

AN ABSTRACT OF THE THESIS OF

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Effect of Vitamin B-6 Supplementation on Lymphocyte Responsiveness
in Independently-Living Elderly Persons.

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The effect of pyridoxine (PN) supplementation on lymphocyte responsiveness was investigated in 15 elderly volunteers (aged 65-81 years) by measuring lymphocyte proliferation to T and B cell mitogens, lymphocyte subpopulations with monoclonal antibodies (T3, T4, T8) and plasma pyridoxal 5'-phosphate (PLP) concentration at pre-supplementation and after 1 and 2 months of daily supplementation. Eleven subjects received 50mg of PN-HCl and 4 received a placebo. Dietary histories were also evaluated for intake of vitamin B-6, protein and kilocalories. Mitogens used for the stimulation of lymphocyte proliferation were

phytohemagglutinin (PHA); concanavalin A (Con A), pokeweed mitogen (PWM), and Staphylococcus aureus Cowain I (SAC). Plasma PLP was measured by a radiotracer method. Before supplementation, mean PLP of the 15 subjects was 31.7 +/- 14.1nM; 5 PN and 3 placebo treated subjects had low PLP levels. After 1 and 2 months of PN-HCl supplementation, the PLP levels increased by 195 +/-88nM and 201 +/-84nM, respectively. Lymphocyte proliferation in response to PHA, PWM, and SAC increased significantly ($p < 0.05$) with PN supplementation. Among PN-treated subjects, lymphocyte blastogenesis was significantly greater in response to Con A and PWM in individuals whose initial PLP was low. Percentages of T3+ and T4+, but not T8+ cells increased significantly in PN-treated individuals. These results suggest that vitamin B-6 status is important in maintaining immunocompetence in the elderly.

**The Effect of Vitamin B-6 Supplementation
on Lymphocyte Responsiveness in
Independently-Living Elderly Persons**

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THE EFFECT OF VITAMIN B-6 SUPPLEMENTATION ON LYMPHOCYTE
RESPONSIVENESS IN INDEPENDENTLY-LIVING ELDERLY PERSONS

INTRODUCTION

Aging is a process which all persons experience from conception to old age. As one approaches old age, physiological changes occur in the body ranging from alteration in body composition to increased susceptibility to infection and disease to altered metabolic processes. In our society, the proportion of persons over the age of 65 years is growing rapidly. At the turn of the century 4% of the population was over 65 and by 1980 this figure had increased to 12% (1). Thus, it has become more important to understand the problems and mechanisms involved in aging with hopes of improving the health and quality of life in aged persons.

One of the changes associated with aging is a decline in immune function. 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 (2-6). At the same time elderly persons tend to be at a greater risk for vitamin B-6 (B-6) deficiency (7-13). B-6 plays an important role both in the

humoral and cell-mediated arms of immunological response (14-22). A deficiency of B-6 appears to alter lymphocyte function by reducing nucleic acid synthesis (14, 19, 22) which impairs DNA synthesis and subsequent RNA and protein synthesis (22). Lymphocyte reactions to an antigenic challenge, which require a proliferative response, are compromised in B-6 deficiency. Despite this relationship, little research has been done on the effects of nutrition on immunosenescence, and much less on the effect of vitamin B-6 on the impaired immune response in elderly persons. This research project was designed to test whether increasing vitamin B-6 intake would improve immune status of elderly persons. Elderly volunteers were given daily for 2 months 50mg of pyridoxine hydrochloride (PN-HCl) or a placebo. Changes in lymphocyte subsets, lymphocyte proliferation in response to mitogens, and plasma pyridoxal 5'-phosphate (PLP) concentration were monitored before supplementation and after 1 and 2 months of PN supplementation.

THE AGE-RELATED CHANGES IN SUBSETS AND FUNCTIONS OF LYMPHOCYTES
AND VITAMIN B-6 STATUS IN HUMANS: A REVIEW OF
LITERATURE

Alterations in lymphocyte function and vitamin B-6 status occur with aging. How these two factors relate to one another may influence the health of the increasing population of elderly in this country.

Vitamin B-6 (B-6) deficiency impairs both humoral and cell-mediated immunity in humans and animals (14-22). The relationship between B-6 and lymphocyte function has been recently reviewed in detail by Ha (23). The review presented here will focus on describing the changes in lymphocytes and B-6 nutriture associated with aging.

