Pyridoxine (Vitamin B6) Neurotoxicity: Enhancement by Protein-deficient Diet

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Key words: pyridoxine; B6-vitamers; peripheral neuropathy; sensory neuropathy; protein nutrition.

Large doses of pyridoxine cause injury to the primary sensory neurons in trigeminal and dorsal root ganglia of animals and patients subjected to megavitamin therapy. The increased hazard to subjects with reduced renal excretory function has been explored previously. In the present work, the neurotoxicity of pyridoxine for rats was found to be increased by dietary protein deficiency. A mere 3 or 7 days of pretreatment with either of two protein-deficient diets was sufficient to accelerate and intensify the clinical neurological signs and histological lesions from pyridoxine injections. These results are caused, at least in part, by loss of body weight, decreased protein binding in serum and decreased consumption of water and decreased volume of urine, which reduce the urinary losses of the toxicant. The vitamers related to pyridoxine (pyridoxal, pyridoxamine) and the coenzyme (pyridoxal 5-phosphate) did not cause clinical signs or lesions similar to those produced by pyridoxine even when injected in maximum tolerated doses. Neither a protein-deficient diet nor bilateral nephrectomy changed the results with the vitamers. Copyright © 2004 John Wiley & Sons, Ltd.

INTRODUCTION

Although the water-soluble B-vitamins are often considered to be non-toxic, there are many reports of neurotoxicity from large doses of pyridoxine (vitamin B6) prescribed for the treatment of various diseases (‘megavitamin’ therapy). Pyridoxine is especially toxic to the peripheral nervous system (Antopol and Tarlov, 1942; Krinke et al., 1985; Windebank et al., 1985; Xu et al., 1989; Jortner, 2000). This aspect of the vitamin has special interest because it is not known if its toxicity is related to one or more of its numerous physiological functions or to a still unknown metabolite of the original molecule or of the other vitamers to which it may be converted in vivo. In addition, large doses of pyridoxine cause severe damage to primary sensory neurons and nerves that mimic axonal reaction with central chromatolysis. This feature makes it a very useful model for neurotoxicology research.

Recently, we reported that reduced renal excretion of pyridoxine could contribute to the development of neurotoxicity in rats (Levine and Saltzman, 2002). In the course of that work, we noticed that food deprivation could influence the toxic effects of large doses of this vitamin. In the present work we have explored the effects of various types and durations of dietary restriction on the neurotoxicity of pyridoxine.

Pyridoxine is interconvertible in vivo with the vitamers pyridoxal and pyridoxamine (McCormick and Chen, 1999). In order to find the mechanism for the neurotoxicity of pyridoxine, it was important to determine if these vitamers caused similar clinical signs and histological lesions as the parent vitamin.

MATERIALS AND METHODS

Lewis rats of both sexes, bred in this laboratory, were kept in plastic cages in groups of four with hardwood litter and were fed Rodent Diet 5001 (PMI Feeds, St Louis, MO) ad libitum until they weighed 150–250 g. At that time, metal grids were inserted in the cages to reduce coprophagy, and dietary restrictions were imposed. Water was always freely available and lights were on from 6 a.m. to 6 p.m. The dietary restrictions were:

(i) The usual diet (5001) was provided but only every other day.
(ii) A semi-synthetic, protein-free diet (based on AIN 76A) (Dyets, Bethlehem, PA) was freely available every day as the sole nutrient.
(iii) Sucrose cubes (Domino Dots, Domino Sugar Corp., New York) were freely available every day as the sole nutrient.

Each experiment included a control group in which no dietary restrictions were imposed. On the third or seventh day after the start of dietary restrictions the rats were treated with pyridoxine hydrochloride (Sigma Chemical Company): 600 mg kg⁻¹ dissolved in distilled water at 60 mg ml⁻¹ or dissolved in a solution of 25 mg ml⁻¹ sodium bicarbonate. This additive reduced the acidity of the hydrochloride and thereby avoided the mild peritoneal irritation caused by the hydrochloride. Alternatively, an equimolar amount of the free pyridoxine base (500 mg kg⁻¹) was dissolved at 50 mg ml⁻¹ in water. A preliminary experiment

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showed that all three preparations caused identical neuro-
toxicity. The dose was 10 ml kg \(^{-1}\) body weight injected
intraperitoneally in the morning and again in the afternoon
on three or four consecutive days. Dietary restrictions were
continued during the treatment period.

