Journal of the Neurological Sciences, 122 (1994) 140–143

JNS 4251

Ultra-high dose methylcobalamin promotes nerve regeneration in experimental acrylamide neuropathy

Tetsuya Watanabe, Ryuji Kaji *, Nobuyuki Oka, William Bara and Jun Kimura

Department of Neurology, Kyoto University Hospital, Shogoin, Sakyoku, Kyoto 606-01, Japan

(Received 11 June, 1993)
(Revised, received 19 October, 1993)
(Accepted 28 October, 1993)

Key words: Methylcobalamin; Acrylamide neuropathy; Nerve regeneration; Therapy; Compound muscle action potential

Summary

Despite intensive searches for therapeutic agents, few substances have been convincingly shown to enhance nerve regeneration in patients with peripheral neuropathies. Recent biochemical evidence suggests that an ultra-high dose of methylcobalamin (methyl-B12) may up-regulate gene transcription and thereby protein synthesis. We examined the effects of ultra-high dose of methyl-B12 on the rate of nerve regeneration in rats with acrylamide neuropathy, using the amplitudes of compound muscle action potentials (CMAPs) after tibial nerve stimulation as an index of the number of regenerating motor fibers. After intoxication with acrylamide, all the rats showed equally decreased CMAP amplitudes. The animals were then divided into 3 groups; rats treated with ultra-high (500 μg/kg body weight, intraperitoneally) and low (50 μg/kg) doses of methyl-B12, and saline-treated control rats. Those treated with ultra-high dose showed significantly faster CMAP recovery than saline-treated control rats, whereas the low-dose group showed no difference from the control. Morphometric analysis revealed a similar difference in fiber density between these groups. Ultra-high doses of methyl-B12 may be of clinical use for patients with peripheral neuropathies.

Introduction

In spite of the common belief that peripheral nerves are capable of extensive regenerative growth after injury, clinical recovery seldom is complete in patients with peripheral neuropathy. Various agents are known to enhance peripheral nerve regeneration, but not many have proved helpful in clinical settings (Horowitz 1989).

Vitamin B12 has an important role in methyl transfer reactions through folate metabolism, and a deficiency of it causes hematologic and neurologic disorders such as megaloblastic anemia and peripheral neuropathy. Neurologic manifestations however are not always accompanied by hematologic abnormalities nor are they reversible by folate supplement (Shorvon 1980; Lindenbaum 1988). Vitamin B12 may have a specific action on the nervous system, in addition to its action on the hematopoietic system.

Recent biochemical evidence suggests that methylcobalamin (methyl-B12) acts directly as a methyl donor in DNA metabolism (Pfohl-Leszkowicz 1991). More importantly, ultra-high concentrations (> 1 μM or 1.34 μg/ml of methyl-B12) up-regulate gene transcription, which may in turn increase protein synthesis for nerve regeneration.

We have used physiological and morphological methods to investigate the effect of ultra-high (500 μg/kg) versus low (50 μg/kg) doses of methyl-B12 on peripheral nerve regeneration in experimental acrylamide neuropathy.

Methods

Animal treatment

Twenty-six male Wistar rats (body weight 250–300 g) were treated with acrylamide (25 mg/day i.p. for 5 days a week for 4 weeks). All animals developed clinical signs of neuropathy such as hind limb weakness and ataxia by the end of administration (day 0). The animals were allowed to recover for 90 days (day 0–90), and divided into 3 groups: 12 rats treated with ultra-high doses of methyl-B12 (Eisai Co. Ltd., Tokyo, Japan) (high-dose group; 500 μg/kg body weight/day, i.p.), 5 treated with low doses methyl-B12 (low-dose group; 50 μg/kg, i.p.) and 9 treated with normal saline (control group). Methyl-B12 or saline was injected 5 days a week for 12–13 weeks after day 0.

* Corresponding author. Tel.: 075-751-3770; Fax: 075-761-9780.

CMAP measurement

Physiological evaluations were made before and immediately after acrylamide administration and 3 times during the recovery phase; during the intervals from day 25–35 (indicated as "day 30"), day 55–65 ("day 60") and day 85–95 ("day 90").

