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METABOLIC DISEASES

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Methionine synthase deficiency without megaloblastic anaemia

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Abstract We report findings on a child presenting with neonatal homocystinuria, hypomethioninaemia and severe neurological symptoms, including developmental delay and seizures. Methylmalonic aciduria was not present. The activity of methionine synthase in fibroblasts was severely deficient and formation of methylcobalamin from ^{57}Co labelled cyanocobalamin was very low. The patients cells complemented with those of a cblE patient but not with those of two cblG patients. No biochemical or clinical response to injections of hydroxycobalamin was found. Both off treatment and on betaine and methionine supplementation the patient, at age 8 years, has not developed megaloblastic anaemia. In addition, the patient is homozygous for the C677T polymorphism in the 5,10 methylenetetrahydrofolate reductase (MTHFR) gene and the concomitant existence of this mutation with the methionine synthase defect may prevent folate «trapping» and thus anaemia.

Conclusion We report the lack of megaloblastic anaemia in a patient with severe methionine synthase deficiency who is also homozygous for C677T in MTHFR, hypothesize that the MTHFR polymorphism protects the patient against anaemia and speculate that homozygosity for MTHFR C677T could cause the dissociation between haematological and neurological disease seen in some patients with vitamin B12 deficiency.

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Key words Methionine synthase · 5,10 Methylenetetrahydrofolate · Megaloblastic anaemia · Polymorphism · Homocysteine

Abbreviations *MS* methionine synthase · *MeCbl* methylcobalamin · *AdoCbl* adenosylcobalamin · *OH-Cbl* hydroxycobalamin · *CN-Cbl* cyanocobalamin · *PEG* polyethyleneglycol · *MTHFR* 5,10 methylenetetrahydrofolate reductase · *THF* tetrahydrofolate · *MMA* methylmalonic aciduria

Introduction

The remethylation of homocysteine to methionine is catalysed by the cytoplasmic enzyme 5-methyltetrahydrofolate:homocysteine methyltransferase (EC 2.1.1. 13) also called methionine synthase (MS) (Fig. 1). The enzyme requires methylcobalamin (MeCbl) for activity. Another cobalamin derivative, adenosylcobalamin (AdoCbl), is used as cofactor for the mitochondrial enzyme methylmalonyl-CoA mutase.

Functional MS deficiency is characterised by homocystinuria and hypomethioninaemia in the absence of methylmalonic aciduria (MMA). Patients with functional MS defects are divided into two classes, cblE and cblG, on the basis of complementation analysis [23, 24]. CblE patients have low MS activity in fibroblasts only when reducing conditions are suboptimal, while cblG patients show low activity under all conditions. Among 11 reported CblE and G patients all have, at least to some degree, responded biochemically and clinically to therapy with hydroxycobalamin (OH-Cbl), some also to cyanocobalamin (CN-Cbl) [24]. All the patients have had various neurological symptoms and megaloblastic anaemia, although one patient presented in adulthood mainly with neurological problems [3].

The currently more accepted hypothesis for the occurrence of megaloblastic anaemia in patients with

vitamin B12 deficiency and genetic MS defects is «folate trapping» [11]. Since the reduction of 5,10-methylenetetrahydrofolate (5,10-methyleneTHF) to 5-methyltetrahydrofolate (methylTHF) by 5,10-methylenetetrahydrofolate reductase (MTHFR) is essentially irreversible under physiological conditions folate is «trapped» as methylTHF. Subsequent lack of regeneration of folate coenzymes including 5,10-methyleneTHF leads to reduced thymidylate synthesis (Fig. 1). In patients with vitamin B12 deficiency neurological symptoms are generally considered to be late manifestations of cobalamin deficiency, although it is well recognised that some patients may have a neurological disorder without megaloblastic anaemia [15].

Recently a polymorphism in the MTHFR gene resulting in a thermolabile enzyme was reported [8]. Individuals homozygous for this polymorphism have slightly elevated homocysteine levels, indicating some impairment of the enzyme in vivo. The frequency of the homozygous state for the polymorphism was 5% in a Dutch population [8] and 18% in a British population [1].

