Polyamine Concentrations in the Brain of Vitamin B_{12}-Deficient Rats

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To study the pathophysiology of the neuronal degeneration in vitamin B_{12} deficiency, we investigated the concentrations of the polyamines putrescine, spermidine, and spermine in brain regions and liver using high-performance liquid chromatography with fluorescence detection. Male Wistar rats were fed either a control or vitamin B_{12}-deficient diet for 20 weeks. No remarkable behavioral changes were observed. Serum vitamin B_{12} and hepatic methionine concentrations were significantly lower and hepatic homocysteine was elevated in rats fed vitamin B_{12}-deficient diet than in controls. Vitamin B_{12} deficiency was associated with decreased concentrations of spermidine, spermidine in liver and some regions of brain, although there were no observed abnormalities in behavior. These results suggest that vitamin B_{12} deficiency may play a role in neuronal degeneration through the disturbance of polyamine concentrations in rat brain. Exp Biol Med 228:1069–1071, 2003

Key words: polyamine; putrescine; spermidine; spermine; Vitamin B_{12} deficiency

Vitamin B_{12} deficiency is usually associated with pernicious anemia and characterized pathologically by degeneration of the white matter of the spinal cord. Neural pathology is most evident in the posterior and lateral columns in the peripheral nerves and brain, and clinical expressed as paresthesia, impairment of position and joint sense, sensory ataxia, and paraparesis. Subacute combined degeneration was described by Leichtheim in 1887 and the first complete clinical and pathological account was provided by Russel *et al.* in 1900 (1).

Vitamin B_{12} (cobalamin) has a complex ring structure, similar to a porphyrin, with a cobalt ion complexed at its center. The vitamin is synthesized exclusively by microorganisms and localized primarily in liver, where it exists as methylcobalamin, adenosylcobalamin, and hydroxocobalamin. Methylcobalamin serves as a coenzyme in the combined conversion of homocysteine to methionine and methyl-tetrahydrofolate to tetrahydrofolate. L-methionine first condenses with ATP to form S-adenosylmethionine (2). Ornithine decarboxylase and S-adenosylmethionine decarboxylase are involved in the regulation of polyamine synthesis in mammals (3). Also, polyamine oxidase is present in liver peroxisomes where it oxidizes spermine to spermidine, which is further oxidized to putrescine (4). The polyamines putrescine, spermidine, and spermine are present in almost all cells and play important roles in protein synthesis, cell division, and cell growth. In addition, specific interactions of polyamines with a number of different types of ion channels have recently been reported (5–11).

Below we summarize results from a study aimed at examining the possible relationship between vitamin B_{12} deficiency and alterations in polyamine concentrations in brain and liver in the rat.

Materials and Methods

Experimental Animals. Male Wistar rats were bred and maintained in plastic cages. Animals were randomly divided into two dietary groups. Group 1 (vitamin B_{12} deficient) consisted of 10 rats fed a vitamin B_{12}-deficient diet (Oriental Yeast Co., Ltd, Tokyo, Japan). Group 2 (control) consisted of 10 rats fed the identical diet as that fed to Group 1 except that it was supplemented with vitamin B_{12} (5.1 μg/100 g diet). Feeding of the test diets was initiated one month after birth and continued for 20 weeks. Animals were killed by decapitation under anesthesia with chloroform inhalation. Experiments were conducted using procedures approved by the Aichi Medical University Committee on the Guide for the Care and Use of Laboratory Animals and in accordance with NIH guidelines.

Determination of Serum Vitamin B_{12} Level. Levels of vitamin B_{12} were determined using a modified version of the method of Kihara *et al.* (12). In brief, serum (100 μl) was mixed with 1 ml of tracer (^{57}Co-vitamin B_{12}). The mixture was vortexed, and placed in a boiling water bath for 15 min, while being protected from light. After cooling to room temperature, 500 μl of binding proteins was mixed and incubated for 1 hr at room temperature. After

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centrifugation (10 min, 3,000 rpm), the supernatants were decanted and subjected to a gamma counter.