Rats were anesthetized with a mixture of ketamine (100 mg/kg, i.p.), xylazine (10 mg/kg, i.p.) and atropine (0.05 mg/kg, i.p.). A pair of needle electrodes (Nihon Kohden NE233S) was placed at the ankle with the cathode on the medial aspect. A supramaximal stimulating current was applied to the tibial nerve at the ankle with a constant voltage stimulator (Medelec MS6). We recorded the compound muscle action potentials (CMAPs) from the small hind foot muscles with a recording needle electrode (Nihon Kohden NE233S) on the dorsum and a reference at the second digit. CMAP amplitudes, which reflect the number of innervated muscle fibers, were measured from the initial negative to positive peaks. Recordings usually were made on one side, the same side being used throughout the study. The other side, however, was used whenever the reproducibility of the potential became insufficient because of local hematoma formation. Repetitive nerve stimulation was performed at 3 Hz, whenever possible, in order to assess the neuromuscular transmission (Kimura 1989). The skin temperature at the hind limb, which was monitored throughout the recording session, was kept between 30 and 32°C.

Morphometry

The morphological study used specimens of the tibial nerve at the midcalf taken from 3 animals on day 60, each specimen being representative of a specific group with CMAP amplitude values within 1 SD of the mean. The analysis method used has already been described elsewhere (Kaji 1989).

Statistical analysis was made with a microcomputer (Macintosh II) with StatView® software (Abacus Concepts, Inc., Berkeley, CA, USA).

Vitamin B12 assay

Serum and CSF concentrations of vitamin B12 were measured in two animals from high-dose and low-dose groups during the day 90 period by competitive protein biding assay, which was performed at SMI Bristol Laboratories, Tokyo, Japan, on a commercial basis. Normal serum levels in the rat are 683 ± 63 pg/dl (mean ± SD, $n = 4$) (Sagawa et al. 1987).

Results

CMAP amplitudes

The CMAP amplitudes decreased sharply after acrylamide treatment (Before and After in Fig. 1, Table 1). These amplitudes did not differ statistically among the groups ($P = 0.861$ for Before; $P = 0.416$ for After; one-way ANOVA). In the recovery phase, the high-dose group showed a significantly higher amplitudes than the other groups on day 30 ($P < 0.001$; one-way ANOVA, Fisher's PLSD), day 60 ($P < 0.001$), and day 90 ($P = 0.001$). The low-dose and the control groups did not show significant difference ($P > 0.05$). Representative recordings from each group are shown in Fig. 2. Repetitive nerve stimulation at 3 Hz produced no significant decrement (< 20%) in CMAP amplitudes, suggesting that the decreased amplitudes are not caused by impaired neuromuscular transmission but represent the reduced number of motor fibers innervating the muscle.

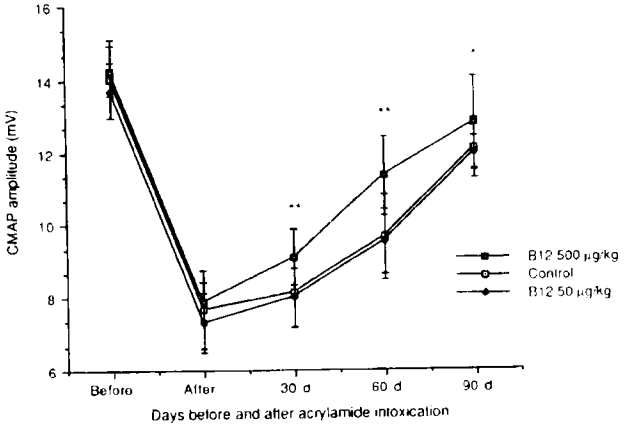

Fig. 1. Comparison of CMAP changes among the groups. Bars indicate standard deviations. $^{**}P < 0.001$, $^{*}P = 0.001$

Body weight

The three groups showed body weight changes which did not significantly differ during the experiment (ANOVA $P > 0.05$; Fig. 3).

TABLE 1

CMAP CHANGES (mean + SD, mV)

Groups	Control	High-dose	Low-dose
Before	13.92 ± 1.16	13.89 ± 0.84	13.72 ± 0.77
$P = 0.86$ *	($n = 9$)	($n = 12$)	($n = 5$)
After	7.76 ± 1.20	7.82 ± 0.94	7.32 ± 0.81
$P = 0.42$ *	($n = 9$)	($n = 12$)	($n = 5$)
Day 30	7.70 ± 1.11	9.32 ± 0.83	7.99 ± 0.80
$P < 0.001$ *	($n = 9$)	($n = 11$)	($n = 5$)
Day 60	9.30 ± 0.98	11.67 ± 0.99	9.51 ± 0.89
$P < 0.001$ *	($n = 8$)	($n = 10$)	($n = 5$)
Day 90	11.41 ± 0.79	12.8 ± 0.98	11.93 ± 0.46
$P = 0.001$ *	($n = 5$)	($n = 8$)	($n = 2$)

* P-value of the statistical significance in the difference among 3 groups (one-way ANOVA). n: number of samples. Some animals were lost due to anesthetic accidents during the observation period.

