

High Dose Dietary Pyridoxine Induces T-Helper Type 1 Polarization and Decreases Contact Hypersensitivity Response to Fluorescein Isothiocyanate in Mice

Chie Kobayashi, Kohta Kurohane, and Yasuyuki Imai*

Laboratory of Microbiology and Immunology and the Global COE Program, University of Shizuoka School of Pharmaceutical Sciences; 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan.

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Pyridoxine (vitamin B₆) is commonly used as a dietary supplement and beneficial effects of it are expected. However, excess ingestion of pyridoxine has been shown to cause a severe sensory neuropathy in humans and experimental animals. We have been studying the linkage between the nervous and immune systems using a fluorescein isothiocyanate (FITC)-induced contact hypersensitivity (CHS) mouse model. We have found that activation of transient receptor potential ankyrin 1 (TRPA1), which is expressed on sensory neurons, enhances skin sensitization to FITC. Another feature of FITC-induced CHS is its dependence on T helper 2 (Th2) type responses. We hypothesized that the excess intake of pyridoxine may affect sensitization to FITC and influence helper T-cell polarization. We examined FITC-induced CHS in BALB/c mice fed a diet containing excess pyridoxine (120 mg/kg diet) for 3 weeks. We found that mice fed on the excess-pyridoxine diet exhibited a lower response as to FITC-induced CHS compared with ones fed on a diet with a standard pyridoxine content (6.0 mg/kg diet). Moreover, the interferon (IFN)- γ /interleukin (IL)-4 ratio produced by draining lymph node cells was significantly higher with the excess-pyridoxine diet. This suggested that the cytokine balance was shifted toward Th1 with the excess-pyridoxine diet. Consistently, Th1-dependent oxazolone-induced CHS was enhanced with the excess-pyridoxine diet. These results suggested that an excess pyridoxine intake actively influences the immune system by altering helper T cell polarization.

Key words pyridoxine; contact hypersensitivity; cytokine

The use of vitamins for several medical conditions is quite usual based on the expectation of many people that vitamins have beneficial effects and are harmless. In the absence of professional supervision, self-medication by patients and healthy people could cause an overuse of vitamins, and their adverse effects are often disregarded. Pyridoxine, a form of vitamin B₆, is commonly used as a dietary supplement and therapeutic agent. The current tolerable upper intake level of vitamin B₆ is 100 mg per day in human adults in the United States.¹⁾ A high dose of pyridoxine is administered in conditions such as the premenstrual and carpal tunnel syndromes, and has been used as a treatment upon ingestion of the false morel mushroom, *Gyromitra esculenta*.²⁾ In the synthesis of the neurotransmitter gamma-aminobutyric acid, the mushroom toxin monomethylhydrazine competitively inhibits a pyridoxine-dependent step. This provides the rationale for this treatment.³⁾

A neurologic disease that presented in individuals consuming large quantities of vitamin B₆ for prolonged periods of time drew attention in the 1980s. An association between chronic abuse of oral pyridoxine supplements, and a severe sensory neuropathy of insidious onset and course has been revealed.⁴⁾ One may develop progressive sensory neuropathy manifested by sensory ataxia, diminished distal limb proprioception, paresthesia, and hyperesthesia with daily oral doses of up to 6 g per day for 12–40 months.^{4,5)} The mechanism of action has not been fully elucidated, and studies of pyridoxine neuropathy in experimental animals have yielded diverse results. Pyridoxine intoxication appears to produce a neuropathy characterized by necrosis of sensory neurons in the dorsal root ganglia, and degeneration of peripheral and central sensory projections. Neurons of large diameter are particularly affected.⁴⁾

It has been demonstrated that the immune system has some

connection to the peripheral nerve system.^{6,7)} Anatomically, epidermal Langerhans cells have been shown to be closely associated with calcitonin gene-related peptide (CGRP)-containing nerve fibers.^{6,8)} Functionally, it has been shown that contact hypersensitivity (CHS) is abolished through systemic deletion of capsaicin (CAP)-sensitive nerve fibers.⁶⁾ These studies indicated that peptidergic nerve fibers are involved in the CHS response.