We report here on a patient with severe MS deficiency belonging to the cblG complementation class, who is also homozygous for the polymorphism in the MTHFR gene. The patient differs from previously reported patients with defective MS, clinically by the lack of megaloblastic anaemia and by the lack of a biochemical response to cobalamin administration. The biochemical characteristics may be explained by the severely reduced MSmRNA found in this patient [10].

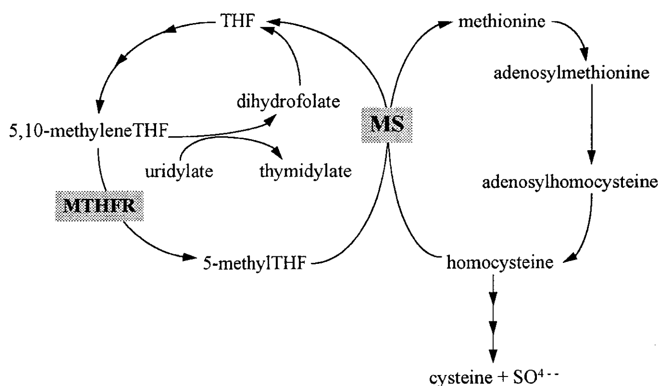

Fig. 1 A simplified schematic representation of the interrelation between vitamin B12 and folate dependent enzymes

Case report

The patient is the third child of healthy, unrelated, Norwegian parents. The pregnancy and delivery was normal and the birth weight 3100 g. Shortly after birth the boy showed failure to thrive and he was admitted to hospital at 6 weeks of age after two episodes of seizures. On investigation the child was dystrophic, severely hypotonic and showed no reaction to visual or auditory stimuli. Biochemical investigations revealed homocystinuria (66–208 μmol/mmol creatinine), homocystinaemia (31–42 μmol/l; severe hypomethioninaemia (2–3 μmol/l; normal 14–30) and no MMA. The initial haemoglobin value, 11.7 g/dl, and other haematological parameters were close to normal for age (Table 1). Between 8 and 14 weeks of age the patient received two blood transfusions due to declining haemoglobin values. Also three injections of 1 mg of OH-Cbl and oral folic acid (1 mg/day) was given within a 2-week period, but with no definite clinical or biochemical response. The reticulocyte count, however, which was 4% immediately before the OH-Cbl treatment, increased to 12% 1 week after the last injection of OH-Cbl. At the age of 14 weeks the haemoglobin value stabilised at 10 g/dl and therapy was started with 3×0.5 g of betaine, increasing to 3×1 g/day, and folic acid, 3×5 mg/day. Plasma homocystine (free) decreased from 20 to 3 μmol/l and methionine increased from 2 to 14 μmol/l within 2 days, and he became less lethargic and fed better. He continued on this treatment until 17 months of age when folic acid was stopped, although betaine treatment was continued. His haematological status did not alter after stopping folic acid. An examination of bone marrow at the age of 22 months showed a normal erythropoiesis and no hypersegmentation of neutrophils or giant metamyelocytes. A second trial of OH-Cbl therapy, 1 mg daily for 5 weeks, was undertaken at the age of 23 months, but again with no apparent clinical and negligible biochemical effect and it was therefore discontinued after 5 weeks. L-Methionine supplementation (50 mg/kg/day) was started at the age of 2 years 7 months following the finding of low levels of adenosylmethionine 124 nmol/l (normal 131–450) and methionine 0.8 μmol/l (normal 1.1–13.5) in CSF (analysis performed by Dr. R. Surtees, London) [20].