A. Age-Related Alterations in Subsets and Functions of Lymphocytes

1. Thymic involution

The thymus is a gland composed of a cortex, medulla and epithelial cell network. The thymus serves two major functions in the body. Immature T cells formed in the bone marrow migrate to the cortical region of the thymus where they divide and differentiate into functionally mature and distinct T lymphocyte subsets. Additionally, the epithelial cells of the thymus secrete several polypeptide hormones important in the differentiation of

pre- and post-thymic lymphocytes (24).

Thymic involution precedes other immunological alterations in aging. Thymic atrophy begins at the age of sexual maturity and reaches its climax in man by the age of 45 or 50 years when only 5 to 10 % of its maximum mass remains (25). The decrease in thymic size is due primarily to atrophy of the cortex followed by atrophy of epithelial cells, which then reduces the secretion of thymic hormones (24). With aging, fewer precursor T lymphocytes migrate to the thymus, but the percentage of immature lymphocytes in the thymus increases (26). In general, the cortex of the involuted thymus is more sparsely populated with lymphocytes, which are replaced by macrophages and lipid granules (24, 27). The thymus maintains serum levels of thymic hormones until an age of 20 or 30 years. After this, a gradual decline in serum concentration occurs until about the age of 60 years when thymic hormone levels can no longer be measured (24). Because thymic involution precedes other alterations in lymphocyte function, it may be responsible for the subsequent decline in T cell function seen in aging (2, 4, 5).

2. T lymphocyte subsets

Mature T lymphocytes are differentiated into functionally distinct subpopulations. These subsets consist of T helper cells, T suppressor cells and cytotoxic T cells. Conflicting data have

been presented on changes which occur in T cell subpopulations with aging. Using spontaneous rosette formation of lymphocytes to sheep red blood cells as a means of identifying T lymphocytes, a decline (28-31) or little change (32) in the total number of T cells has been observed. According to Kishimoto et al. (31), this discrepancy may be due to the methodology employed for quantitating T rosettes. Those who report a decrease generally did not include fetal calf serum (FCS), while those noting no change included FCS in the culture media.

Using monoclonal antibodies to membrane receptors found on all mature T lymphocytes (e.g. OKT3 or Leu 4) most investigators (33-36) reported a modest, but significant decline in the percentage of mature T cells in peripheral blood with aging. Two studies reported a sex difference in the decline in T cells, with older men showing a more significant decline in comparison to young men, and older women showing no significant decline in total T cells compared to young women (33, 34). This sex difference was not confirmed in other studies (35, 36).

Even though the total number of T lymphocytes may be unchanged as a function of aging, changes in the balance of immunoregulatory T subpopulations could affect immune responsiveness in the elderly. In this regard, Moody et al. (37) reported that inducer/helper T cells, as quantitated by the monoclonal antibody OKT4, were elevated in the elderly while

Mascart-Lemone et al. reported a decrease in the percentage of T4 positive (T4+) cells in older men (34). However, the majority of studies have found no significant differences in the percentage of T4+ cells between young and elderly humans (33, 35, 36).

The suppressor/cytotoxic T cell subpopulation as identified with the monoclonal antibodies OKT8 or Leu 3a (T8+ cells) has been reported to decrease with age in humans (33, 35-37). Nagel, Chrest and Adler (33) noted this change particularly among aged men, while Mascart-Lemone et al. (34) found an increase in T8+ cells among older men but not women.

When the T4+/T8+ ratio is considered, the results of Nagel et al. (33) showed a significantly higher T4+/T8+ ratio in elderly male subjects as compared to young male subjects. However, in a follow-up study, Nagel et al. (36) found no significant difference in helper/suppressor ratios in older people, a result confirmed by Ligthart, Schuit and Hijmans (35). An imbalance in T4+/T8+ cells may contribute to impaired immune function, but whether or not it contributes to the age-related decline in immune response requires further study.

In lymphocyte subset analysis, another interesting phenomenon has been described. Significantly greater numbers of non-T, non-B, non-monocyte mononuclear cells have been observed in the elderly both by monoclonal antibody analysis (35) and by the number of non-E-rosetting, non-surface Ig positive lymphocytes

(28). This increase in "null" cells may reflect an increase in circulating immature T and B cells or an increase in natural killer cells (35).