We have been studying the linkage between the nervous and the immune system in a fluorescein isothiocyanate (FITC)-induced mouse CHS model. It has been recognized that skin sensitization to FITC is markedly enhanced in the presence of various phthalate esters.^{9–11)} The enhancing effect of phthalate esters is associated with enhanced trafficking of FITC-presenting dendritic cells (DC) from the skin to draining lymph nodes.¹⁰⁾ The enhancing effect of phthalate esters is correlated with the ability to activate transient receptor potential ankyrin 1 (TRPA1).¹¹⁾ TRPA1 is a calcium permeable cation channel expressed in subsets of sensory neurons that act as nociceptive receptors.¹²⁾ The agonistic activity of phthalate esters has been demonstrated by the calcium uptake in TRPA1-responsive sensory neurons of dorsal root ganglia as well as in TRPA1-expressing Chinese hamster ovary (CHO) cells.¹¹⁾ In contrast, desensitization of TRPA1 by local pre-treatment with a TRPA1 agonist, allyl isothiocyanate (AITC), at the site of FITC application suppressed sensitization to FITC. Such treatment also inhibited the trafficking of FITC-presenting mature DCs to draining lymph nodes.¹³⁾ Furthermore, local pre-treatment with a CGRP antagonist suppressed sensitization to FITC.¹³⁾

FITC-induced CHS is also known to depend on T helper 2 (Th2)-type immune responses.¹⁴⁾ Phthalate esters have been shown to facilitate such a polarization tendency.¹⁵⁾

* To whom correspondence should be addressed. e-mail: imai@u-shizuoka-ken.ac.jp

From this evidence showing a link between sensory neurons and the immune system, we speculated that excess pyridoxine intake might affect sensory neurons that control the immune system. Decreased nociception through impaired sensory neurons may lead to insufficient sensitization in FITC-induced CHS. We also examined how excessive pyridoxine intake could affect the immune system in the CHS model. We found that excess pyridoxine in the diet facilitated interferon (IFN)- γ production by draining lymph nodes, which might change the balance between T helper 1 (Th1) and Th2 type responses in FITC-induced CHS. In agreement with this, Th2-type FITC-induced CHS was suppressed whereas Th1-type 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone)-induced CHS was enhanced with the pyridoxine-excess diet.

MATERIALS AND METHODS

Mice Specific pathogen-free female BALB/c mice were purchased from Japan SLC Inc. (Shizuoka, Japan), and were used at 8-weeks of age, weighing 18.6 ± 0.22 g (means $\pm$ S.E.M.). Animal care and experiments were performed in accordance with the guidelines for the care and use of laboratory animals of the University of Shizuoka. Animal studies have been reviewed and approved by the Animal Research Committee of the University of Shizuoka.

Diets American Institute of Nutrition (AIN)-93G without AIN-93-Vitamin mix (VX) (Oriental Yeast Co., Tokyo, Japan), AIN-93-VX without pyridoxine (Oriental Yeast Co., Tokyo, Japan), and pyridoxine (Wako Pure Chemicals, Osaka, Japan) were blended together with a food processor (Panasonic Corp., Osaka, Japan). The compositions of the synthetic diets with standard and excess amounts of pyridoxine are shown in Table 1. The standard-pyridoxine diet contained 6.0 mg/kg of pyridoxine and the excess-pyridoxine one contained 120 mg/kg of pyridoxine (20 times excess). Feeding of these synthetic diets was started at 8-weeks of age (denoted by day 0) and continued during experiments.

Reagents Acetone, dibutyl phthalate (DBP), fluorescein isothiocyanate (FITC), Tween 20, kanamycin, and

2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were purchased from Wako Pure Chemicals (Osaka, Japan). Oxazolone was purchased from Sigma (St. Louis, MO, U.S.A.), RPMI 1640 from Nissui Pharmaceuticals (Tokyo, Japan), *N*-(2-hydroxy-ethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) from Nacalai Tesque (Kyoto, Japan), and fetal bovine serum (FBS) from Hyclone (South Logan, UT, U.S.A.). Purified anti-mouse IFN- γ monoclonal antibody (mAb) (clone XMG1.2, rat immunoglobulin G1 (IgG1)), biotin-conjugated anti-mouse IFN- γ mAb (clone R4-6A2, rat IgG1), purified anti-mouse interleukin (IL)-4 mAb (clone 11B11, rat IgG1), biotin-conjugated anti-mouse IL-4 mAb (clone BVD6-24G2, rat IgG1), purified anti-mouse IL-17A (clone eBio17CK15A5, rat IgG2a), biotin-conjugated anti-mouse IL-17A mAb (clone eBio17B7, rat IgG2a), recombinant mouse IFN- γ , recombinant mouse IL-4 and recombinant mouse IL-17A were purchased from eBioscience (San Diego, CA, U.S.A.). Horseradish peroxidase (HRP)-avidin was purchased from Zymed (South San Francisco, CA, U.S.A.). Dulbecco's minimum essential medium (DMEM) was purchased from Nissui Pharmaceuticals (Tokyo, Japan). R-Phycoerythrin (PE)-conjugated hamster anti-mouse CD11c mAb (clone HL3; IgG1) and PE-conjugated hamster IgG1 isotype control (clone G235-2356) were purchased from BD Biosciences (San Jose, CA, U.S.A.).