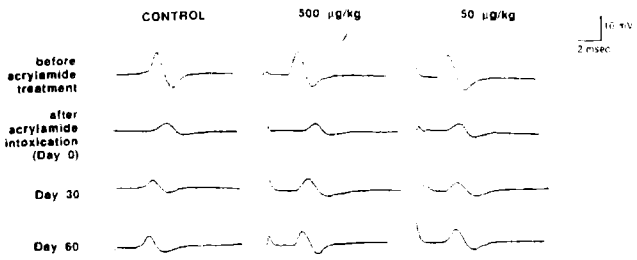

Fig. 2. Representative serial recordings of CMAPs in the same animals.

Morphometry

Results of the morphometry performed on each animal whose CMAP is shown in Fig. 2 are given in Fig. 4. Fiber densities were 2125/mm^2 in the control, 3290/mm^2 in the low-dose, and 5360/mm^2 in the high-dose groups. The histograms showed increased numbers of small and medium-sized myelinated fibers with two prominent peaks at 4 and 9 μm in the high-dose group, as compared to the other groups. The maximum fiber diameter was 15 μm in the high-dose group, the largest among the groups.

Vitamin B12 levels

Serum levels of vitamin B12 were 2500 μg/ml in a rat from the high-dose group, and 180 μg/ml in one from the low-dose group. CSF levels were 15.2 μg/ml in the former, and 1.2 μg/ml in the latter.

Discussion

We have shown here that animals treated with an ultra-high dose of methyl-B12 promptly recovered CMAP amplitude after acrylamide intoxication, as compared to the controls and those treated with a low-dose of methyl-B12. Because the CMAP amplitude represents the number of innervated muscle fibers, its prompt recovery indicates accelerated reinnervation of muscles by axonal regeneration in the ultra-high dose group. In fact, nerve specimens from representative animals in each group provided morphometric evidence of increased numbers of large-diameter fibers in that group. Although the animals lost body weight after acrylamide intoxication, the high dose of methyl-B12 did not act through the improvement of the general nutritional conditions, because the body weight recovery curve did not differ among the three groups.

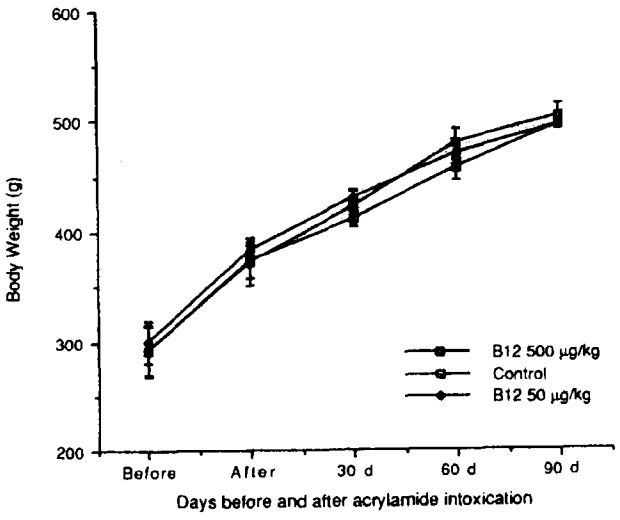

Fig. 3. Body weight change in three groups during the experiment.