Determination of Amino Acids in Liver. Amino acids were determined according to the method of Perry *et al.* (13). Each sample of liver was homogenized in 5 volume of ice-cold 0.2 M perchloric acid at 4°C. The pH of the supernatant was adjusted to 2.2 by the addition of 0.5 M KOH. After being chilled it was centrifuged to remove precipitated potassium perchlorate. Deproteinized aqueous extracts of liver were then stored at −80°C until chromatography on the amino acid analyzer. Amino acids and other ninhydrin-positive compounds were determined on a Beckman System 7300 automatic amino acid analyzer, where lithium citrate buffers were used instead of sodium citrate. The columns were operated at a temperature of 35°C until glutamine had been eluted from the cation exchange resin, and thereafter at 70°C.

Preparation of Samples for Polyamines. The brain was removed and dissected into cerebral cortex, brain stem (pons/medulla), cerebellum, and spinal cord using the atlas of Paxinos and Watson (14). Liver also was removed. Tissues were immediately weighed, quickly frozen by placement on dry ice, and stored at −80°C until analysis. Thawed tissue was homogenized by a sonication setting at 40 watts for 20 sec in cold 0.2 M perchloric acid. The homogenate was centrifuged (15,000 g at 4°C for 15 min) and the residue was passed through a 10,000-molecular-weight-cutoff filter (Millipore, Bedford, MA, USA).

Determination of Polyamine Concentrations. Polyamines such as spermidine, spermine, and putrescine were determined using a sensitive and simple liquid chromatographic assay with fluorometric detection. Polyamines were determined by the modified method of Kabra and Lee (15). Briefly, filtrates prepared as described previously were dansylated as follows. In a polypropylene tube, 50 μl of 1,6-diaminohexane, internal standard (200 pmol per 50 μl), 200 μl of saturated sodium carbonate, and 200μl of dansyl chloride (10 mg/ml) were added to 50 μl of the supernatant. The capped tube was vortexed for 15 sec prior to incubation at 60°C for 60 min. The tube was cooled to room temperature, polyamines were extracted by elution from a Symmetry column C_{18} (4.6 × 75 mm, 3.5 μm particle size; Waters, Milford, USA) and then separated on a reversed-phase column by gradient elution. The complete analysis of putrescine, spermidine, and spermine was completed within 30 min. Aliquots of 100 μl of the solutions were injected into a prepacked ODS 3.5 μm column (4.6 × 75 mm) protected by an ODS precolumn. The dansylated polyamines were eluted with a mobile phase using acetonitrile at a flow rate of 1.5 ml/min with a continuous gradient changing from 50% to 100% for 15 min, 100% for 3 min, and from 100% to 50% for 12 min. Fluorescence of the dansylated polyamines was monitored with a Waters fluorescence detector (excitation filter at 340 nm and emission filter at 515 nm). All statistical comparisons were done using a nonparametric test (Mann-Whitney U test) with P value less than 0.05 considered as a significant difference.

Results

Growth and Serum Vitamin B_{12} Status of Experimental Animals. There was no significant difference in mean body weight of rats fed the control and vitamin B_{12}-deficient diets for 20 weeks (Table I). Moreover, no remarkable behavioral changes were observed in rats chronically fed the vitamin B_{12}-deficient diet. Serum vitamin B_{12} in rats fed the vitamin B_{12}-deficient diet was significantly lower than that of controls (Table I).

Levels of Methionine and Homocysteine in the Liver. The mean concentration of methionine in the liver of vitamin B_{12}-deficient rats was significantly lower than that of controls (Table I), while the mean concentration of homocysteine was significantly higher (Table I).

Levels of Polyamines in the Liver and Brain. Concentrations of spermidine in liver, cerebral cortex, cerebellum, and brain stem were significantly lower in the vitamin B_{12}-deficient rats than in controls. In the spinal cord, the concentration of spermidine tended to be lower in spinal cord of the vitamin B_{12}-deficient rats than in control animals, although the difference was not statistically significant (Table II).

The concentration of spermine was significantly lower in liver, cerebral cortex, and brain stem in vitamin B_{12}-deficient rats than in control rats. Spermine content in cerebellum and spinal cord also tended to be low in the vitamin B_{12}-deficient group compared with controls but the differences were not statistically significant (Table II).

Putrescine was significantly lower in the liver of vitamin B_{12}-deficient rats than in controls (Table II). Concentrations of putrescine were below the limit of detection in cerebral cortex, cerebellum, and spinal cord of both dietary treatment groups.