3. T lymphocyte function

a. Suppressor function. Changes in suppressor T cell function have been observed with aging. Mitogenic doses of concanavalin A (Con A) have been shown to induce a non-specific suppressor function in lymphocyte cultures which then are able to depress responses to mitogens, antigens and allogeneic cells (6). The age of the lymphocyte donor did not seem to prevent Con A activation of a suppressor effect; however, the degree of suppression produced was lower with cells from elderly than those from young adults (6). A decline in suppressor activity of cells from aged persons on blastogenesis of allogeneic cells (6) and on the proliferative response of a cloned cell line (38) have also been reported. At the same time, percent suppression on autologous responder cells was shown to be elevated in in vitro cell cultures from elderly as compared to young subjects (38, 39). These results suggest that lymphocyte production of suppressor activity declines with age, but that sensitivity to suppression may be increased in lymphocytes from the elderly (38, 39). Con A activated suppressor activity in the elderly (71 to 99 years old) has also been shown to be inversely correlated with subsequent

mortality. Persons surviving 2 years after the study had a mean suppressor activity of 20.6%, while nonsurvivors had a mean suppressor activity of 0.1% (40).

Two groups of investigators have observed a maintenance (41) or increase (31, 42) in the T cell dependent B cell secretion of immunoglobulins (Igs) in response to pokeweed mitogen (PWM) in elderly persons, suggesting a possible decline in suppressor function with aging. Kishimoto et al. (42) tested this hypothesis by measuring the effect of Con A activated suppressor cells on Ig production. They found that T suppressor cells from older persons took longer in culture to reach a maximum suppressive effect and had less suppressor activity on Ig secretion than T cells from young donors. Treatment of the population with low doses of irradiation and mitomycin C prior to culturing them with B cells resulted in overall enhanced Ig production, with greater enhancement of IgG and IgM secretion in cell cultures of newborns and young adults than in cultures from aged adults. These results support the concept that aging is associated with a loss of T suppressor function (42).

Another method of measuring suppressor function of T cells involves the activation of cells by T cell specific anti-human brain associated thymocyte antigen (anti-BAT). Abe et al. (43) found that lymphocytes from young individuals, cultured with anti-BAT and complement in the presence of phytohemagglutinin

(PHA), Con A, or PWM, showed a dramatic decrease in proliferation only in response to Con A. Lymphocytes from elderly donors, however, showed no significant change in response to the mitogens with the addition of anti-BAT. The effect of anti-BAT and complement on Ig synthesis in response to PWM stimulation was to enhance Ig synthesis in cultures with cells from young persons, but not with lymphocytes from old donors. These results indicate that a suppressor function found in lymphocytes from young subjects is eliminated with the addition of anti-BAT, and that this suppressor function is lacking in lymphocytes from elderly subjects.

A decreased T suppressor population and/or function would be consistent with the increased frequency of autoantibodies seen in the elderly population and might also correspond to the increased incidence of diseases such as multiple myeloma and "benign" monoclonal immunoglobulinemia in the aged (33).

b. Cell-mediated immunity. Many studies have found impaired cell-mediated immunity (CMI) in the elderly (2, 4, 5, 44). Several in vitro and in vivo techniques have been utilized to measure the decline in T cell function and to understand more fully the mechanisms involved in the senescence of these functions.

The in vitro response of T lymphocytes to specific

mitogens, e.g. PHA and Con A is a common means of assessing mature T cell activity. Many investigators have reported impaired proliferative capacity of lymphocytes from elderly donors (> 60 years of age) to T cell mitogens and antigens (such as Mycobacterium tuberculosis and Varicella-Zoster virus) as determined by reduced tritiated thymidine (3H-thymidine) incorporation (34, 45-50). Hicks, et al. found a gradual decrease in T cell responsiveness with age, beginning in childhood and continuing throughout the life span (51). Using a variety of methodologies, several researchers (45, 47-49, 52) have demonstrated both a reduced number of mitogen responsive cells in preparations from elderly donors and an impaired ability of these cells to divide sequentially in culture. For example, after 96 hours in culture with PHA, the number of T cells from elderly donors which had divided for a third time was 1/4 of that of cultures of lymphocytes from young individuals. The number of cells of elderly dividing a second time was 1/2 that of cells of young persons. The number of cells dividing for the first time in cultures from both young and old was similar (47).

