblastic anemia and mental retardation associated with a decreased hepatic activity of methionine synthase. However, enzyme activity was not assayed in lymphocytes and bone marrow cells. Moreover, neither infection nor immune abnormality was described in this child.

Methionine synthase deficiency appears to be different from inborn errors of cobalamin metabolism despite certain clinical and hematological similarities (1, 2, 22). In these inherited defects of cobalamin metabolism, a megaloblastic anemia, which is found in most of the cases, is associated with a varying degree of physical and mental retardation. However, homocystinuria is present with or without methylmalonylaciduria. Dillon et al. (1) reported a case of an abnormality of cobalamin metabolism consisting of an inability to maintain normal tissue concentrations of adenosyl and methyl cobalamin associated with homocystinuria and methylmalonylaciduria. In this case, fibroblasts also failed to grow in a medium deprived of methionine. However, methionine synthase activity, which was very low when assayed without methylcobalamin, became almost normal after addition of this coenzyme to the reaction mixture. In addition, serum methionine levels were normal. The patient reported by Schuh et al. (2) presented with a megaloblastic anemia with homocystinuria consequent to a defect in methylcobalamin retention. Cultured fibroblasts also showed an absolute requirement of methionine for growth but methionine synthase activity in cell extracts was normal, as were methionine levels in plasma.

More recently, a neonatal megaloblastic anemia with homocystinuria and reduced levels of methionine synthase was reported (23). This case seems different from ours since fibroblasts grew at a rate sufficient to allow enzyme assays. Moreover treatment with cobalamin and folate resulted in rapid hematologic and clinical improvement. Homocystinuria would have been expected in our patient. The absence of homocystinuria could in part be explained by a remethylation of homocysteine by betaine homocysteine methyltransferase. Indeed, this enzyme, present in large amounts in the liver, could compensate for the deficient activity of methionine synthase. This situation has been reported in rats after exposure to nitrous oxide.
which inactivates methionine synthase (24). In addition homocysteine is also converted to
cystathionine by cystathionine synthase. Recently, Finkelstein & Martin (25) have shown
that in mammals, metabolism of homocysteine is distributed between these competing path-
ways. This case of methionine synthase deficiency was also found to be associated with ab-
normalities of immunologic functions. Throughout his life, L. B. suffered from severe and re-
peated infections. Immunological abnormalities were characterized by an hypergamma-
globulinemia with 2 oligoclonal peaks, and by a fluctuating lymphopenia with a profound cel-
llular immunodeficiency. It is not possible to determine whether this immunodeficiency was
responsible for the occurrence of the severe measles interstitial pneumonitis or whether this
viral infection induced secondary immunosuppression.

The immune deficiency observed in our patient is somewhat different from that associated
with transcobalamin II deficiency as reported by Hitzig et al. (26). The cellular immune ab-
normalities associated with this case of methionine synthase deficiency are reminiscent of
those observed in purine nucleoside phosphorylase or orotate phosphoribosyl transferase de-
ciciencies (27, 28). In these metabolic disorders, cellular immunodeficiencies are predomin-
ant and worsen progressively. They are often associated with the presence of autoantibodies
and monoclonal immunoglobulins.

In fact, methionine synthase is a crucial enzyme in preventing methyltetrahydrofolate trap
and in regenerating tetrahydrofolate. This coenzyme is needed for DNA, RNA, protein
synthesis and other cell functions. A functional folate deficiency can be expected to lead to
an impairment of mitotic activity which is primarily manifest in rapidly proliferating cells,
those in, for example, the hematopoietic, gastrointestinal and immunologic systems. This
suggests that the immune disturbances observed in this child may not have been merely coin-
cidental.

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