Discussion

As expected, weaned rats fed a diet deficient in vitamin B_{12} for 20 weeks had significantly lower mean concentrations of vitamin B_{12} and hepatic methionine and elevated hepatic homocysteine without inducing evident behavioral

Table I. Body Weights, Serum Vitamin B_{12}, and Hepatic Methionine and Homocysteine Concentrations in Rats Fed Vitamin B_{12}-Deficient and Control Diet for 20 Weeks[a]

Rats	Control	Vitamin B_{12} deficient	U Test
Body weights (g)	351 ± 9.4	361 ± 6.0	$P = 0.38$
Vitamin B_{12} (pg/ml)	1139 ± 91	153 ± 14	$P = 0.0001$[b]
Methionine (mmol/g)	118 ± 10	8.2 ± 3.6	$P = 0.0002$[b]
Homocysteine (mmol/g)	5.0 ± 1.0	16.9 ± 3.4	$P = 0.0042$[b]

[a] Data are mean ± SEM for 9–10 experimental animals.
[b] Denotes statistically significant difference (Mann-Whitney U test).

Table II. Spermidine, Spermine, and Putrescine Concentrations in Tissues from Rats Fed Vitamin B_{12}-Deficient and Control Diets[a]

	Control rats (N = 10)	Deficient rats (N = 10)	U test
Liver			
Spermidine	113.7 ± 23.1 (μg/g)	9.4 ± 2.1	$P = 0.0003$[b]
Spermine	130.4 ± 28.6	9.4 ± 2.1	$P = 0.0005$[b]
Putrescine	3.0 ± 0.5	1.4 ± 0.1	$P = 0.0018$[b]
Cerebral cortex			
Spermidine	49.9 ± 10.2	9.9 ± 2.7	$P = 0.0046$[b]
Spermine	42.3 ± 12.4	11.0 ± 3.3	$P = 0.026$[b]
Cerebellum			
Spermidine	11.3 ± 2.8	2.6 ± 0.9	$P = 0.0086$[b]
Spermine	7.9 ± 3.7	1.3 ± 0.5	$P = 0.0968$
Brain stem			
Spermidine	14.2 ± 3.7	4.9 ± 1.2	$P = 0.0175$[b]
Spermine	9.6 ± 4.0	1.0 ± 0.2	$P = 0.0275$[b]
Putrescin	18.1 ± 10.0	0.8 ± 0.4	$P = 0.0525$
Spinal cord			
Spermidine	4.2 ± 1.6	1.4 ± 0.6	$P = 0.1054$
Spermine	0.7 ± 0.4	0.5 ± 0.3	$P = 0.7071$

[a] Mean ± SEM for N = 10 tissue samples.
[b] Denotes statistically significant difference (Mann-Whitney U test).

changes. The putrescine portion of spermidine and spermine is derived from L-ornithine, while the diaminopropane portion originates from L-methionine via the intermediate formation of S-adenosylmethionine. These changes were associated with decreases in the concentrations of the polyamines in liver and some regions of the brain. Recently, endogenous polyamines, and especially spermine, have been found to cause neural blockade and modulation of a number of types of ion channel (5–11). Intracellular spermine is responsible for the intrinsic gating and rectification of strong inward rectifier K^+ channels by directly plugging the ion channel pore. Intracellular spermine also causes inward rectification at some subtypes of Ca^{2+} permeable glutamate receptors in the central nervous system, again by plugging the receptor channel pore, and spermine can even permeate the ion channel of these receptors. Extracellular spermine has multiple effects at the N-methyl-D-aspartate subtype of glutamate receptor, including stimulation that increases the size of NMDA receptor currents and voltage-dependent block. Interactions of polyamines with other types of cation channels have been reported.

Overall, vitamin B_{12}-deficiency results in a methionine decrease and a homocysteine increase in the liver, and consequently in decreases of polyamines concentrations such as spermidine, spermine, and putrescine in the liver and brain. These results suggest that the changes in endogenous polyamine concentrations may play a significant role in the neuronal degeneration of vitamin B_{12} deficiency. Scalabrino *et al.* (16) reported that gastrectomized rats are deprived of intrinsic factor and develop a spongy demyelination in the white matter of the spinal cord and changes in L-ornithine decarboxylase activity and polyamine contents. Further investigations are needed to elucidate the relationship between vitamin B_{12} status, polyamine metabolism, and the impact of this interaction on neurological function.

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