To identify the mechanisms involved in the impaired responsiveness to mitogens of lymphocytes from elderly donors, several investigators (45-47) compared the length of the cell cycle of mitogen stimulated lymphocytes from old and young donors. Tice, et al. (45) found that lymphocytes from aged persons entered

the pool of PHA stimulated cells more slowly than lymphocytes from young persons. The minimum length of the cell cycle was the same in both young and old, but the mean and maximum lengths of replication were longer in lymphocytes from aged subjects. An increased cell cycle time was supported by another study which observed that after 4 days of culture, PHA-induced proliferation of T cells from elderly subjects was impaired, but after 8 days of culture, no decline in proliferation was observed (52). On the other hand, Hefton et al. found no significant difference between young and aged individuals in the length of time required for PHA stimulated lymphocytes to complete the cell cycle, but found that fewer lymphocytes from elderly subjects proliferated in response to mitogens (47). In a more recent study, Staiano-Coico et al. followed the progression of the cell cycle phases more closely, and found that, although again fewer lymphocytes from elderly donors initiated cell division, the progression through the phases of mitosis, G-1 to S to G-2 and M, occurred at comparable rates for both groups and the total length of the cell cycle did not vary significantly between groups (46).

Another possible mechanism involved in the impaired mitogen responsiveness of T lymphocytes from elderly humans may be the changes observed in the production and sensitivity to T cell growth factor or Interleukin-2 (IL-2). IL-2 is an essential factor in mitogen-induced blastogenesis of T cells, as

proliferation depends on the ability of lymphocytes to produce and respond to IL-2 (53). IL-2 production and secretion is induced by mitogen stimulation of a subset of T cells in conjunction with adherent monocytes. A second subset of T cells, also sensitized and activated by the mitogen, binds IL-2 and proliferates. Progeny cells of IL-2 responsive lymphocytes maintain sensitivity to IL-2 allowing continued growth with IL-2 present. Stimulated T lymphocytes from elderly donors do not produce as much IL-2 activity as lymphocytes from young donors (3, 47, 53, 54). Gillis et al. (53) explored the possibility that depressed levels of IL-2 in cultures from elderly may be due to suppression of IL-2 production. However, when a mixed culture of cells from both young and old donors was compared, the IL-2 activity and ³H-thymidine incorporation from the mixed culture were not significantly different from the levels predicted by either cell population alone indicating no suppression of IL-2 production (53). The effect of the addition of exogenous IL-2 on the proliferation of T lymphocytes from young and old individuals has also been studied. Gillis et al. (53) found that all young donors but only 2 of 10 elderly donors demonstrated an increase in ³H-thymidine incorporation when exogenous IL-2 was added to the culture media. Kennes, Brohee and Neve (54), however, observed an increase in ³H-thymidine incorporation in IL-2 supplemented cultures from both young and aged in PHA, Con A and PWM stimulated cultures, although

the response of cultures from young significantly exceeded the response from aged persons. The level of response of lymphocytes from the elderly increased after IL-2 addition nearly to the level of response of young donors before IL-2 addition. These results indicate that IL-2 production is impaired in the elderly and that the elderly may have fewer IL-2 sensitive cells.

Another way to assess in vitro CMI is to measure cell-mediated cytotoxicity (CMC). When T lymphocytes are exposed to cells which are genetically dissimilar and consequently have "foreign" determinants on the cell surface, the lymphocytes proliferate and differentiate into cytotoxic effector cells which directly attack the "foreign" cells (55). CMC is measured routinely in 2 ways: by measuring a degree of target cell lysis (cell-mediated lympholysis, CML) or by measuring the degree of proliferation of lymphocytes to foreign cells (mixed lymphocyte reaction, MLR) (55).

Several studies found a decline in CML with aging (31, 56, 57) while another study found no significant difference in CML between young (< 45 years) and old (> 70 years) subjects (50). Becker et al. (56) tested the hypothesis that the decline observed in CML with aging was due to increased monocyte-mediated suppression of the cytotoxic reaction in older persons. However, they found that cytotoxicity did not increase consistently in monocyte-depleted cultures in cells from elderly subjects and

thus, could not account for the decline in CML. The authors concluded that the decline in CML was due to an intrinsic decline in number and/or function of cytotoxic T cells or non-monocyte mediated negative modulation.

Several investigators reported no decline in MLR to irradiated allogeneic cells with aging (31, 37, 50). Furthermore, Charpentier et al. (50) found no change in the ability of irradiated lymphocytes from old donors compared to young donors to stimulate a MLR. On the other hand, Moody et al. (37) observed a decline in the MLR of T cells from elderly persons to non-T cell autologous cells as compared to cell cultures from young persons.