Sensitization and Elicitation of a Contact Hypersensitivity (CHS) Reaction The method used for contact sensitization to FITC was based on that used in earlier studies^{10,11} with modifications. After five days feeding on the synthetic diets (Table 1), mouse forelimbs were shaved using small animal clippers. Two days later, mice were anaesthetized by intraperitoneal injection of pentobarbital, and then 160 μ L of an FITC solution (0.5%, w/v) in a 1:1 (v/v) mixture of acetone and DBP (A/DBP) was epicutaneously applied to the shaved forelimbs (day 7). On day 14, the same amount of the FITC solution in the same solvent was applied again. On day 21, the baseline ear thickness (0h) in each animal was measured using a dial thickness gauge (Mitutoyo, Kanagawa, Japan). Mice were challenged by applying 20 μ L of an FITC solution (0.5% in A/DBP) on the right auricle. The left auricle was treated with 20 μ L of A/DBP alone as a control. Ear thickness was measured 24 and 48 h later. For sensitization with oxazolone,¹⁶ mice were sensitized on day 7 by applying 240 μ L of an oxazolone solution (0.1%, w/v) in acetone. On day 12, mice were challenged by applying 20 μ L of an oxazolone solution (0.5% in acetone) on the right auricle. The left auricle was treated with 20 μ L of acetone alone as a control. Ear thickness was measured after 24–72 h later. Ear swelling at Xh is defined as follows: [(thickness of the right ear at Xh)–(thickness of the right ear at 0h)]–[(thickness of the left ear at Xh)–(thickness of the left ear at 0h)].

Flow Cytometry Twelve days after feeding on a synthetic diet, standard- or excess-pyridoxine, mouse forelimbs were shaved using small animal clippers. On day 14, mice were epicutaneously sensitized with 160 μ L of a solution of FITC (0.5%, w/v). Twenty-four hours after sensitization, brachial lymph nodes from 2 mice were pooled for each experimental group. Single-cell suspensions of lymph nodes were prepared in DMEM by gentle teasing with needles. Total cell numbers in lymph nodes were determined by cell counting. After washing in phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) and 0.1% Na₂S₂O₃ (B-PBS), 2×10^6

Table 1. Synthetic Diet Compositions with Standard Pyridoxine and Excess Pyridoxine

Ingredient	Standard	Excess
	(g/kg diet)	
Casein ^{a)}	200	200
L-Cystine	3.00	3.00
Corn starch	397	397
α -Corn starch	132	132
Sucrose	100	100
Soybean oil	70.0	70.0
Cellulose powder	50.0	50.0
Mineral mix	35.0	35.0
(AIN-93G-MX)		
Vitamin mix (AIN-93-VX) (pyridoxine omitted)	10.0	10.0
Choline bitartrate	2.50	2.50
Butylhydroquinone, mg	14.0	14.0
Pyridoxine, mg	6.00	120

a) Vitamin-free casein.

cells were incubated with $2\mu\text{g}$ of PE-conjugated anti-CD11c or PE-conjugated isotype control mAb for 15 min each at 4°C in $100\mu\text{L}$ of B-PBS. After washing in B-PBS twice, the cells were resuspended at 2×10^6 cells/mL in B-PBS. A total of 5×10^5 cells were examined with a flow cytometer (BD FACS Canto II; BD Biosciences) using gates for forward and side light scatter to collect signals of cell-associated fluorescence. The isotype control was used to define the threshold for CD11c-positive cells. The threshold for FITC⁺ cells was set so as to make sure that 99.98% of cells are FITC-negative in unsensitized lymph node cells, because the proportion of FITC⁺ cells are small in draining lymph nodes even in FITC-sensitized mice. As for CD11c-axis (PE), the threshold was set so as to make sure that 99.9% of cells are PE-negative after staining with isotype control antibodies.

Lymph Node Cell Culture for Cytokine Production

After 5 d feeding on the synthetic diets, mouse forelimbs were shaved using small animal clippers. On days 7 and 14, mice were epicutaneously sensitized with $160\mu\text{L}$ of a solution of FITC (0.5%, w/v). Twenty-four hours after the second sensitization (on day 15), brachial lymph nodes were collected, and single-cell suspensions were prepared for each individual mouse. The lymph node cells (2.5×10^6 cells/1 mL/well) were cultured for 48 and 72 h in the wells of a flat-bottomed 24-well culture plate (Falcon #3047) in RPMI 1640 supplemented with 10 mM HEPES (pH 7.2), 10% FBS and $30\mu\text{g/mL}$ kanamycin at 37°C under a humidified atmosphere of 5% $\text{CO}_2/95\%$ air. Two wells were used for each mouse. The culture supernatants were individually collected from the wells.

