Cobalamin neuropathy

Is *S*-adenosylhomocysteine toxicity a factor?

Erica VIEIRA-MAKINGS,* Jack METZ,* Justin VAN der WESTHUYZEN,* Teodoro BOTTIGLIERI†‡ and Israel CHANARIN

*Department of Haematology, School of Pathology of the South African Institute for Medical Research and the University of Witwatersrand, P.O. Box 1038, Johannesburg 2000, South Africa, and †Section of Haematology, MRC Clinical Research Centre, Northwick Park Hospital, Harrow, Middx., U.K.

Cobalamin neuropathy was produced in cape fruit bats (*Rousettus aegyptiacus*) by a cobalamin-free diet combined with intermittent exposure to nitrous oxide, which inactivates cobalamin. There were no significant differences in *S*-adenosylmethionine/*S*-adenosylhomocysteine ratios in the central nervous system of cobalamin-deficient and cobalamin-replete bats. Taken with other data there are no grounds of support for a hypothesis that cobalamin neuropathy is the result of impaired methylation, however produced.

INTRODUCTION

Cobalamin (Cbl) neuropathy has been produced in several animal models, including the monkey (Agamanolis *et al.*, 1976; Scott *et al.*, 1981), fruit bat (Green *et al.*, 1975) and, most recently, the pig (Weir *et al.*, 1988). In these the development of neuropathy has been considerably ameliorated by adding methionine to the diet (Scott *et al.*, 1981; Van der Westhuyzen *et al.*, 1982; Weir *et al.*, 1988). The synthesis *de novo* of methionine requires Cbl as a coenzyme, and hence it was suggested that the defect in Cbl function producing neuropathy was in relation to an impairment of methionine metabolism.

The second biochemical pathway in mammals requiring Cbl is the conversion of methylmalonic acid (MMA) to succinic acid. MMA is derived from various precursors, including valine. Recently it has been found that the neuropathy in the Cbl-deficient cape fruit bat (*Rousettus aegyptiacus*) is ameliorated by valine (Vieira-Makings *et al.*, 1989), casting some doubt as to which pathway is involved in the development of neuropathy.

Much of the available methionine is converted into *S*-adenosylmethionine (AdoMet), which is a general donor of methyl groups in transmethylation reactions. Arnstein & Neuberger (1953) showed that Cbl was required for methyl-group synthesis and for the normal transfer of methyl into choline (transmethylation). Impaired transmethylation has remained a possible explanation for some of the consequences of Cbl deficiency, including neuropathy (Scott *et al.*, 1981). However, the concentration of AdoMet in the brain of Cbl-deficient animals, including those dying with Cbl neuropathy, is generally normal or is even raised (Lumb *et al.*, 1983; Van der Westhuyzen & Metz, 1983). This has made it less likely that impairment of transmethylation was involved. Indeed, McLoughlin & Cantrill (1986) found no change in labelled-methyl-group incorporation into brain phospholipids in Cbl-deficient fruit bats and no change in

synaptosomal and myelin lipid methylation. Further, there was no impairment of methylation of arginine in myelin basic protein from fruit bats dying of Cbl neuropathy (Deacon *et al.*, 1986).

After methyl-group transfer, AdoMet is converted into AdoHcy (*S*-adenosylhomocysteine), AdoHcy loses its adenosine group and the homocysteine remaining is regenerated into methionine by accepting a methyl group from methyl-Cbl (methionine synthetase reaction). This pathway is largely non-functional in Cbl deficiency. Thus brain has no mechanism for disposing of homocysteine. AdoHcy, by inhibiting transmethylation reactions, is held to be toxic. AdoHcy competes with AdoMet for binding sites on transmethylation enzymes. Elevation of AdoHcy levels was present in nerve tissues from Cbl-deficient pigs, and stress was laid on the decreased AdoMet/AdoHcy ratio, which was significantly lower than that in controls (Weir *et al.*, 1988). Less severe accumulation of AdoHcy was present in Cbl-deficient brain of rat, a species that does not get neuropathy.

