

Pyridoxine supplementation: effect on lymphocyte responses in elderly persons¹⁻⁴

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ABSTRACT The effect of pyridoxine supplementation on lymphocyte responsiveness was investigated in 15 persons aged 65–81 y. Eleven subjects received 50 mg/d pyridoxine HCl (PN). Four subjects received a placebo. Lymphocyte proliferation to T and B cell mitogens, lymphocyte subpopulations with monoclonal antibodies, and plasma pyridoxal 5'-phosphate (PLP) were measured before and after 1 and 2 mo of supplementation. After 1 and 2 mo plasma PLP levels increased by 195 ± 88 nM and 201 ± 84 nM, respectively, in subjects receiving PN. With PN supplementation, lymphocyte proliferation increased significantly in response to phytohemagglutinin ($p < 0.01$), pokeweed mitogen ($p < 0.01$), and *Staphylococcus aureus* (Cowain I) ($p < 0.05$). For PN-treated subjects with low presupplement plasma PLP levels, lymphocyte blastogenesis also increased significantly ($p < 0.01$) in response to concanavalin A. Percentages of T3⁺ and T4⁺ but not T8⁺ cells increased significantly ($p < 0.05$) in PN-treated subjects. These results suggest that improving vitamin B-6 status is important in stimulating immunocompetence in the elderly. *Am J Clin Nutr* 1987;46:659–64.

KEY WORDS Lymphocytes, pyridoxine, pyridoxal 5'-phosphate, immunocompetence, aged

Introduction

One of the changes associated with aging is a decline in immune function. As previously reviewed (1–3), the elderly have an increased number of autoantibodies, an impaired ability to respond to new antigens, a reduced in vitro responsiveness of lymphocytes to mitogens, and an impaired skin reactivity to antigens. At the same time, elderly persons tend to be at a greater risk for vitamin B-6 deficiency (4–6). Vitamin B-6 deficiency was shown to have a depressive effect on both humoral (7, 8) and cell-mediated (9–11) immune function. A deficiency of vitamin B-6 appears to alter lymphocyte function by reducing nucleic acid synthesis (12). Thus, lymphocyte reactions to an antigenic challenge which require a proliferative response are compromised in vitamin B-6 deficiency.

Little is known about the influence of vitamin B-6 on immunosenescence in aged people. The purpose of this study is to determine whether increasing vitamin B-6 intake will improve the immune status of older persons.

Materials and methods

Subjects

Fourteen Caucasian women aged 65–81 y and one Caucasian man aged 74 y participated as subjects. They were living independently in their own homes and eating their usual diets. Criteria for selection included freedom from any known immu-

nodeficiency, liver, kidney, or metabolic disorder; not taking any drugs which are known to be immunosuppressive or affect vitamin B-6 metabolism; and not taking any vitamin or mineral supplements which might have any known effect on the study. Table 1 describes the physical characteristics of the subjects and lists any nutritional supplements they were taking. Each subject signed an informed consent form before participating in this study. This investigation was approved by the Oregon State University Committee for Protection of Human Subjects.

Experimental design

Of the 15 subjects, 11 received 50 mg of pyridoxine HCl (PN) (McKesson Laboratories, Dublin, CA) daily. To obtain control values for our methods, the remaining four subjects took a placebo (Stayner Corporation, Berkeley, CA or Eli Lilly and Co, Indianapolis, IN) daily. The subjects were not aware of which preparation they received. Compliance was verified by providing a 14-d supply of tablets at a time, counting any remaining tablets

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TABLE 1
General information on subjects

Subject	Sex	Age	Ht	Wt	Supplements*	Group†
		y	cm	kg		
1	F	76	158	55.5	Ca	Placebo
2	F	81	170	55.9	Vitamin C, Ca	PN
3	F	73	165	67.3	—	PN
4	F	75	160	59.1	Ca, Zn	Placebo
5	F	77	158	49.1	—	PN
6	F	72	172	86.4	K, Vitamin C	PN
7	F	65	158	77.7	—	PN
8	F	68	158	53.2	—	PN
9	F	69	168	70.9	—	PN
10	M	74	178	75.0	—	PN
11	F	69	155	55.0	—	Placebo
12	F	81	158	72.7	—	PN
13	F	65	170	71.4	—	PN
14	F	69	170	68.2	Ca, Vitamin D	Placebo
15	F	77	160	75.0	Ca	PN

* Nutritional supplements taken as reported by the subjects.