Cytokine Detection by Enzyme-Linked Immunosorbent Assay (ELISA)

The concentrations of IFN- γ , IL-4 and IL-17A in the culture supernatants were determined by means of a sandwich ELISA. The wells of an ELISA plate (Costar #9018; Corning Inc., NY, U.S.A.) were coated with capture antibodies, $0.1\mu\text{g}$ of purified anti-IFN- γ (clone XMGI.2), anti-IL-4 (clone 11B11), or anti-IL-17A (clone eBio17CK15A5), in $100\mu\text{L}$ PBS for 15 h at 4°C . After washing with PBS, nonspecific binding sites were blocked with 1% BSA-PBS for 3 h at 20°C . After washing 3 times with PBS containing 0.05% Tween 20 (PBS-Tween), $100\mu\text{L}$ aliquots of culture supernatants or cytokine standards diluted with 10% FBS-RPMI 1640 were added to the wells. After the plate had been incubated for 1 h at 20°C , the wells were washed 3 times with PBS-Tween. The bound cytokines were detected with the respective detection antibodies, *i.e.*, $0.1\mu\text{g}$ of biotin-anti-IFN- γ (clone R4-6A2), biotin-anti-IL-4 (clone BVD6-24G2), or biotin-anti-IL-17A (clone eBio17B7), diluted with $100\mu\text{L}$ of 0.1% BSA-PBS-Tween. After the plate had been incubated for 1 h at 20°C , the wells were washed 3 times with PBS-Tween. To detect the binding of biotinylated antibodies, $0.1\mu\text{g}$ of HRP-avidin, diluted with $100\mu\text{L}$ of 0.1% BSA-PBS-Tween, was added to the wells and then the plate was incubated for 30 min at 20°C . After the wells had been washed 3 times with PBS-Tween, $100\mu\text{L}$ of 1 mM ABTS dissolved in 0.1 M citrate buffer (pH 4.2) containing 0.03% H_2O_2 was added. After 10–30 min incubation at 20°C , absorbance readings were made with a microplate reader (Rainbow Reader; Tecan, Salzburg, Austria) at 405 nm. The cytokine concentration of each culture supernatant was determined based on a dose-response curve generated with standard recombinant cytokines.

Statistical Analyses For two-group comparisons,

Student's *t* test or Welch test was employed.

RESULTS

Body Weight Changes Feeding of the synthetic diet containing excess pyridoxine or standard pyridoxine was started at 8-weeks of age (denoted by day 0). We measured body weights from 8 to 11-weeks of age. The body weights of mice in each group increased similarly during the experiments (Fig. 1).

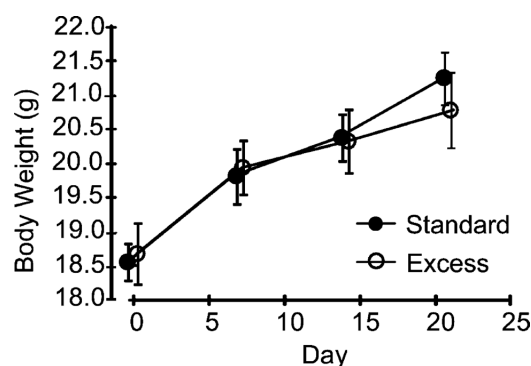


Fig. 1. Body Weight Changes

BALB/c mice were fed on a synthetic diet with a standard (closed circles) or an excess amount of pyridoxine (open circles) during experiments. Feeding of a synthetic diet was started at 8-weeks of age (day 0). Body weights were measured on days 0, 7, 14 and 21. The results are shown as the mean $\pm$ S.E.M. for each group ($n=8$).

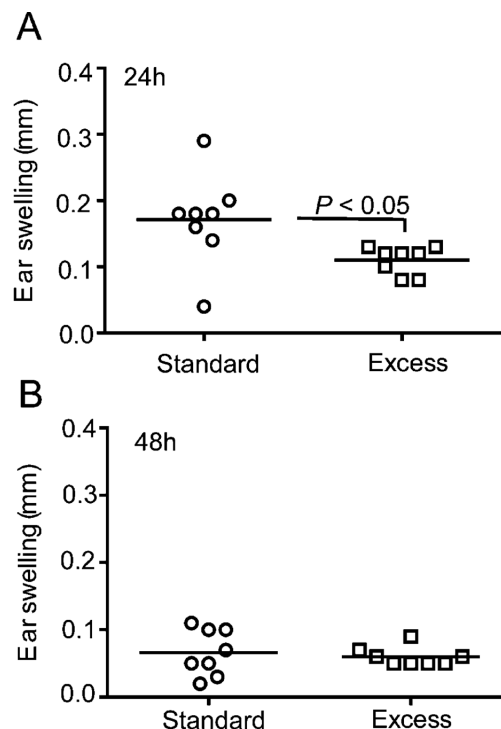


Fig. 2. Effect of a Diet Containing Excess Pyridoxine on FITC-Induced Contact Hypersensitivity

After 1 week feeding on a synthetic diet with or without excess pyridoxine, BALB/c mice (9-weeks old) were epicutaneously treated with 0.5% FITC in acetone/DBP on days 7 and 14. On day 21, the mice were challenged, and the ear swelling at 24 h (A) and 48 h (B) was determined. The values for individual mice are plotted, and the mean for each group ($n=8$) is shown by a bar. Statistical significance (compared with the standard-pyridoxine diet) was analysed by means of the Welch test. The results are representative of two experiments.