This study was planned to investigate the significance of a raised AdoMet level in Cbl-deficient fruit-bat brain and, in view of the report by Weir *et al.* (1988), to assess the role of AdoHcy.

MATERIALS AND METHODS

Experimental animals

Cape fruit bats (*Rousettus aegyptiacus*), caught in the wild, were maintained on a pest-free diet of freshly washed or peeled fruit (bananas and papayas). The animals were fed *ad libitum*, were supplied with clean tap water daily and their cage floors were kept clean. A multi-vitamin preparation (Abidec; Parke-Davis, Cape Town, South Africa) lacking in vitamin B₁₂ was given orally to all the bats to ensure adequate intake of vitamin A, vitamin B₆, thiamin, riboflavin and niacin. Control animals were given intramuscular vitamin B₁₂ (5 µg of

Abbreviations used: Cbl, cobalamin; MMA, methylmalonic acid; AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine.

‡ To whom correspondence and reprint requests should be addressed.

Fig. 1. H.p.l.c. separation of AdoHcy and AdoMet

(a) Standards containing 800 pmol of AdoHcy and 400 pmol of AdoMet; (b) control liver; (c) control brain cortex.

cyano-Cbl/kg body wt.) every 2 weeks to maintain normal vitamin B₁₂ status.

Groups

The bats were randomly divided into four groups: one control and three experimental groups, each comprising four animals. The experimental groups were depleted of vitamin B₁₂ and received the standard fruit diet, either unsupplemented (group 1) or supplemented with L-methionine (99%, E. Merck, Darmstadt, Germany) in the proportion of 0.6 g/kg of fruit (group 2) or with L-valine (99%, Riedel-de Haen) in the proportion of 0.4 g/kg of fruit (group 3) or in the proportion of 0.55 g/kg of fruit (group 4) throughout the study. The methionine and valine were equimolar.

Experimental procedure

The protocol for this study was approved by the Animal Ethics Committee of the University of Wit-

watersrand, Johannesburg, South Africa. Before commencement of the experiment, all the bats were examined clinically, sexed and weighed. Animals were bled by cardiac puncture for measurement of vitamin B₁₂ by radioisotopic dilution assay (Simultrac-SNB kit; Becton-Dickinson, New York, NY, U.S.A.). The bats were bled again at 6 weeks, 12 weeks and 18 weeks, at which time the experiment was terminated. The three experimental groups were depleted of vitamin B₁₂ by exposure to an atmosphere of nitrous oxide (N₂O)/O₂ (1:1) for 90 min/day in a specially constructed environmental chamber in which water vapour and CO₂ were controlled (Arnstein & Neuberger, 1953). After the exposure period the bats were returned to their cages. Animals were examined weekly for development of neurological impairment. Flight characteristics were also recorded. After 6 weeks of N₂O exposure the experimental animals were vitamin B₁₂-deficient and exposure to N₂O was stopped. These animals continued to receive

Table 1. Cbl, AdoMet and AdoHcy levels, and the AdoMet/AdoHcy ratio, in livers of fruit bats

Statistical significance: **P* < 0.05 compared with controls; ***P* < 0.05 compared with Cbl-deficient group only. Abbreviations: Cbl-def, Cbl-deficient; Cbl-def, Met, Cbl-deficient, but supplemented with methionine; Cbl-def, Val, Cbl-deficient, but supplemented with valine.

Treatment	Plasma cobalamin (pmol/l)		Level in liver (nmol/g)		AdoMet/AdoHcy ratio
	Initial	18 weeks	AdoMet	AdoHcy	
Control	1890 ± 169	1990 ± 171	76.7 ± 14.8	24.0 ± 3.7	3.3 ± 1.2
Cbl-def	1997 ± 173	8 ± 4	62.9 ± 18.0	36.2 ± 8.3*	1.9 ± 0.9
Cbl-def, Met	1690 ± 190	18 ± 2	1268 ± 284**	166.7 ± 76.5**	9.1 ± 4.8
Cbl-def, Val	1750 ± 34	15 ± 2	52.8 ± 31.4	41.4 ± 19.1	1.7 ± 1.7

the vitamin B₁₂-deficient diet until the experiment was terminated at 18 weeks.