At the age of 3 years 4 months his gross and fine motor skills were estimated as equivalent to a developmental stage of 10 months. His mental ability was difficult to evaluate, he could not speak and he uttered only a few sounds. Vision and hearing had not been investigated by function tests. He had continuous rotating eye movements, but grasped at toys held before him and responded towards auditory stimuli. He recognised his parents, showed discrimination and had a continuous development of skills. His weight was 12.2 kg (< 10th percentile) and height 90 cm (< 2.5th percentile). A cerebral CT scan showed evidence of slight atrophy but this was less marked than at the age of 8 weeks. The EEG was normal. Essential haematological parameters are given in Table 1. Total homocysteine in plasma was 113/μmol/l. Other parameters at the age of 3 years 4 months were (normal values in parenthesis): leucocytes 12.4×10^9/l (6–17.5), platelets 487×10^9/l (150–400), red cell count 3.7×10^{12}/l (3.9–5.3), reticulocytes 1.3% (< 2), vitamin B12 (total) 1001 pmol/l (100–700), serum folate > 54 nmol/l (4–20), red cell folate 1185 nmol/l (340–1150), serum iron 17 μmol/l (8.9–21.5), serum ferritin 19 mg/l (7–140). A peripheral blood smear showed no obvious pathological changes.

The patient was lost to follow up from the age of 4–8 years. At the age of 8 years he was readmitted to hospital with seizures. His

Table 1 Haematological parameters at presentation, age 6 weeks, and at age 3 years 4 months and 8 years (treatment is with betaine 1.5 g × 3/day and L-methionine 50 mg/kg/day). Normal values in parenthesis

	Hb g/100 ml	Hct %	MCV fl	MCH pg	MCHC g/100 ml
6 weeks	11.7 (9–14)	33 (28–42)	98 (77–115)	35 (26–34)	36 (29–37)
3 years 4 months	10.3 (11.5–13.5)	31 (34–40)	82 (75–87)	27 (24–30)	33 (31–37)
8 years	12.4 (11.5–15.5)	37 (35–45)	90 (77–95)	30 (25–33)	33 (31–37)

plasma methionine level was undetectable and total homocysteine in plasma was 100 μmol/l, but his haematological status was normal (Table 1). Information on compliance to treatment prior to hospitalisation was not available. On anticonvulsive treatment he recovered quickly. He has never developed any language, but can walk, recognises his parents and grasps at objects held before him. His weight is 18.4 kg (10th percentile), height 116 cm (<2.5th percentile) and head circumference 49 cm (<2.5th percentile).

Methods

Amino acids and homocystine (free) in plasma and urine were analysed using a Biotronik LC 5001 amino acid analyser with ninhydrin detection. For *total* homocysteine in plasma the procedure was as follows: 0.5 ml of plasma was mixed with 0.5 ml Li-citrate buffer, 0.2 M pH 8.2, dithiotreitol, 3 mg, was added and the pH adjusted to 8.0–8.5 with $NaCO_3$. After 1 h at room temperature protein was precipitated with sulphosalicylic acid, 50 mg, the sample was centrifuged and the supernatant injected on the Biotronik amino acid analyser.

Fibroblasts were cultured in Eagle minimum essential medium (MEM) with 10% fetal calf serum as previously reported [6]. MS activity in fibroblasts was measured in cell extracts incubated with ^{14}C methyltetrahydrofolate, homocysteine, S-adenosylmethionine, β-mercaptoethanol (150 mmol/l, high reducing conditions) and methylcobalamin, essentially as described [18]. Labelled methionine was separated on a Dowex 1 column. Methionine and serine formed from ^{14}C formate in fibroblasts were measured in cells in monolayer incubated with ^{14}C formate and homocysteine for 16 h as previously described [6] but using ^{14}C formate as the labelled compound [2]. Labelled methionine and serine were determined in precipitated proteins after oxidation and hydrolysis by high voltage electrophoresis [7]. Analysis of cobalamin coenzyme distribution in plasma was performed with HPLC and a radioisotope dilution assay as previously reported [22]. The blood sample was collected in the dark in tubes containing EDTA. The plasma was stored at −20°C, shielded from light. The synthesis of cobalamin coenzymes in cultured fibroblasts was studied as reported [12]; cells were grown for 3–5 days in MEM, then the medium was changed to MEM containing human serum and ^{57}Co labelled CN-Cbl and then incubated for a further 4 days. The cells were harvested in the dark, cobalamins extracted with ethanol (80°C), separated by HPLC and radioactivity measured in fractions corresponding to marker cobalamins. Complementation studies were performed in fibroblasts fused with polyethyleneglycol (PEG) essentially as previously reported [9]; then methionine formation was measured as described above. Radioactively labelled compounds were purchased from Amersham, UK.