The experiments were evaluated by clinical signs: 1+,
slight weakness or minimal splaying of the hindlimbs; 2+,
moderate weakness and splaying of hindlimbs and slight
weakness and splaying of forelimbs; 3+, severe weakness
and immobility or lateral recumbency. Hindlimb exten-
sions was impaired, as described by Jolicœur et al. (1979),
but crossed legs and curled toes were not observed.

One day after the last treatment, the rats were anesthet-
ized with CO\(_2\) and exsanguinated through the inferior vena
cava. Serum was analyzed in a Roche-Hitachi model 747–
100 analyzer. The skull was opened, the brain removed
and the trigeminal nerves and ganglia were fixed in situ with Bouin’s fluid. The skulls were decalcified and
coronal sections taken at two levels through the ganglia.
Paraffin-embedded blocks were cut at 5 µm and stained
with hematoxylin and eosin or cresyl violet for Nissl bodies.
Histological lesions of the large trigeminal neurons were
loss of Nissl bodies (central chromatolysis), peripheral dis-
placement of nuclei and irregular or indented nuclear
membranes, as described by others (Antopol and Tarlov,
1942; Krinke et al., 1985; Windebank et al., 1985; Xu et al.,
1989; Jortner, 2000). When these changes were present in
all large neurons, the lesions were designated as severe. In
moderate lesions, chromatolysis and nuclear displacement
were common but nuclear deformation was rare. In mild
lesions, nuclear displacement was less common and chro-
matolysis was less severe. Evaluations were done at 440 × magnification, on randomized slides, without knowledge
of the treatment.

Experiments on the vitamers were done as described
above, but in addition anephric rats were utilized as
described previously (Levine and Saltzman, 1997).

RESULTS

Control rats fed their usual diet of 5001 pellets with-
out restriction, studied 1 day after 3 days of pyridoxine
injections i.p., and no clinical signs, had only minimal
histological lesions in the trigeminal ganglia. Even 4 days
of pyridoxine treatment caused only slight (or no) clinical
signs (Table 1) but the ganglia had lesions, albeit mild.

Consumption of the usual diet restricted to every other
day, starting 3 or 7 days before pyridoxine treatment and
continuing to the end, did not enhance the neurotoxicity
(Table 1).

In contrast, rats fed the protein-free semisynthetic diet,
or only sucrose cubes, starting 3 or 7 days before pyridoxine
and continuing through the treatment period, already
had mild neurological signs after 3 days of pyridoxine
injections that progressed to severe signs after the fourth
day of treatment (Table 1). Their histological lesions in
trigeminal ganglia were moderate after 3 days and severe
after 4 days of treatment. In three additional experiments
(not included in Table 1) two groups of rats were given
only half the usual dose for the four daily pyridoxine injec-
tions i.p., or they were given the full dose by oral gavage
instead of i.p. In all these experiments the sucrose-fed rats
had more severe signs and/or lesions than the control rats

on the unrestricted diet. Thus, enhancement by dietary
restriction was not limited to the particular pyridoxine dose
and route used for the experiments recorded in Table 1.

Rats pretreated with sucrose as the sole nutrient might
have been subjected to vitamin deficiency during the
pretreatment period while they were losing weight. In
an additional experiment (not included in Table 1), this
potential lack was avoided by twice-daily subcutaneous
injections of a multivitamin preparation during the pre-
treatment period. Sucrose-fed rats given the additional
multivitamin pretreatment responded in the same way as
sucrose-fed rats from Table 1 to the subsequent challenge
with 4 days of the usual dose of pyridoxine. In accordance
with the experiments in Table 1, they all developed severe
clinical signs, in contrast to the lack of signs in the control
cats fed their usual diet who also had been pretreated
with vitamins. Therefore, the vitamin status during the pre-
liminary period of dietary restriction had no influence on
the subsequent neurotoxicity of large doses of pyridoxine.

Relation of body weight changes to pyridoxine
neurotoxicity

Rats fed the sucrose diet for 7 days before inception
of pyridoxine injections had lost 27 g (average) of body
weight during that period (16% of initial weight). Rats
fed sucrose for only 3 days had lost only 21 g (average
12% of initial body weight). Rats in both of these sucrose-
fed groups lost an additional 20 g during 4 days of pyridox-
ine treatment (12% of initial body weight). Rats on the
protein-free diet also lost considerable body weight during
the preliminary 7 days and during the treatment period.
All these rats developed clinical signs and severe lesions
after the pyridoxine treatments. The control rats fed their
usual diet gained rather than lost weight before the injec-
tions started and they lost only 5 g after 4 days of treat-
ment with pyridoxine. They did not develop clinical signs.