Development of neurological impairment

After 6 weeks all bats in the unsupplemented group began to show signs of neuromuscular impairment, manifested by reluctance to use the hind limbs. By 9 weeks the bats were no longer climbing, the legs being held passively, and the clutching reflex was lost. After 11 weeks flight was reduced to short hops. By 17 weeks they had ceased to fly, and spasticity was evident by head retraction and rigidity of the back.

Bats receiving supplemental methionine remained clinically normal up to 17 weeks, when they started showing signs of impaired hind-limb use.

The bats that were exposed to N₂O but received supplementation with valine remained clinically well up to 18 weeks. Control bats remained well throughout the experimental period.

At the end of the study the animals were killed by decapitation, and liver, brain and spinal cord removed immediately. Brain tissue was dissected on ice into cortex, cerebellum and brain stem. All tissues were then deproteinized with 5 vol. of cold 0.4 M-HClO₄, centrifuged, and the clear HClO₄ extracts were stored at -20 °C until assayed.

Measurement of AdoMet, AdoHcy, methionine and valine by h.p.l.c.

AdoMet and AdoHcy were separated on an h.p.l.c. system (Pye-Unicam PU4010) using a reverse-phase column (25 cm × 5 cm) containing OD-Hypersil (5 µm particle size) (Shandon Southern, Runcorn, Cheshire, U.K.). Isocratic elution of the compounds was achieved with a mobile phase of 0.1 M-sodium acetate and 0.5 M-heptanesulphonic acid adjusted to pH 4.5 and containing 4.2% acetonitrile. Detection was carried out with u.v. (254 nm)-detecting system (Pye-Unicam). H.p.l.c.-grade chemicals and solvents were used throughout. Tissue HClO₄ extracts (40–80 µl) were injected directly into the h.p.l.c. system, separation of both AdoMet and AdoHcy occurring within 14 min (Fig. 1). Calibration of the method was carried out with standards of AdoHcy, obtained from Sigma Chemical Co., and AdoMet, generously given by Dr. G. Stramentinoli, Bioresearch Laboratories, Milan, Italy.

Methionine, valine and other large neutral amino acids were separated by reverse-phase h.p.l.c. and detected by fluorescence after pre-column *o*-phthalaldehyde derivatization as described previously (Bottiglieri, 1987). The HClO₄ extracts were neutralized with phosphate buffer and NaHCO₃ before derivatization.

Statistical methods

The data were analysed by multiple comparison of means after one-way analysis of variance and significance limits obtained from Dunnett's *t*-distribution.

RESULTS

The data (Tables 1 and 2) show that, in spinal-cord and brain tissue, there were no significant differences in AdoMet and AdoHcy levels in control as compared with Cbl-deficient bats. Nor were there any differences in the AdoMet/AdoHcy ratios. In the liver there was a small,

Table 2. AdoMet and AdoHcy levels and AdoMet/AdoHcy ratios in the central nervous system of fruit bats

Results are means ± s.d. Statistical significance: **P* < 0.05 compared with controls; ***P* < 0.05 compared with Cbl-deficient group only. Abbreviations are as defined in Table 1.