† Treatment group. The pyridoxine HCl (PN) group received 50 mg of PN-HCl daily; the placebo group received a placebo daily.

every 2 wk, and by verbal reminders every 1–2 wk throughout the 2-mo period.

The study consisted of three testing periods: before supplementation (baseline), after 1 mo of PN or placebo supplementation, and after 2 mo of PN or placebo supplementation. At each of the three testing periods the following assays were performed: lymphocyte proliferation in response to mitogens, lymphocyte subset analysis, plasma pyridoxal 5'-phosphate (PLP) concentration, hemoglobin (Hb), hematocrit (Hct), white blood cell (WBC), and differential counts. Because of an anticipated intraperson variability, the lymphocyte proliferation assay was conducted 1 wk before the testing period and again on each full test day and the mean of the two responses was used to represent the response at that test period. In total, blood was drawn six times from each subject. Subjects were tested on 2 d within the same week to facilitate efficient handling of samples. On each test day two placebo- and five or six PN-treated individuals were tested. Blood was drawn between 0700 and 0900 from the antecubital vein of fasting subjects into evacuated tubes containing heparin. When all of the tests were performed, 30 mL of blood were drawn; for the blastogenesis assay alone, 10 mL were obtained. Except for PLP, all analyses were performed on the same day that the blood was collected. Plasma was stored at -40°C and assayed for PLP after the completion of the lymphocyte studies.

Isolation of mononuclear cells

Whole blood was diluted 1:1.5 with RPMI 1640 (GIBCO, Grand Island, NY) in 50 mL centrifuge tubes; 18–20 mL of Ficoll-Paque (Pharmacia, Piscataway, NJ) were layered under the diluted blood using 6-in blunt needles (Popper and Son, New Hyde Park, NY). Blood was centrifuged at $400 \times g$ for 30 min at 4°C . Mononuclear cells (MNC) were removed from the interface, placed in 15 mL tubes, and washed twice in medium.

Blood cell counts

White blood cells were counted using a Coulter Counter (Model ZBI; Coulter Electronics, Inc, Hialeah, FL). Smears were

made using a 1:500 dilution of whole blood for a cytocentrifuged preparation. A differential leukocyte count was made using a Wright-Giemsa stain.

Lymphocyte proliferation

Lymphocyte proliferation was measured by ^3H -thymidine incorporation following culture with the T-cell mitogens phytohemagglutinin (PHA) and concanavalin A (Con A), the T-cell-dependent B-cell mitogen pokeweed mitogen (PWM), and the T-cell-independent B-cell mitogen *Staphylococcus aureus* Cowan I (SAC).

Dilutions of each mitogen were made and plated in triplicate, 0.1 mL per well, into 96-well, flat-bottomed microtiter plates (Linbro, McLean, VA). All dilutions were made in RPMI supplemented with 10% fetal calf serum (10% RPMI). PHA (Wellcome, Beckenham, UK) was diluted to concentrations of 10, 2.5, and $0.625 \mu\text{g/mL}$; Con A (Calbiochem, LaJolla, CA) to 40, 10, and $2.5 \mu\text{g/mL}$; PWM (Sigma, St Louis, MO) to 2.0 and $0.5 \mu\text{g/mL}$; SAC (Calbiochem, La Jolla, CA) to 3.2×10^8 , 8×10^7 , and 2×10^7 killed bacteria/mL. PHA, Con A, and PWM were plated and frozen at -70°C until the day before testing when SAC was plated. Wells containing 10% RPMI alone were included to determine background responses.