T lymphocyte proliferation has been shown to involve a cytoplasmic factor which is capable of stimulating DNA synthesis in quiescent nuclei. This activator of DNA replication (ADR) is present only in PHA stimulated peripheral blood lymphocytes (PBL) and has been proposed as an intracellular signal for PHA-induced blastogenesis. Gutowski et al. (58) investigated the ability of PHA-stimulated lymphocytes from old and young subjects to produce ADR activity capable of inducing DNA synthesis in quiescent, isolated, frog nuclei. They found that although the PBL from elderly persons did not proliferate as well as PBL from young in response to PHA, the ADR from both groups was able to induce DNA synthesis comparably in quiescent nuclei. These results suggest that impaired functional capacity of T lymphocytes from elderly

persons is not a function of reduced ADR activity or lack of this intracellular "second messenger".

In vivo T cell function has also been studied in humans to some degree. The most common method of testing CMI in vivo in humans is by testing delayed type hypersensitivity (DTH) reactions to skin antigens (e.g. streptodornase, streptokinase, Candida). In general, DTH reactions to such antigens decreases with age, although some discrepancies have been observed (2, 44, 59). These discrepancies may be more a problem of test methodology than a denial of reduced DTH reactions with aging. Antigens and populations chosen may have been inappropriate (5). One study did not show DTH energy, but elderly subjects required second strength antigens to get a response in 16% of the cases (2). For common skin test antigens or secondary DTH reactions, the reduced response of the elderly may reflect altered response to antigenic challenge or loss of immunological memory or both (4). To control for differences in time between sensitization and challenge, one can expose young and aged populations to a "new" antigen (e.g.; dinitrochlorobenzene, DNCB) and then challenge the subjects with the same antigen after the same period of time. Using this protocol, a striking difference is seen between reactions of the old and young (2, 5, 60). One study revealed that 31% of those older than 70 years of age failed to respond to the challenge while only 6% of persons less than 70 failed to respond (60).

Another method which measures CMI in vivo is graft versus host (GVH) reactions to transplantations. GVH testing has been done most frequently in experimental animals although a cutaneous model of GVH reactions, lymphocyte transfer, has been done in humans. Elderly subjects were less able to induce a positive transfer reaction than young subjects when a cutaneous lymphocyte transfer test was given (61).

4. B lymphocyte population and functions

Results concerning changes in the B cell population with aging are conflicting. Using antisera to surface immunoglobulins, some researchers (28,31) have observed no change while others (35, 52) have found a small but significant decrease in B cell numbers and proportion in peripheral blood.

On the other hand, B lymphocyte function appears to be impaired in aged individuals. Using in vivo antibody (Ab) response to vaccines as a means of testing B lymphocyte function, several investigators have reported a decline in the antibody response of elderly persons to agents such as Japanese B encephalitis virus, tetanus toxoid, and salmonellae flagellin (2, 4, 6). Using in vitro antibody production as a means of assessing B cell function, investigators have also observed decreased immunoglobulin (Ig) production to both T dependent (41, 59, 62, 63) and T independent (41) antigenic stimuli in aged

humans. One study showed no decline in Ab response to PWM (a T dependent B cell mitogen) with age, but found an increase in the incidence of non-responders to PWM with age (64). Using PWM, Wrabetz et al. (41) found a decline in IgM, but a preserved IgG secretion in old donors (65 to 94 years old) versus young controls (20 to 38 years old). In response to the T-independent antigen, *Salmonella paratyphi B*, they found a decline in IgG production and an even greater loss of IgM secretion in cells from old persons (41). These results suggest that an intrinsic defect in B cell antibody synthesizing capacity occurs with aging. Antonaci et al. (65) found old donors (65 to 84 years old) had a reduced ability to produce antibody to staphylococcal protein A stimulation as determined by a hemolytic plaque assay. They also found, as did Antel et al. (63), that removal of adherent cells (monocytes) or pretreatment with indomethacin (an inhibitor of prostaglandin synthesis) significantly increased the antibody response of old donors, although not to the levels of young donors. These data support the hypothesis that part of the decline in antibody production seen in older persons may be due in part to monocyte suppression, mediated through prostaglandin secretion (65). In addition, Antel et al. (63) showed that Ig-secreting B cells from elderly persons were more sensitive to Con A induced T cell suppression than B cells from young donors.

Conflicting evidence has been reported concerning changes in

circulating levels of Ig subclasses with aging. Some note a gradual increase (31, 66) while Phair et al. (44) observed no change in serum IgG and IgA. Both a decline (4, 44) and no change (31, 66) in serum IgM levels with age have also been reported. This may be due to the relative insensitivity of most assays for measuring serum titers (e.g., hemagglutination) (67).