The activity of MTHFR in fibroblasts was determined as previously reported [17]. Analysis of the C677T mutation in the MTHFR gene was performed as described [8].

Results

Table 2 shows MS activity ("total" enzyme with high reducing conditions) in fibroblasts from the patient with different concentrations of MeCbl in the assay. The patient's cells revealed very low enzyme activity with negligible response to high concentrations of MeCbl regardless of whether cells were grown in medium supplemented with OH-Cbl. Methionine and serine formation from labelled formate, (Table 3), is low in intact cells from the patient and there is no increase above expected variation of the assay following addition of very high concentrations of OH-Cbl, MeCbl or folate to the culture medium. The results of the complementation study with cells from the patient and cblE and cblG patients are shown in Fig. 2. Complementation was obtained with cells from a cblE patient; after fusion the formation of methionine increased approximately 15 times. No complementation was observed with cells from two cblG patients. Fig. 3 shows the formation of cobalamin coenzymes from ^{57}Co labelled CN-Cbl in fibroblasts from the patient and also the percentage distribution of cobalamin coenzymes in the patients plasma at the age of 1.5 years. The total cobalamin uptake in fibroblasts from the patient was normal, 127 pg/mg

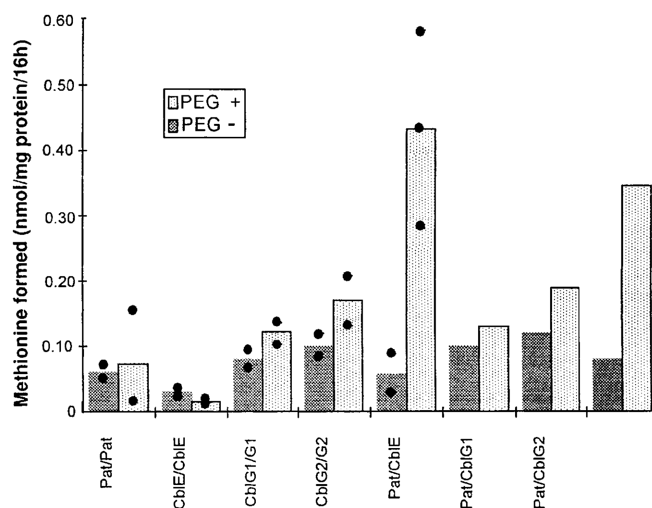

Fig. 2 Complementation studies between the cells from the patient and cells from one cblE and two cblG patients (cblG1 and cblG2). In experiments performed in duplicates or triplicates (Pat/CblE) the dots represent the single values. Intact methionine biosynthesis was measured by the incorporation of ^{14}C formate as described in Methods. Fusion was performed by exposing the cells to PEG. Note that the individual cells treated with PEG may show an increased methionine formation of 23%–89%

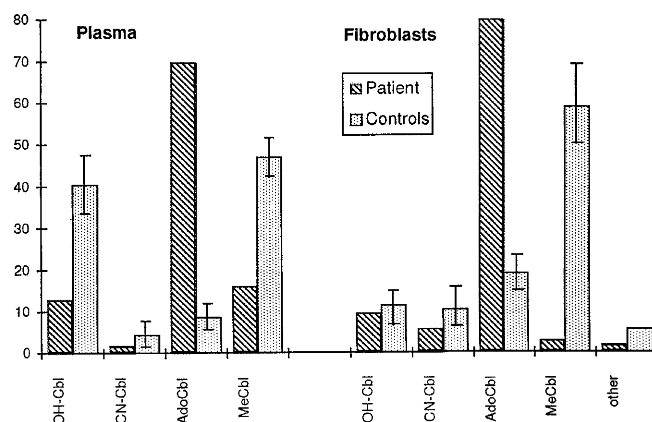

Fig. 3 Synthesis of cobalamin coenzymes in cultured fibroblasts from the patient and the distribution of cobalamin coenzymes in plasma, compared with controls (fibroblasts, n = 10, plasma n = 15). The *vertical bars* (control values) represent mean ± SD. Plasma was sampled at age 1.5 years on treatment with betaine 1 g × 3/day. Folic acid was stopped 3 weeks prior to the investigation. The methods are as described in the text