Separated MNC were resuspended in 10% RPMI at 2×10^6 cells/mL, and 0.1 mL was plated into microtiter plates. All plates were incubated 96 h at 37°C in an atmosphere of 5% CO_2 and 95% humidity. Twenty-four hours before termination of the incubation, $0.5 \mu\text{Ci } ^3\text{H}$ -thymidine (New England Nuclear, Boston, MA) in $20 \mu\text{l}$ was added to each well. The plates were frozen at -70°C until harvested.

Cells were harvested onto glass microfiber filter paper (Whatman, Madestone, UK) using an ADAPS (ADAPS, Dedham, MA) cell harvester. Filter disks were placed in prelabeled minivials (Wheaton, Millville, NJ) and allowed to dry. Liquid scintillation fluid (Instagel®; Packard Instruments, Downers Grove, IL) was added to each vial and the vials were counted in a liquid scintillation counter. Data were recorded as cpm using a preset tritium channel. Of each triplicate culture, the median cpm was selected as the response level. The median cpm of the unstimulated cultures of each subject was subtracted from each of the respective mitogen responses.

Lymphocyte subpopulations

The monoclonal antibodies used were T3, T4, and T8 (Coulter Immunology, Hialeah, FL). Purified mouse immunoglobulin (Coulter Immunology) served as a control for non-specific staining. Separated MNC were resuspended in medium and adjusted to a concentration of $5\text{--}10 \times 10^6$ cells/mL. Of the cell suspension, 0.1 mL was pipeted into $12 \text{ mm} \times 75 \text{ mm}$ plastic tubes. The appropriate mouse antihuman monoclonal antibody was added to the MNC. The tubes were lightly mixed with a vortex mixer and incubated for 30 min at 4°C . The cells were washed twice and resuspended in 0.1 mL of cold medium. After $5 \mu\text{l}$ of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse F(ab')_2 antibody (New England Nuclear) were added, the lymphocytes were incubated and washed as before. The cells were resuspended in 0.9 mL of phosphate buffered saline (PBS) and fixed with 0.1 mL 10% formaldehyde solution. Lymphocyte fluorescence was analyzed using a Coulter Epics V flow cytometer (Coulter Electronics, Inc) with the laser light tuned to 488 nm. The lymphocyte population was determined by the log of the 90° light scatter of incident light (proportional to the degree of heterogeneity or granularity of the cell) vs forward angle light scatter (proportional to the cross-sectional area of the cell or cell

size). Subsequent analyses were made by gating on this population.

Plasma PLP

Plasma PLP concentration was determined by measuring the $^{14}\text{CO}_2$ evolved during the decarboxylation of L-tyrosine-1- ^{14}C by tyrosine apodecarboxylase (13). The coefficient of variation of a control sample analyzed with each assay was 2.3%; the mean percent recovery of PLP added to plasma was 95.8 ± 6.9 (SD)%.

Collection of dietary information

Before initiation of testing, each subject completed a 3-d dietary record. The subjects' nutrient intake was calculated using the Ohio State University nutrient data base (14).

Statistics

The data were analyzed for statistical significance by the use of PROPHET, a national time-shared computer network of the Biotechnology Resources Program, NIH. The effect of pyridoxine supplementation on lymphocyte responsiveness was assessed by the Neuman-Keuls' multiple-range test to compare the response between the PN- and placebo-treated groups and by the paired *t* test when each subject's response to supplementation (PN or placebo) was compared with her/his value before supplementation (15). Correlation coefficients were calculated to determine relationships between dietary intake of vitamin B-6 or B-6:protein ratio and plasma PLP (15). Unless stated otherwise, data are expressed as mean \pm SD. Values for $p < 0.05$ were considered statistically significant.