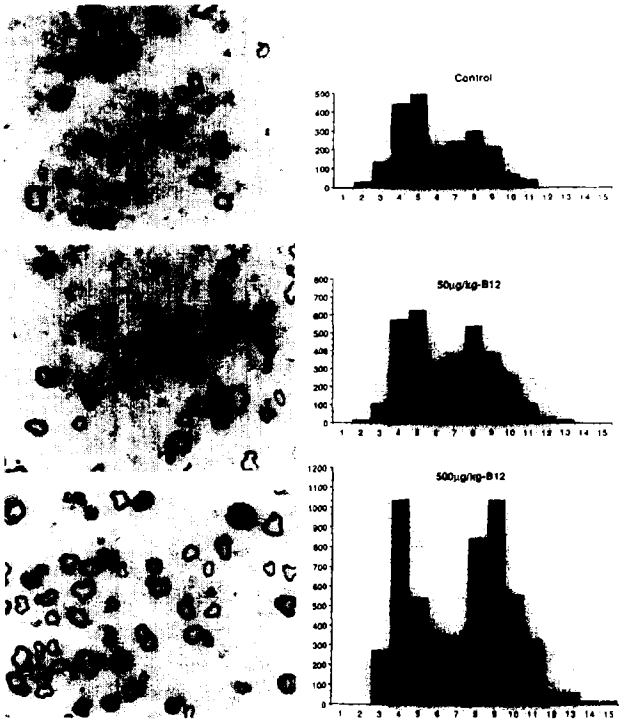

Fig. 4. Morphometric study in the groups in day 60. Representative micrographs used for analysis are shown on the left.

The response of a neuron to axonal damage is best exemplified by wallerian degeneration. Soon after axonotomy, the soma undergoes a process called central chromatolysis, which probably represents a metabolic shift from supporting transmitter production and synaptic function to production of materials needed for nerve regeneration (Seckel 1990). This change should involve a surge in transcription of an entirely different set of genes.

Acrylamide neuropathy is regarded as a prototype of distal axonal neuropathy (Spencer and Schaumberg 1974). It pathogenic mechanism has been intensively studied (Pleasure et al. 1969; Prineas 1969; Spencer and Schaumberg 1974; Spencer and Schaumberg 1977). A demise of the metabolism in the soma results in a length-dependent dying back of the distal axon (Prineas

1969). Impaired axoplasmic flow may result in pathologic changes most pronounced in the distal part of the axon (Pleasure et al. 1969). Acrylamide may directly damage the axonal membrane (Spencer and Schaumberg 1977). Whatever the mechanism, the neuron should sustain a metabolic shift similar to that after wallerian degeneration after acrylamide intoxication.

The role of vitamin B12 in neuronal metabolism is a matter of controversy. The dissociation of neurological and hematological improvements after folate supplement in vitamin B12 deficiency (Shorvon 1980; Lindenbaum 1988) is unexplained because most of the previously known metabolic pathways requiring B12 are through transmethylation utilizing tetrahydrofolate and *S*-adenosylmethionine. A recent biochemical study has demonstrated that methyl-B12 acts directly as a methyl donor in DNA methylation which normally down-regulates gene expression (Cedar 1988; Pfohl-Leszkowicz 1991). Interestingly, at high concentration (> 1 mM or 1.34 μg/ml), methyl-B12 may up-regulate gene transcription possibly by competitive inhibition of DNA methylation with *S*-adenosylmethionine (Pfohl-Leszkowicz 1991). The CSF levels of vitamin B12 in low and high dose groups in our study fell exactly into the ranges that down- or up-regulate gene expression, respectively.

Although the precise biochemical mechanism of action of ultra-high doses of methyl-B12 is unknown, we interpret the present results as reflecting its important physiologic action on metabolism. Because acrylamide neurotoxicity is not likely to be through the known metabolic pathways of vitamin B12, we speculate that the effect of ultra-high dose methyl-B12 is not limited to acrylamide neuropathy but is seen in other axonal neuropathies.

The high but not the low dose group showed accelerated nerve regeneration. The biologic activity of methyl-B12 may not be limited to that of a vitamin which corrects metabolic derangements only when there is a deficiency of it. Methyl-B12 also may have a profound effect on metabolism when present in sufficient amounts. Results of recent clinical trials (Okawa 1990) that used high doses of vitamin B12 to treat sleep–wake rhythm disorder have indicated it has biologic action even in the non-deficient condition.

We conclude that ultra-high doses of methyl-B12 may provide a therapy that enhances peripheral nerve regeneration in various human neuropathies.

Acknowledgments This work was supported by Scientific Research grants (A-01144096, C-04670487, A-04404043) from the Japanese Ministry of Education, Science and Culture and Grants-in-Aid for amyotrophic lateral sclerosis and peripheral neuropathy from the Japanese Ministry of Health and Welfare. We are indebted to Dr. M. Kameyama for helpful advice.

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