Rats fed their usual diet every other day for 7 days
before treatment lost much less weight, only 5 g (average),
and those fed every other day for only 3 days managed to
gain a small amount of weight (2 g) before pyridoxine

treatment. They did not develop clinical signs. Therefore, in all

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Table 1—Pyridoxine neurotoxicity is enhanced by prior dietary restrictions

<table>
<thead>
<tr>
<th>Dietary restriction (and start time)</th>
<th>Clinical signs, average</th>
</tr>
</thead>
<tbody>
<tr>
<td>After 3 days of i.p. pyridoxine</td>
<td>After 4 days of i.p. pyridoxine</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>Usual diet, no restriction</td>
<td>0 (n = 4)</td>
</tr>
<tr>
<td>Usual diet q.o.d. (^{a})</td>
<td>0.6 (n = 8)</td>
</tr>
<tr>
<td>Protein-free diet</td>
<td></td>
</tr>
<tr>
<td>Day 3</td>
<td>0.5 (n = 4)</td>
</tr>
<tr>
<td>Day 7</td>
<td>0.8 (n = 10)</td>
</tr>
<tr>
<td>Sucrose diet</td>
<td>0.8 (n = 10)</td>
</tr>
</tbody>
</table>

\(^{a}\) Start time: number of days before first pyridoxine injection when the diet was started. Diet was continued until the end of the experiment.

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the experiments loss of body weight correlated well with enhanced neurotoxicity of pyridoxine.

Analysis of serum after 3 or 4 days of pyridoxine treatment did not reveal any differences between sucrose-fed rats and 5001-fed controls in levels of glucose, urea, creatinine, electrolytes or enzymes. However, total protein levels were lower in the sucrose-fed rats (4.80 g dl$^{-1}$, SD = 0.43, $n = 18$) than in the 5001-fed controls (5.40 g dl$^{-1}$, SD = 0.33, $n = 13$), as could be expected.

**Table 2—Toxicity of pyridoxine and vitamers**

<table>
<thead>
<tr>
<th>Vitamer Pretreatment</th>
<th>Non-lethal dose (mmol/kg$^{-1}$)</th>
<th>Clinical signs and histological lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridoxine-HCl</td>
<td>None</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td>Sucrose diet</td>
<td>2.3–5.9</td>
</tr>
<tr>
<td></td>
<td>Bilateral nephrectomy</td>
<td>0.30–0.73</td>
</tr>
<tr>
<td>Pyridoxal-HCl</td>
<td>None</td>
<td>1.8–2.4</td>
</tr>
<tr>
<td></td>
<td>Sucrose diet</td>
<td>1.5–1.8</td>
</tr>
<tr>
<td></td>
<td>Bilateral nephrectomy</td>
<td>0.4–1.5</td>
</tr>
<tr>
<td>Pyridoxal 5-phosphate</td>
<td>None</td>
<td>1.2–2.9</td>
</tr>
<tr>
<td></td>
<td>Sucrose diet</td>
<td>0.6–1.7</td>
</tr>
<tr>
<td></td>
<td>Bilateral nephrectomy</td>
<td>0.6</td>
</tr>
<tr>
<td>Pyridoxamine-2HCl</td>
<td>None</td>
<td>3.5–11.6</td>
</tr>
<tr>
<td></td>
<td>Sucrose diet</td>
<td>0.3–11.6</td>
</tr>
<tr>
<td></td>
<td>Bilateral nephrectomy</td>
<td>0.3–2.3</td>
</tr>
</tbody>
</table>

- Bilateral nephrectomies were preceded by the sucrose diet, as described.
- Doses listed were injected on four successive days without mortality. Where a range of doses is indicated, 2–7 levels were tested, each level in 2–4 rats. Doses are specified as mmol kg$^{-1}$ to facilitate comparisons. Larger doses of pyridoxal-HCl and pyridoxal 5-phosphate were tested but are not included in the table because they were lethal after one, two or three daily doses.

### DISCUSSION

Large doses of pyridoxine were required in order to demonstrate neurotoxicity. These doses are relevant to megavitamin therapy of patients with various diseases, some of whom have also developed neurotoxicity.