Results

Vitamin B-6 intake and plasma PLP

The 15 subjects' dietary intake of vitamin B-6 assessed before supplementation was 1.51 ± 0.70 mg/d, $\sim 75\%$ of the recommended dietary allowances (RDA) (16). Expressed as mg vitamin B-6/g protein, the subjects' mean intake was 0.022 ± 0.006 . One-third of the subjects had an intake < 0.02 mg/g protein, the standard used for setting the RDA for vitamin B-6. The subjects' protein intake was adequate.

The mean plasma PLP concentration before supplementation of the 11 PN-treated subjects was 33.1 ± 15.4 nM (range, 13.5–53.3 nM) and of the 4 placebo-treated subjects of 28.2 ± 10.8 nM (range, 16.7–42.1 nM). No correlation was found between the 15 subjects' plasma PLP concentration and their dietary vitamin B-6 intake expressed as mg/d or mg/g protein.

With pyridoxine supplementation, plasma PLP concentrations increased significantly ($p < 0.01$) in the PN group (Fig 1). After rising to a mean increase of 195 ± 88 nM following one month of PN supplementation, PLP concentration had little further increase. Plasma PLP concentrations did not change in the subjects receiving a placebo.

Hematological status

The initial mean hemoglobin concentration and hematocrit for the women were 151 ± 9 g/L and 0.43 ± 0.02 , respectively; for the man they were 167 g/L and 0.44,

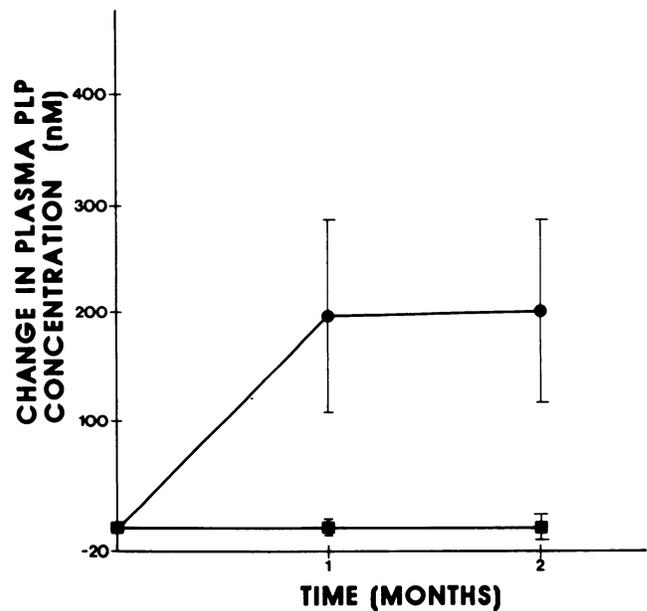


FIG 1. Changes in plasma PLP concentration in response to supplementation. Each subject's presupplement PLP value was subtracted from the values after 1 and 2 mo supplementation. ● represents mean change in PLP of 11 subjects who received 50 mg of pyridoxine hydrochloride (PN) daily and ■ represents the mean change in 4 subjects who received a placebo daily. Vertical bars represent SD.

respectively. The subjects' hemoglobin and hematocrit remained relatively constant throughout the 2-mo study. White blood cell and differential counts of the subjects were within normal ranges and did not change significantly during the 2 mo of supplementation.

Lymphocyte proliferation

The mean of the two blastogenesis responses done for each mitogen for each subject at each of the three time periods (baseline, 1 mo and 2 mo postsupplementation) was used to represent the response at a particular point in testing. Inasmuch as the subjects showed peak responses of ^3H -thymidine incorporation at different mitogen concentrations, peak responses independent of mitogen concentration were used to compare lymphocyte proliferation between groups. In Figure 2 the responses of the PN and placebo groups to T- and B-cell mitogens are compared after correcting for individual variation by subtracting each subject's presupplement value from the values at 1 and 2 mo of supplementation. Compared with the placebo group, the subjects receiving pyridoxine showed a significant increase in their response to PHA at 1 mo ($p < 0.01$), to SAC at 2 mo ($p < 0.05$), and to PWM at 1 mo ($p < 0.05$) and at 2 mo ($p < 0.01$). Because there was an increased response to Con A by some but not all of the subjects in the PN group, the difference between the PN and placebo groups was not statistically significant (Fig 2b). The response of the placebo group to PWM and Con A (Fig 2b, d) decreased with time. Because of the small