Particular subclasses of Ig may not change with aging, but the frequency of monoclonal Igs increases with age (66). This alteration reflects disordered immunoregulation of normal B cells (4). In addition, the incidence of autoantibodies also increases with age (3, 68-70). Using a T independent polyclonal B cell activator (Epstein-Barr virus), Fong et al. (70) reported an increased ability to induce an IgM anti-IgG response and an antithyroglobulin response in older people (75 to 90 years of age). Others (66, 69) have noted an increased incidence of autoantibodies in elderly to nuclear factors, lipoproteins, rheumatoid factors and thyroglobulin. One study (68) noted an increased incidence of anti-T cell antibodies in sera of aged individuals (60 to 99 years of age) and these autoantibodies were also reactive with a subset of T cells similar to that identified by sera from patients with juvenile rheumatoid arthritis. Increases in autoantibodies with age appears to have a negative correlation to survival (71) which may reflect the possible contribution of autoantibodies and the circulating immune

complexes to tissue and organ damage with age (4).

5. Summary

The changes that occur in both T and B lymphocytes with aging appear to be complex and interrelated. A variety of factors may be involved. Thymic involution and subsequent decline in T cell differentiation may lead to a loss of T helper and/or T suppressor activity. Alterations in T regulatory function in turn likely affect both cellular and humoral immunity. Increased suppressor activity of monocytes and macrophages or increased sensitivity of lymphocytes from elderly to suppressor activity may result in impaired lymphocyte responsiveness. Lymphocytes may also have an intrinsic decline in cellular function which results in a decreased ability to proliferate and respond to immunologic challenges with increasing age of the individual. Other environmental factors may also influence the loss of lymphocyte function.

B. The Effect of Nutrition on Lymphocyte Function in Elderly Persons

One factor which can influence lymphocyte function and is frequently overlooked by researchers is the nutritional status of their subjects. It has been established that protein-calorie

malnutrition (PCM) as well as deficiencies of several vitamins and minerals cause an impairment of immune function (reviewed in 16). The elderly are a group which may be at risk for nutritional deficiency. Chandra et al. (72) examined 51 persons, all over the age of 60, and found 21 who showed some evidence of PCM on the basis of clinical features, anthropometric measurement and biochemical assessment. Using these 21 with PCM for further study, they found a reduction in the number of rosette-forming T cells as well as in T3+ cells, and 15 of the 21 showed DTH anergy. In these subjects, T4+ and T8+ subsets were low. Lymphocyte proliferation to PHA was reduced. After a general nutritional supplement (Ensure, Ross Laboratories) which provided approximately 500 kcal, 17.5g protein, 0.75mg of vitamin B-6 and other essential vitamins and minerals was given for 2 months in addition to the self-selected diets of the subjects, the immune functions of these elderly improved significantly. The lymphocyte stimulation index to PHA increased, DTH reactivity improved, and T3+, T4+ and T8+ subpopulations increased. Nutritional status measurements (serum albumin, transferrin, zinc, retinol-binding protein, prealbumin concentrations) also showed improvement. This study suggests that nutritional status should be considered when immune parameters are studied and that part of the impaired functional capacity observed in elderly persons may be due to poor nutritional status.

C. Vitamin B-6 Status in the Elderly

Little is known of the nutritional requirements of older persons, particularly of those over 65 or 70 years of age (73), even though this segment of the population is growing rapidly. A number of factors may contribute to possible malnutrition in elderly persons: social and physical isolation, mental and physical disability, low socioeconomic status, and lack of family support, as well as physiological changes which may alter the need for different nutrients. A poor diet may lead to subclinical or overt malnutrition which could aggravate existing health problems.

Vitamin B-6 (B-6) is a nutrient frequently overlooked in the American diet. B-6 plays an essential role in the metabolism of protein, carbohydrates, and lipids, and in the synthesis of neurotransmitters and nucleic acids (74). Deficiency of this nutrient can lead to impaired immune responsiveness in animals and humans (16, 19). Yet, the elderly population tends to consume diets low in B-6 and to be at risk for biochemical deficiency of this nutrient.