Table 2 MS activity in extracts of cultured fibroblasts from the patient grown with (+) or without (−) OH-Cbl supplementation (1 mg/l, 750 nmol/l), and assayed with and without addition of MeCbl (mean of 2–5 determinations). The activities of two cblG patients (cblG1 and cblG2) and of controls (mean ± SD and range) are also given

		MS activity (pmol/min/mg protein) (mean ± SD; range)
Patient,	−OH-Cbl, −MeCbl	1.0
	+OH-Cbl, −MeCbl	1.3
	−OH-Cbl, MeCbl 50 μmol/l	1.5
	+OH-Cbl, MeCbl 50 μmol/l	1.6
	−OH-Cbl, MeCbl 2000 μmol/l	2.0
cblG1,	−OH-Cbl, MeCbl 50 μmol/l	31
cblG2,	−OH-Cbl, MeCbl 50 μmol/l	19
Controls (n = 14),	−OH-Cbl, MeCbl 50 μmol/l	98 ± 21 (60–150)
Controls (n = 11),	+OH-Cbl, MeCbl 50 μmol/l	320 ± 170 (140–810)

Table 3 Methionine and serine formed from ^{14}C formate in fibroblasts from the patient with cells grown in normal medium (four determinations) and in cofactor supplemented medium

		Methionine	Serine
		(nmol/mg protein/16h) (mean ± SD; range)	
Patient's cells in normal medium		0.1 ± 0.04 (0.03–0.15)	0.35 ± 0.32 (0.15–0.91)
OH-Cbl	1 mg/l	0.14	
OH-Cbl	100 mg/l	0.08	
folate	100 mg/l	0.14	
MeCbl	1 mg/l	0.21	
Control cells (n = 22)		3.5 ± 1.1 (2–7)	5.2 ± 2.1 (3–10.2)

protein (controls n = 10, 113, range 40–305), but the fraction of MeCbl in these cells was only 2.7% compared to 40%–76% in controls, whereas the fraction of AdoCbl was highly increased. Also in plasma there was a low percentage of MeCbl and a highly increased fraction of AdoCbl. The concentration of MeCbl in plasma was, however, 126 pmol/l which is within the normal range 182 ± 50 (mean ± SD). AdoCbl concentration in plasma was highly increased, 548 pmol/l (normal 32 ± 10). The recovery of cobalamin coenzymes extraction from plasma was 87%.

The MTHFR activity in fibroblasts from the patient was 2.4 nmol/h per milligram protein in the absence of added FAD (controls, n = 24, mean 6.9, range 3.0–16) and 3.9 with added FAD (controls, n = 32, mean 8.5, range 3.2–16). The activity of methylmalonylCoA mutase was normal (results not shown).

The patient was homozygous for the C677T mutation in the MTHFR gene (Fig. 4). Assay of MTHFR activity in fibroblasts confirmed a thermolabile enzyme. In a population of 423 healthy Norwegians, 18–70 years of age, we found 8.6% to be homozygous for this mutation.

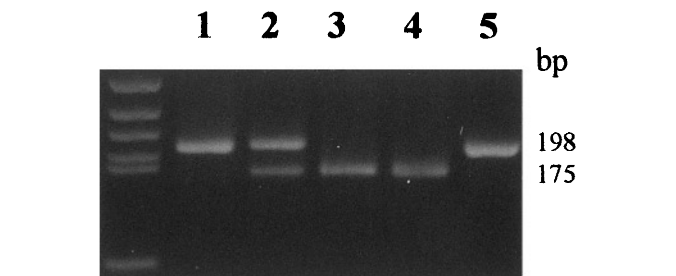

Fig. 4 Agarose gel electrophoresis of a 198 bp, HinfI digested, PCR product across the C677T mutation in the MTHFR gene from the patient and controls. The C677T mutation introduces a restriction site for HinfI and the mutated allele is digested into fragments of 175 and 23 bp, whereas alleles without the mutation remain undigested. *Lane 1*, control without the C677T mutation; *lane 2*, control heterozygous for C677T; *lane 3*, control homozygous for C677T; *lane 4*, the patient and; *lane 5*, undigested PCR product from the patient. The 23 bp fragment is not seen