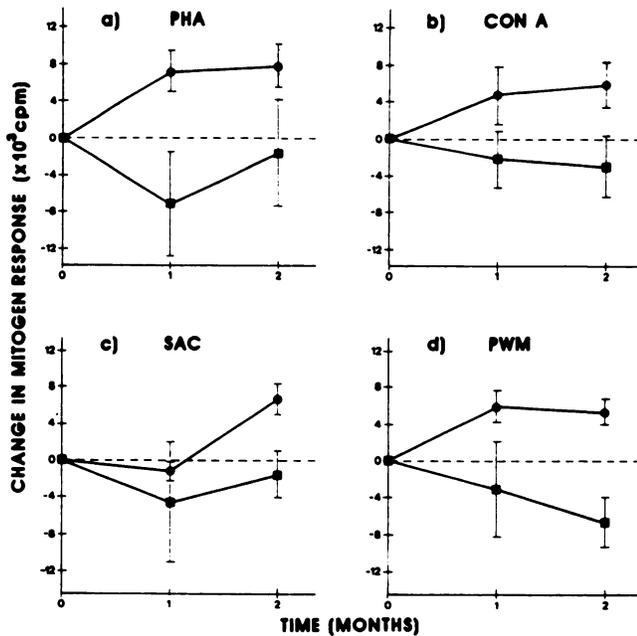


FIG 2. Changes in peak lymphocyte incorporation of ^3H -thymidine in response to the mitogens a) phytohemagglutinin (PHA), b) concanavalin A (Con A), c) *Staphylococcus aureus* Cowain I (SAC), and d) pokeweed mitogen (PWM). \bullet represents the mean of the 11 subjects who received pyridoxine HCl (PN) daily for 2 mo and \blacksquare the mean of 4 subjects who received a placebo daily. Vertical lines represent SEM.

number of subjects in the placebo group and large variation, these decreases were not statistically significant.

The effect of supplementation on lymphocyte proliferation was also analyzed within each group of subjects by comparing peak levels of ^3H -thymidine incorporation

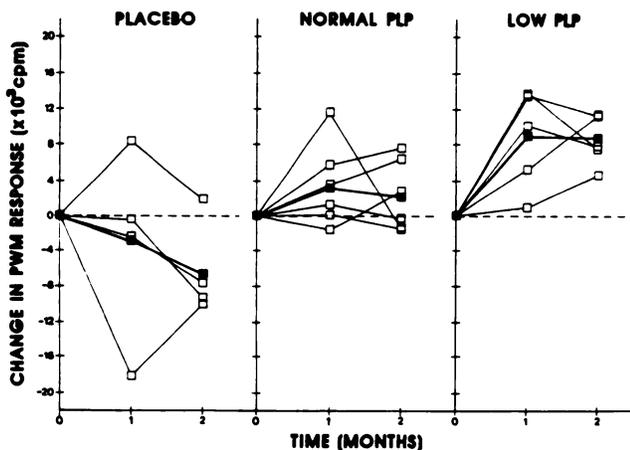


FIG 3. Influence of presupplement plasma pyridoxal 5'-phosphate (PLP) concentration on pyridoxine HCl (PN)-induced changes in lymphocyte incorporation of ^3H -thymidine in response to pokeweed mitogen (PWM). Division of the PN-treated subjects into normal-PLP and low-PLP subgroups was on the basis of the subject's plasma PLP concentration before receiving PN (see text). \square represents individual data; \blacksquare , mean data for group or subgroup.