1. Vitamin B-6 metabolism

Vitamin B-6 (B-6) is a generic term used to describe the

derivatives of 3-hydroxy-2-methylpyridine which have biological activity (75). The major forms of the vitamin include: pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM); and their phosphorylated forms, pyridoxine 5'-phosphate (PNP), pyridoxal 5'-phosphate (PLP), and pyridoxamine 5'-phosphate (PMP). The predominant urinary metabolite of B-6 is 4-pyridoxic acid (4PA). The metabolism of vitamin B-6 is summarized in Fig. 1.

PLP is a cofactor for over 100 enzymes (76). The primary function of B-6 is in the metabolism of protein, wherein PLP is required for transaminases, decarboxylases, and other enzymes catabolizing amino acids. Other PLP dependent enzymes are also required for the production of the neurotransmitters, serotonin and dopamine, and in the synthesis of a precursor of porphyrin, essential to hemoglobin synthesis (74). More detailed information concerning vitamin B-6 can be found in extensive reviews (77-81). The purpose of this review of vitamin B-6 is to summarize its metabolism and function.

2. Recommended Dietary Allowance

The Recommended Dietary Allowance (RDA) of vitamin B-6 for adult females is 2.0 mg and for adult males, 2.2 mg (80). A ratio of 0.02 mg B-6 /g of protein is used as the basis for calculating the RDA. On this basis, 2.0 mg of B-6 and 2.2 mg B-6 per day would be adequate for consumption of up to 100 g and 110 g of

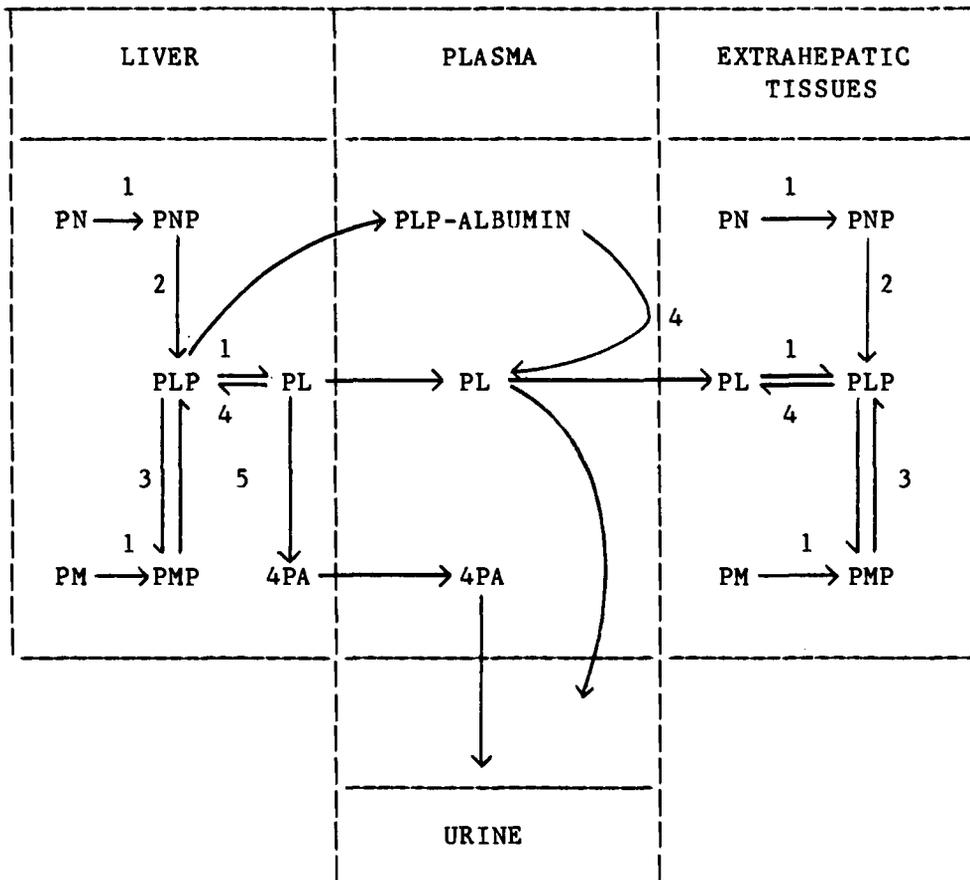


Fig. 1. Pathways for the metabolism of vitamin B-6. Numbers indicate enzymes catalyzing the reactions: 1 = pyridoxal kinase, 2 = PNP oxidase, 3 = transaminase, 4 = alkaline phosphatase, 5 = aldehyde oxidase and perhaps, aldehyde dehydrogenase. Abbreviations are given in the text. Figure adapted from Lumeng et al. (82).

protein, respectively. Protein intake in the United States generally exceeds the RDA (0.8g/kg of body weight or 44g of protein for females weighing 55kg and 56g for 70kg males) and the levels of protein used for the B-6 RDAs represent average intakes (80).