Temperature	Spinal cord			Cortex			Cerebellum			Brain stem		
	Level (nmol/g)		AdoMet/AdoHcy ratio	Level (nmol/g)		AdoMet/AdoHcy ratio	Level (nmol/g)		AdoMet/AdoHcy ratio	Level (nmol/g)		AdoMet/AdoHcy ratio
	AdoMet	AdoHcy		AdoMet	AdoHcy		AdoMet	AdoHcy		AdoMet	AdoHcy	
Control	18.6 ± 2.8	2.5 ± 0.7	7.7 ± 1.6	15.4 ± 0.4	2.6 ± 0.7	5.8 ± 1.7	15.3 ± 2.1	2.5 ± 0.7	6.7 ± 2.5	13.5 ± 2.8	2.0 ± 0.3	6.7 ± 1.4
Cbl-def	17.4 ± 2.5	3.1 ± 1.0	6.1 ± 1.4	17.5 ± 1.2	3.5 ± 0.5	5.2 ± 0.9	14.5 ± 2.0	2.0 ± 0.3	7.4 ± 0.8	13.2 ± 2.4	2.2 ± 0.4	6.1 ± 0.7
Cbl-def, Met	25.0* ± 1.4	6.9* ± 2.4	3.9*** ± 1.1	20.7* ± 2.1	5.1* ± 0.7	4.1 ± 0.5	19.5 ± 4.1	4.2* ± 1.1	4.7 ± 0.7	19.2 ± 16.6	5.0* ± 1.2	3.9* ± 1.6
Cbl-def, Val	19.1 ± 3.5	4.7* ± 1.1	4.4* ± 1.5	21.1 ± 5.5	4.1* ± 0.5	5.1 ± 1.1	150 ± 2.4	2.4 ± 0.3	6.3 ± 1.5	19.5 ± 6.9	3.0 ± 1.4	6.9 ± 1.2

but significant, rise in AdoHcy and no change in AdoMet levels in control as compared with Cbl-deficient bats. Methionine supplementation caused a rise in AdoMet levels in liver and much less so in spinal cord and cortex, with a generally insignificant fall in the ratio. Amino acid analysis showed the expected rise in brain-cortex methionine in the methionine-supplemented group (803 ± 144 nmol/g) compared with the control (38 ± 8 nmol/g) and the expected rise in valine (430 ± 62 nmol/g) in the valine-supplemented group as compared with the unsupplemented group (203 ± 55 nmol/g). There were no other significant changes.

DISCUSSION

The pig showed marked falls in AdoMet/AdoHcy ratios when Cbl-deficient and developed neuropathy. The Cbl-deficient rat showed a smaller fall in ratio without developing any neuropathy. This study shows that neuropathy develops in the Cbl-deficient fruit bat without any change at all in AdoMet/AdoHcy ratios. It is clear that the development of neuropathy is unrelated to methylation, confirming other studies outlined in the Introduction.

Methionine can influence Cbl metabolism in other ways than just providing methyl groups (Chanarin *et al.*, 1985, 1989) (Scheme 1). Methionine is a precursor of polyamines. A product of this pathway is 5'-methylthioadenosine, which is reconverted into methionine and 'active' formate. Formate from this source joins the single-carbon-unit pool and is donated via the folate coenzyme in the synthesis of adenine and guanine. 5'-Methylthioadenosine, indeed, is more active than methionine in overcoming Cbl deficiency. Methionine is thus

a source of formate and this can be impaired in Cbl deficiency. In the rat at least, Cbl deficiency leads to considerable impairment in the utilization of formate. The evidence can be summarized as follows: (1) in Cbl deficiency there is a rise of endogenous formate in liver and blood as well as in brain (J. Perry, R. Deacon, M. Lumb & I. Chanarin, unpublished work); (2) formyltetrahydrofolate levels in tissues are very low in Cbl deficiency; (3) the enzyme formyltetrahydrofolate synthetase, which adds formate to tetrahydrofolate, is induced in Cbl deficiency; (4) the effect of Cbl deficiency is reversed by supplying preformed formyltetrahydrofolate, but not by supplying tetrahydrofolate; (5) substances which supply active formate, such as methionine or 5'-methylthioadenosine, reverse Cbl deficiency.

Thus it would seem sensible to explore formate metabolism in Cbl-deficient brain, and the defect in the brain may be essentially the same as that found in liver and marrow cells.

The amelioration of Cbl neuropathy by valine does put a defect of MMA metabolism in the central nervous system back into play, and this aspect, too, needs exploration. As valine supplementation would aggravate a lesion in the MMA pathway, the effect of valine is at present difficult to understand.

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Scheme 1. Metabolic pathways showing the metabolism of methionine and AdoMet

Abbreviations; CHO, formyl; CH₂, methylene; CH₃, methyl; H₄, tetrahydro.

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