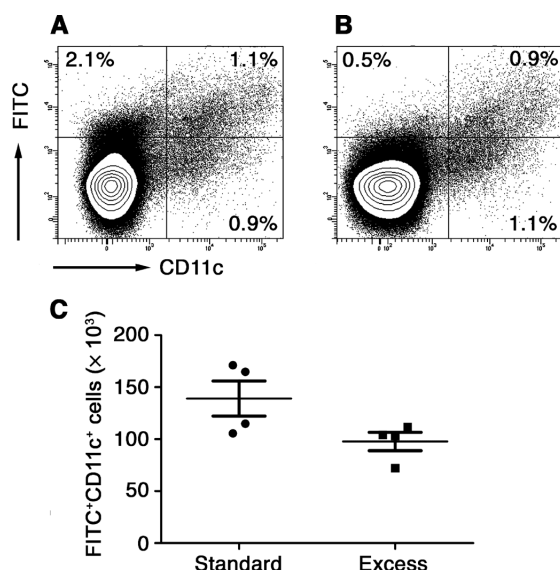


Fig. 3. Effect of a Diet Containing Excess Pyridoxine on the Trafficking of FITC-Presenting DCs

After 2 weeks feeding on a synthetic diet, BALB/c mice (9-weeks old) were epicutaneously treated with 0.5% FITC in acetone/DBP. Twenty-four hours after sensitization, brachial lymph nodes were pooled from 2 mice (4 pools for each diet). Lymph node cells were prepared and stained with PE-anti-CD11c or PE-isotype control (not shown) antibodies and then analyzed with a flow cytometer. (A, B) Representative results showing the cell-associated FITC fluorescence (y-axis) and CD11c levels (x-axis) of lymph node cells from mice on a diet containing the standard amount of pyridoxine (A), and ones on a diet containing excess amount of pyridoxine (B). The number in each region of the panels represents the percentage of cells in the region relative to the total lymph node cell number. (C) Summary of experiments showing the number of FITC⁺CD11c⁺ cells in the draining lymph nodes of each pool (2 mice per pool). The individual values for each pool, means and S.E.M. ($n=4$) for each dietary condition are shown.

Effect of the Pyridoxine Content in the Diet on FITC-Induced CHS We examined whether or not the pyridoxine-excess diet had an influence on FITC-induced CHS, for which a Th2 response is required. Mice were fed with a synthetic diet with either excess pyridoxine or the standard amount of pyridoxine for up to 23 d. During this period, the mice were epicutaneously sensitized with FITC twice on days 7 and 14. After 21 d on the synthetic diets, mice were challenged with FITC on an ear. The increase in ear thickness was determined (Fig. 2). The ear-swelling response peaked at 24 h after challenge. Mice on the diet with excess pyridoxine exhibited a significantly lower ($p<0.05$) ear-swelling response at 24 h after challenge. No difference ($p>0.05$) was seen at 48 h after challenge when the ear-swelling response was already diminished.

Effects on Dendritic Cell (DC) Trafficking We examined whether the lower ear-swelling response in mice on the diet with excess pyridoxine was associated with decreased DC trafficking. Mice were fed on a synthetic diet with either excess pyridoxine or the standard amount of pyridoxine for 14 d, and then epicutaneously sensitized with FITC. On day 15, draining lymph node cells were prepared, and the numbers of FITC⁺CD11c⁺ cells were determined with a flow cytometer (Fig. 3). The numbers of FITC⁺CD11c⁺ cells in the draining lymph nodes tend to be lower in the excess-pyridoxine condition than those in the standard-pyridoxine condition ($p=0.07$ by t test).