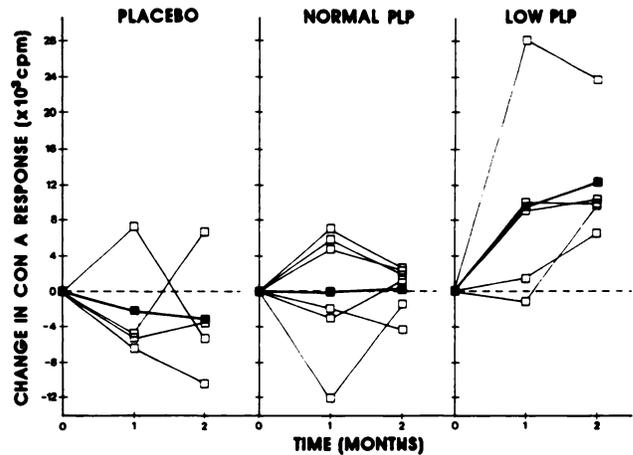


FIG 4. Influence of presupplement plasma pyridoxal 5'-phosphate (PLP) concentration on pyridoxine HCl (PN)-induced changes in lymphocyte incorporation of ^3H -thymidine in response to concanavalin A (Con A). Placebo group, normal- and low-PLP subgroups are same as described for Fig 3. \square represents individual data; \blacksquare , mean data for group or subgroups.

in response to each mitogen after 1 and 2 mo of supplementation with the groups' respective presupplement values (data not given). Viewed this way, the results also showed that the PN group had significantly higher ($p < 0.01$) responses to PHA and PWM at 1 and 2 mo and to SAC at 2 mo. Con A responses were not significantly altered over time in PN-treated subjects. The placebo group showed no significant changes from any of the mitogens.

To determine if vitamin B-6 status before PN supplementation affected the subjects' responses to the mitogens, the PN-treated subjects were divided into two subgroups based on the distribution of the subjects' presupplement plasma PLP concentration. The low PLP subgroup (five subjects) had plasma concentrations of 13.5–22.3 nM (mean, 18.0 ± 3.2 nM) and the normal PLP subgroup (six subjects) had values of 36.0–53.3 nM (mean, 45.6 ± 6.8 nM). After 2 mo of PN supplementation, subjects in the low-PLP subgroup had a significantly higher response to PWM (Fig 3) than the subjects in the normal-PLP subgroup ($p < 0.05$) and placebo group ($p < 0.01$) and significantly higher ($p < 0.01$) response to Con A (Fig 4) than the subjects in the normal-PLP subgroup or placebo group. In contrast both the low- and normal-PLP subgroups showed increased responses to PHA and SAC stimulation (data not shown).

Lymphocyte subset analyses

A considerable degree of nonspecific staining was observed with cells from several of the subjects from each group, making a reliable estimate of the percent positive cells difficult. For this reason only data which showed a clear separation between background (negative) fluorescence and higher intensity (positive) fluorescence at both pre- and postsupplementation testing periods were in-

cluded in the analyses. Thus, for five subjects within the PN-treated group before and after 2 mo of supplementation, proportions of T3⁺ cells increased, respectively, from 43.4 ± 1.7 (SE) to 54.2 ± 3.3 ($p < 0.05$), of T4⁺ cells from 32.3 ± 3.7 to 44.4 ± 3.1 ($p < 0.05$), and of T8⁺ cells from 13.9 ± 2.8 to 11.8 ± 2.2 ($p > 0.05$). The placebo-treated subjects revealed no significant changes in the proportions of T3⁺, T4⁺, and T8⁺ cells.

Discussion

In animals, vitamin B-6 deficiency results in reduced antibody production (8, 17), delayed-type hypersensitivity (DTH) reaction (17), T-cell cytotoxicity (18, 19), mixed lymphocyte reactions (9, 11), and response to T-cell mitogens (11). In humans depleted of vitamin B-6, antibody production is decreased (7) and lymphocytopenia is produced (20). In this study, a group of elderly persons who were likely to have a depressed vitamin B-6 status (4–6) and reduced immunocompetence (1–3) were studied to determine whether PN supplementation could improve lymphocyte function. Each subject served as her/his own control so that changes in immune responsiveness before and after pyridoxine supplementation could be evaluated. A placebo group was included to monitor nonspecific changes in the variables over time.