Discussion

The biochemical findings in the patient demonstrate a functional MS deficiency in the cblG complementation class. However, both clinically and biochemically the patient differs from previously reported patients with functional MS deficiency. In our patient the in vitro deficiency of MS activity, only 1% (of mean control) residual activity, is more severe than the 8%–18% reported in most other cblG patients [24] and the enzyme activity of our patient could not be stimulated by supplementation of cobalamin in the medium of cultured cells, or by addition of cofactors in the assay. In contrast, all but one of the seven previously reported cblG patients have shown increased fibroblast MS activity after supplementation of the culture medium with OH-Cbl [23, 24]. The lack of in vitro stimulation by OH-Cbl is in agreement with the poor biochemical response to therapy with OH-Cbl. Also the distribution of cobalamin coenzymes formed from labelled CN-Cbl in fibroblasts of the patient was even more abnormal than in previously reported patients [24], with very low MeCbl formation and highly increased AdoCbl.

The MS gene was recently cloned [13, 14] and the mRNA level studied in two CblG patients, one of them being the present patient. In contrast to the other CblG patient studied our patient had a severe reduction of MSmRNA. The resulting lack of MS protein may explain the severe biochemical defect together with the lack of clinical response to OH-Cbl.

Despite severe neurological symptoms, the patient had not developed overt megaloblastic anaemia by the age of 8 years. The lack of megaloblastic anaemia is not easily explained on the basis of the molecular defect of MS. Presumably the lack of anaemia might be due to an effect of the treatment or to other constitutional factors. It is possible that folic acid treatment may have masked an early development of megaloblastic anaemia but against this is the fact that he has not developed anaemia in the absence of folic acid for more than 6 years.

Chanarin et al. [4, 5] claim that formate resulting from conversion of methionine to methylthioribose acts as a supply of one carbon units for the folate cycle. According to this hypothesis the betaine treatment and methionine supplementation given to the patient could restore the folate cycle and overcome the assumed deficiencies of folate coenzymes caused by the MS defect. Also the normal plasma concentration of MeCbl might indicate maintenance of the folate cycle despite a lack of methionine formation from homocysteine in some tissues.

A possible intriguing explanation of the lack of anaemia in this MS deficient patient is the modulating effect by the concomitant homozygosity for the C677T mutation in the MTHFR gene. This mutation renders the enzyme somewhat impaired and possibly more of the folate derivatives remain in the reduced folate coenzyme pool rather than being trapped by conversion to methylTHF, thus keeping the cellular level of 5,10-methyleneTHF sufficient for adequate thymidylate synthesis. Furthermore, the presumed improved level of S-adenosylmethionine due to the betaine treatment and methionine supplementation may have added to a reduced conversion of 5,10-methyleneTHF to methylTHF since S-adenosylmethionine is an allosteric inhibitor of MTHFR. If the absence of megaloblastic anaemia in this MS deficient patient is mainly due to the concomitant existence of the mutated MTHFR gene, a similar explanation may account for the dissociation of neurological and haematological disease seen in some patients with acquired vitamin B12 deficiency [15].

In contrast to the lack of megaloblastic anaemia the neurological symptoms of the patient were severe from early infancy. Initiation of betaine treatment improved the clinical symptoms, and possibly methionine supplementation may also have had a beneficial effect. This might be explained by the presumed subsequent increase of S-adenosylmethionine formation, shown by Surtees et al. [21] to be deficient in CSF of patients with various inborn errors of the methyl-transfer pathway, and to be associated with demyelination.

Determination of the exact molecular defect of the present and other MS deficient patients will help to delineate the various clinical and biochemical phenotypes of MS defect. Further investigations into the relationship between genetic or acquired MS defects and the polymorphism in the MTHFR gene may also give new insight into the complex interaction of folate and vitamin B12 metabolism. Finally the unusual features of this patient emphasise the need to screen patients with neurological disorders, but without megaloblastic anaemia, for homocystinuria due to MS deficiency.

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