Cytokine Production from Lymph Nodes upon Sensitization to FITC The lower ear-swelling response in the FITC-induced CHS may be mediated through the effect on helper T cell polarization. This can be evaluated as the

production of cytokines by draining lymph nodes. Mice were fed with a synthetic diet with either excess pyridoxine or the standard amount of pyridoxine for 15 d. During this period, the mice were epicutaneously sensitized with FITC twice on days 7 and 14. After 15 d on the synthetic diets, draining lymph nodes were obtained. Lymph node cell suspensions, which contained FITC-presenting DC and FITC-specific T-cells, were cultured. Culture supernatants collected at 48 and 72 h of culture were examined for the accumulation of IL-4, IFN- γ and IL-17A. Both IL-4 ($p<0.05$) and IFN- γ ($p<0.05$) production were higher in mice on the diet containing excess pyridoxine at 48 h (Fig. 4). However, the IFN- γ /IL-4 ratio in the 48-h supernatant was significantly higher ($p<0.01$) under the pyridoxine-excess conditions than that under the standard conditions (Fig. 4E). The ratio in the 72-h supernatant was also significantly higher ($p<0.05$) under the pyridoxine-excess conditions (Fig. 4F). There was no difference in the IL-17A production between the groups on different diets.

Effect of the Pyridoxine Content in the Diet on Oxazolone-Induced CHS If Th1-polarization occurs due to the influence of excess-pyridoxine diets, one may expect enhancement of a CHS response in which a Th1-type response dominates. Oxazolone is known as a hapten that predominantly induces the Th1-type response.¹⁷ Mice were fed on a synthetic diet with either excess pyridoxine or the standard amount of pyridoxine for up to 15 d. Mice were epicutaneously sensitized with oxazolone on day 7. After 12 d on the synthetic diets, mice were challenged with oxazolone on an ear. Then the increase in ear thickness was determined (Fig. 5). The ear-swelling response peaked at 48 to 72 h after challenge. The values representing ear-swelling were significantly higher in the group on the excess-pyridoxine diet at 48 h ($p<0.05$) and 72 h ($p<0.05$) after challenge. The results coincided with the Th1 type polarization with the diet containing excess pyridoxine.

DISCUSSION

It has been reported that excess ingestion of pyridoxine causes sensory neuropathy.³⁻⁵ On the other hand, accumulating evidence suggests that nociception through the TRPA1 channel facilitates the sensitization phase of the FITC-induced CHS mouse model.^{11,13} In the present study, we hypothesized that a diet containing excess pyridoxine might decrease nociception through impairment of sensory neurons, which in turn leads to decreased sensitization in FITC-induced CHS. We found that mice fed on a synthetic diet containing 20-fold excess pyridoxine for 3 weeks exhibited a lower response as to FITC-induced CHS compared with ones fed on a diet with a standard pyridoxine content.

This dietary dose was substantially lower than that with which neurotoxicity was apparently seen in rat experiments.³ In those experiments, 800 mg/kg of body weight/d of pyridoxine was intraperitoneally administered to rats. In the present study, the mice ingested 0.8 mg/kg body weight/d of pyridoxine in the standard diet, while 16.2 mg/kg body weight/d with the excess-pyridoxine diet on average, as judged on analysis of daily food intake.

FITC-induced CHS is initiated by the trafficking of FITC-presenting DC from the skin to draining lymph nodes.¹⁸ It is conceivable that the TRPA1 stimulation on local sen-