Except for common medical problems associated with aging (eg, diverticulitis and various cardiovascular disorders), all subjects were in good health. They had good hematological status as indicated by normal values for hemoglobin, hematocrit, as well as white blood cell and differential counts. None had a condition or was taking a medication that was known to affect the metabolism of vitamin B-6. All subjects were living independently in their own homes and were eating their customary diets. Most of them had adequate intakes of vitamin B-6, protein, and other nutrients. Despite this there was a wide range in plasma PLP concentrations among the 15 subjects during the baseline (presupplement) period. When the PN-treated subjects were categorized according to the distribution of their plasma PLP levels before supplementation, five of the PN-treated subjects (low-PLP subgroup) had plasma PLP concentrations of 13.5–22.3 nM (mean, 18.0 ± 3.2 nM) and the six remaining subjects had levels of 36.0–53.3 nM (mean, 45.6 ± 6.8). According to this classification, two of the four placebo-treated subjects were classified as having low presupplement PLP levels (16.7 nM and 23.8 nM). No direct correlation was found between plasma PLP and dietary vitamin B-6 intake. As expected, the plasma PLP concentration of all subjects in the PN group, who received 50 mg of pyridoxine HCl daily (25 times the RDA), increased significantly (Fig 1). The subjects' hemoglobin, hematocrit, and white blood cell and differential counts were relatively constant during the 2 mo, regardless of supplementation.

To assess the effect of pyridoxine supplementation on immune response in the elderly, standard mitogens were used to stimulate lymphocyte proliferation *in vitro*. *In vitro* lymphocyte proliferation from both T- and B-cell

mitogens increased as a result of pyridoxine supplementation, suggesting that increasing vitamin B-6 intake could improve immune responsiveness of both T and B cells. The low-PLP subgroup of the PN group showed increased response to Con A (Fig 4) and all subjects in the PN group, regardless of their initial PLP concentration, showed increased response to PHA (Fig 2a). Because PHA and Con A may stimulate different subpopulations of T cells (21), these results suggest that different T-cell subsets may have different requirements for vitamin B-6. After 2 mo of PN supplementation, proliferation from SAC, the T-cell-independent B-cell mitogen, increased significantly in the PN group (Fig 2c). Response to the T-cell-dependent B-cell mitogen PWM increased in the low-PLP group, was maintained in the normal-PLP group, and decreased in the placebo group (Figs 2d and 3). The unexpected decline in the placebo group may have resulted from natural variability in individual response in the small number ($n = 4$) of subjects in the placebo group or from variation in cell culture conditions between testing periods. If the latter is assumed, the maintenance of PWM response of the normal-PLP subgroup may be interpreted to represent an increase in response in comparison with the placebo group; the increased response in the low-PLP subgroup may be considered a larger increase in response than that of the normal-PLP subgroup (Figs 2d and 3).

After 2 mo of PN supplementation, there was an increase in the percentages of T3⁺ and T4⁺ cells and no change in the percentage of T8⁺ cells. The increases in T3⁺ and T4⁺ cells suggest that PN supplementation may have influenced the differentiation of immature T cells to mature T cells. However, because interpretable data were obtained from only 5 of the 11 PN-treated subjects, these results must be regarded as preliminary.

The results of this study indicate that PN supplementation improves lymphocyte function in healthy elderly persons and suggest that vitamin B-6 nutriture is important in stimulating immunocompetence in the elderly. In this study both T- and B-cell mitogen responses increased with PN supplementation, particularly in individuals with initially low plasma PLP levels. This suggests that improved vitamin B-6 nutriture in elderly persons may improve *in vivo* cell-mediated and humoral immunity, especially in those with poor vitamin B-6 status. 

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