Dietary restriction has profound consequences for the organism and its response to toxicants (Levin et al., 1993). There are many examples of increased susceptibility to neurotoxins (cyanide, quinacrine, DDT, lead, ergot, etc.) caused by dietary factors, most often low protein content (Holck, 1949). Pyridoxine is an essential nutrient but in high doses it is a neurotoxicant that is as disabling or deadly as any other. In this work, we found that a few days of dietary restriction were enough to cause considerable enhancement of pyridoxine’s neurotoxicity. A diet of sucrose without any other nutrient, and a diet that provided all nutrients except protein, were both effective provided that they were instituted before the large doses of pyridoxine were injected. These two very different types of food deprivation caused loss of body weight. In contrast, a normal diet fed only every other day was not effective and caused hardly any weight loss.

During dietary deprivation, loss of body fat, liver mass and gastrointestinal tract (with contents) exceed losses of other tissues (Peters and Boyd, 1966; Levin et al., 1993). Neural tissue is protected from weight loss, so it becomes, relatively speaking, a larger part of the organism and presumably accumulates a larger amount of the neurotoxin. These considerations may be pertinent to the enhanced neurotoxicity of pyridoxine, but diminished thirst and oliguria during food restriction are probably involved also. Pyridoxine is normally excreted in the urine, and removal of the kidneys increased its toxicity (Levine and Saltzman, 2002). Diminished drinking and oliguria with reduced renal losses could enhance the vitamin’s toxicity. In a preliminary experiment both weight loss and oliguria were induced simultaneously by depriving rats of drinking water during the 4 days of high-dose pyridoxine treatment. As in the previous experiments, this procedure increased the clinical signs and histological lesions compared with control rats having free access to water.

Diminished serum proteins in dietary-restricted rats could lead to reduced protein binding of pyridoxine or a metabolite. Free pyridoxine is more likely to be toxic than protein-bound pyridoxine. Reduced renal excretion and reduced protein binding combined with an increase in the proportion of neural tissue in the body could explain, at least in part, the effects of dietary restrictions in our experiments. However, other (nutritional) factors have not been excluded.

Feeding sucrose should not be thought of as equivalent to starvation. During starvation, body proteins are catabolized to provide amino acids for conversion to glucose (‘gluconeogenesis’). Feeding sucrose avoids the need for gluconeogenesis and thereby conserves body protein. Rats consume sucrose readily and will survive many weeks before a vitamin deficiency (vitamin A) develops (Richter, 1941). Furthermore, our experiment
with prophylactic multivitamin therapy proved that the effects of preliminary sucrose feeding were not due to vitamin deficiency. Preventing gluconeogenesis with a sucrose diet has proved especially valuable in prolonging the life of uremic rats (Levine and Saltzman, 1997, 2000).

Pyridoxine is converted after injection into pyridoxal-5-phosphate, which is the active coenzyme for many enzymes, including amino acid decarboxylase, transaminases, racemase, etc. It is not known if the neurotoxicity of large doses of pyridoxine is caused by the unaltered pyridoxine molecule, by the vitamers or the active coenzyme, by intermediates involved in the conversion to coenzyme or by some unknown derivative. This unsolved problem is important because of the continued, contemporary therapeutic use of large doses of pyridoxine (McCarty, 2000; Lerner et al., 2001). The information on nutritional and renal aspects of pyridoxine neurotoxicity in this and our previous report may facilitate research to identify the active neurotoxic molecule. It is important to be aware that diets, drugs or procedures that reduce renal function or cause loss of body weight can be responsible for a non-specific increase in the neurotoxicity of pyridoxine, which could cause confusion in the search for the active metabolite.

Contributing to this last issue is a study by Windebank (1985), who found almost equal toxicity to cultures of dorsal root ganglia neurons from pyridoxine as from the other B6-vitamers (pyridoxal and pyridoxamine). This observation tended to link pyridoxine toxicity to its coenzyme function. In support of such a linkage, the three vitamers are known to be interconvertible through the activity of pyridoxine oxidase and pyridoxine kinase, yielding the active coenzyme pyridoxal 5-phosphate (McCormick and Chen, 1999).

The importance of the present work is that we have demonstrated that the question of neurotoxicity by the B6-vitamers in vivo was not settled by the in vitro experiments, which found no differences among them (Windebank, 1985). Further study must include assays of each of the vitamers in serum and in the target neural tissues. The incentive for such a study is the possibility that the pyridoxine molecule itself, or a presently unsuspected metabolite, might be responsible for the neurotoxicity.

REFERENCES


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