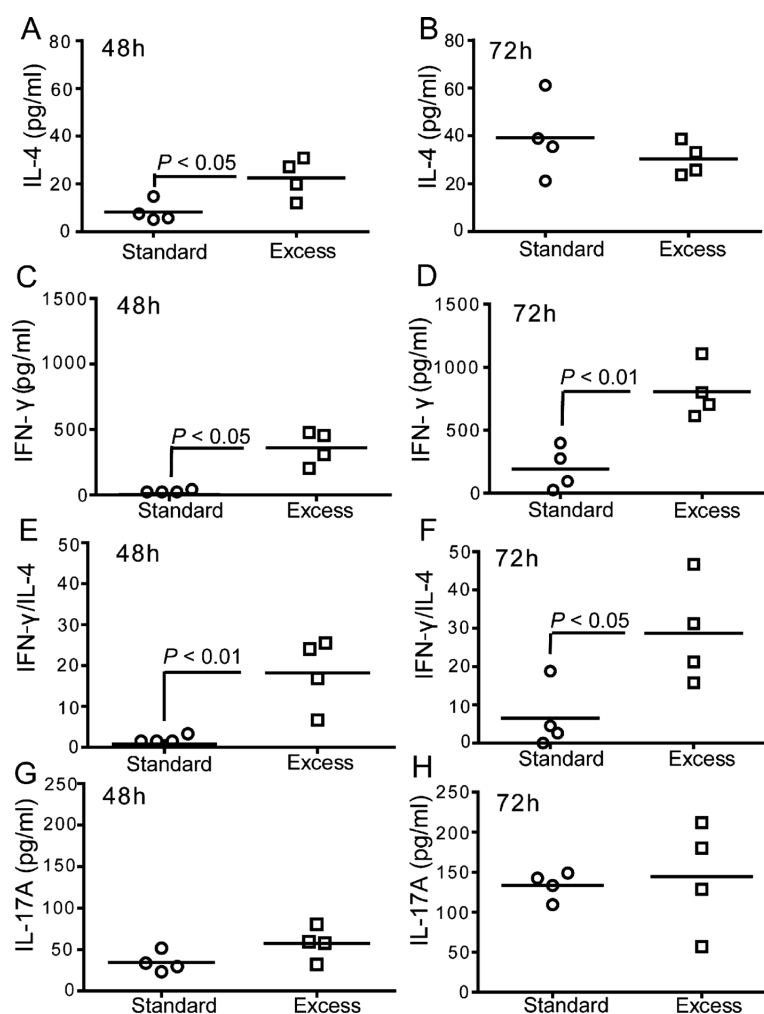


Fig. 4. Effect of a Diet Containing Excess Pyridoxine on Cytokine Production by Lymph Nodes of FITC-Sensitized Mice

After one week feeding on a synthetic diet, BALB/c mice (9-weeks old) were epicutaneously treated with 0.5% FITC dissolved in acetone/DBP on days 7 and 14. On day 15 (24 h after second sensitization), brachial lymph nodes were collected from each individual mouse and single cell suspensions were prepared. The lymph node cells were cultured for 48 h (A, C, E, G) or 72 h (B, D, F, H), and then the amounts of IL-4 (A, B), IFN- γ (C, D), and IL-17A (G, H) in the culture supernatants were determined by means of ELISA. In panels E and F, the IFN- γ /IL-4 ratios are shown. The values for individual mice are plotted, and the means for each group are shown by bars ($n=4$). Statistical significance (compared with the standard-pyridoxine diet) was analyzed by means of the *t* test.

sory neurons facilitates the DC trafficking. This has been supported by the results obtained for FITC-induced CHS, in which FITC-presenting DC trafficking is enhanced if FITC is epicutaneously applied to the skin in the presence of TRPA1 agonistic phthalate esters.¹¹⁾ We examined whether or not the diet containing excess pyridoxine might influence FITC-presenting DC trafficking to draining lymph nodes. The results suggested that the number of FITC⁺CD11c⁺ cells in the draining lymph nodes tend to be decreased with the diet containing excess pyridoxine. The decreased DC trafficking may contribute to the lower response in mice on the diet containing excess pyridoxine. There are some decreases in the FITC⁺CD11c⁺ population in the draining lymph nodes from mice on pyridoxine-excess diet. This trend could reflect a decrease in FITC-presenting resident macrophages in draining lymph nodes. However, the differences are less clear due to the variation among samples. In the case of oxazolone-induced CHS, it is an open question whether or not the DC trafficking is increased in mice on pyridoxine-excess diet. Because oxazolone is not a fluorescent hapten, it is not possible to track oxazolone-presenting DC at present.

Another feature of FITC-induced CHS is its dependence on

Th2 type helper T cell polarization.^{14,15)} Cytokine production by the draining lymph nodes was compared. Despite the trend toward a decrease in FITC-presenting DC trafficking with the diet containing excess pyridoxine, IL-4 accumulation was higher in 24-h supernatant of lymph node cells. Interestingly, IFN- γ production was not only more increased but also an early onset of IFN- γ production was observed with the diet containing excess pyridoxine. Therefore, the ratio of IFN- γ to IL-4 was markedly high with the diet containing excess pyridoxine. This suggests that the Th1/Th2 balance was shifted toward Th1-dominant. It should be noted that the excess-pyridoxine diet did not affect Th17 differentiation, suggesting that the difference in IFN- γ production did not occur by accident due to cell viability differences during cell culture, for example.

If the cytokine balance was shifted toward Th1 with the diet containing excess pyridoxine, the CHS response against haptens, which predominantly induce Th1-type responses, may be increased.¹⁷⁾ As expected, the CHS response to one such hapten, oxazolone, was enhanced with the diet containing excess pyridoxine.

Other studies suggested that the repeated intraperitoneal in-

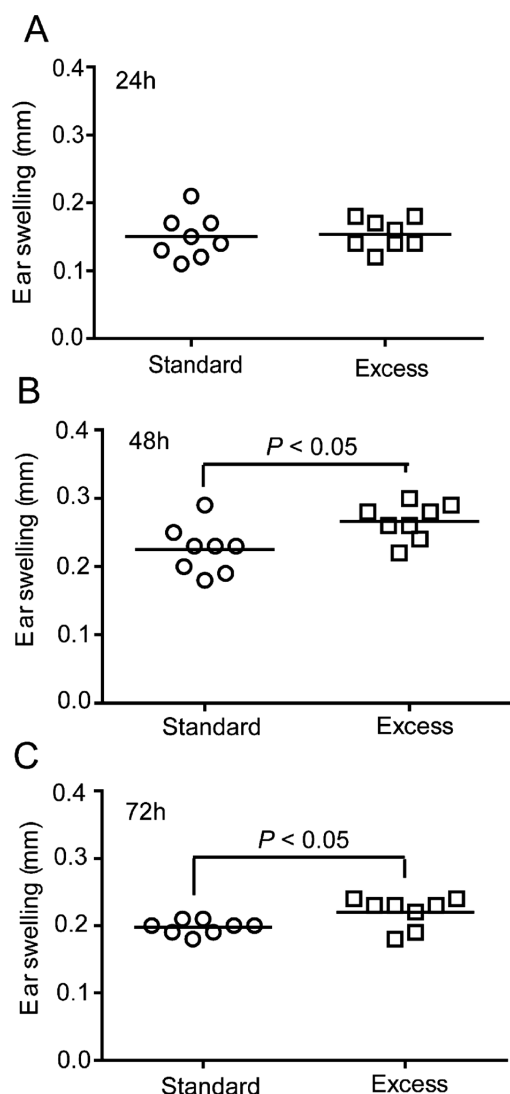


Fig. 5. Effect of a Diet Containing Excess Pyridoxine on Oxazolone-Induced Contact Hypersensitivity

After one week feeding on a synthetic diet, BALB/c mice (9-weeks old) were epicutaneously treated with 0.1% oxazolone dissolved in acetone on day 7. On day 12, the mice were challenged, and the ear swelling at 24h (A), 48h (B), and 72h (C) was determined. The values for individual mice are plotted, and the means for each group are shown by bars ($n=8$). Statistical significance (compared with the standard-pyridoxine diet) was analysed by means of the *t* test. The results are representative of two experiments.

jection of pyridoxine decreases the ovalbumin (OVA)-specific humoral Th2-type immune response. Thus, serum IgE and IgG1 were suppressed, and IL-4 production by spleens of immunized mice was also decreased.¹⁹ Under their experimental conditions, no significant increase in IFN- γ or IgG2a was observed, which is associated with a Th1-type response. However, the overall trend of helper T cell polarization appears to be Th1-biased.

One possible explanation for the suppressive effect on the Th2-type response with the excess-pyridoxine diet may be the inhibition of cathepsin B, a lysosomal enzyme involved in antigen presentation.¹⁹ Pyridoxal phosphate, a coenzyme form of vitamin B₆, is known to strongly inhibit cathepsin B *in vitro* through the formation of hemithioacetal bonds between the aldehyde of pyridoxal phosphate and the sulfhydryl group of cathepsin.^{19,20} However, it is not known whether or not cathepsin B activity in DC is decreased in mice on an

excess-pyridoxine diet. Furthermore, it is hard to imagine why the reduction in antigen processing activity due to cathepsin B inhibition could lead to a selective enhancement of IFN- γ production by draining lymph nodes.

The effect of pyridoxine on the immune system may be observed from the other side. Mice fed on a pyridoxine-deficient diet for 4 weeks were immunized with dinitrophenyl-OVA (DNP-OVA).²¹ DNP-specific serum IgE level was five-fold higher in mice on the pyridoxine-deficient diet (0mg/kg diet) than in ones on the standard-pyridoxine diet (7.0mg/kg diet). IL-4 production in a supernatant of concanavalin A-stimulated spleen cells was four-fold higher in mice on the pyridoxine-deficient diet. This finding suggested that pyridoxine deficiency favors the Th2-type response. This is the opposite tendency from in the case of conditions with excess pyridoxine.

As mentioned above, the effect of pyridoxine in diet has been studied in the context of humoral immunity. We found a similar tendency of helper T cell polarization in cell-mediated immunity using contact hypersensitivity models. One difference is that excess pyridoxine appears not to act in an immunosuppressive manner but to actively enhance IFN- γ production. Another difference is that the effect of excess pyridoxine was seen with the standard protein diet (20% casein), whereas a diet containing excess pyridoxine was tested only with a high protein diet (70% casein) in humoral immunity.¹⁹ It is not clear at present how excess pyridoxine in a diet leads to Th1-polarization. The target could be the activation of helper T cells or DC maturation. Further studies are needed to clarify this issue.

In conclusion, excess pyridoxine in a diet for a short period of exposure has an influence on the immune system. Thus, the excess-pyridoxine diet enhanced antigen driven IFN- γ production by lymph nodes, resulting in a shift toward Th1-type responses. The excess-pyridoxine conditions suppressed the CHS to haptens for which Th2-type responses are required (FITC), while enhanced the CHS to those for which Th1-type responses are required (oxazolone).

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