3. Dietary intake of vitamin B-6

The dietary intake of B-6 by many elderly persons is low. Elsborg et al. found that 83% of 403 elderly persons received less than the RDA (2 mg) (10). However, only 1% consumed less than 0.8mg of B-6. Studying 270 persons over 60 years of age, Garry et al. (83) found that the majority of subjects consumed less than the RDA from diet alone. In this study, 57% of men and 61% of women were taking nutritional supplements, and had median intakes of vitamin B-6 that were about 3 times the RDA. Another study found that 87% of hospitalized elderly who did not take a vitamin supplement consumed diets containing 1 mg of B-6 daily (50% of the RDA) (84).

Since dietary protein plays an important role in determining the B-6 requirement (84), evaluation of dietary B-6 should include dietary protein intake as well. Some studies show a high protein intake (>100% of the RDA) in conjunction with a low B-6 consumption, 50-75% of the RDA on the average (7, 8, 84).

A certain amount of individual variation may exist in vitamin

requirements, and the RDA levels were established with a large margin of safety (80). For these reasons, B-6 intake as a measure of B-6 status may not be adequate. Biochemical measures of adequacy are necessary to give a more complete profile of B-6 nutriture.

4. Biochemical assessment of vitamin B-6 status

One method of biochemical assessment of B-6 status is the stimulation of endogenous transaminases in blood, which require the coenzymatic form of B-6, PLP, to function. The two enzymes used are glutamic-pyruvic transaminase (GPT) and glutamic-oxaloacetic transaminase (GOT). For status assessment, generally, an investigator measures the endogenous or basal activity of the enzyme, then stimulates the activity of the enzyme by the in vitro addition of exogenous PLP. The ratio or percent increase in enzyme activity gives an indication of B-6 status (85). The larger the percent stimulation, the greater the chance of deficiency. The less endogenous PLP available to bind to the enzyme, the less activity the enzyme will show initially and the more it will be stimulated by exogenous PLP. It has been suggested that, in the early stages of B-6 deficiency, the lack of PLP causes a decrease in transaminase activity due to a reduced saturation of apoenzyme. In chronic stages of B-6 deficiency, the lack of coenzyme (PLP) may cause decreased synthesis of the

apoenzyme (11, 86).

Both serum transaminase and erythrocyte transaminase activities have been used as a measure of B-6 status. Erythrocyte transaminases reflect B-6 status more accurately than serum transaminases because serum transaminase levels may be affected by other factors (such as liver disease) (84, 86). Using serum GOT activity, however, Hamfelt (87) found an increasing degree of stimulation of GOT to exogenous PLP with increasing age. Jacobs, Cavill and Hughes (86) found a decreased stimulated level of EGPT, indicating a lack of both apoenzyme and coenzyme. Of 47 hospitalized elderly subjects not taking a B-6 supplement, Vir and Love (84) found that 23, or 49%, had a greater than 15% stimulation of EGPT, indicating B-6 deficiency. The same authors observed that 11% of hospitalized elderly subjects taking 2.5 mg B-6 per day also showed EGPT levels indicating biochemical deficiency. After treating the deficient group with 2.5 mg B-6 per day, the basal EGPT activity increased significantly and the percent stimulation decreased. To some of the deficient group the investigators gave a 50 mg per day supplement of B-6 which improved the response significantly (84). Other researchers have observed biochemical deficiency of B-6 using EGPT stimulation in 24% to 65% of elderly subpopulations depending on the locations and living conditions of the group tested (7, 8, 11, 12, 88). The highest levels of biochemical deficiency were found among groups

of elderly living in a sheltered dwelling facility or residential center in Northern Ireland (11) and institutionalized elderly in Kentucky (12). Biochemical deficiency was reduced in elderly subjects when they were supplemented with vitamin B-6 (88).

Using EGOT, less biochemical deficiency in elderly persons has been shown as compared to EGPT stimulation. Jacobs et al. (86) found no correlation between basal or stimulated EGPT levels with increasing age, but found 30% of unsupplemented elderly at risk for B-6 deficiency at a EGOT ratio >2.0 . Others have shown a risk of B-6 deficiency in 19% of geriatric patients (9). The discrepancy between EGOT and EGPT measures can be explained by the fact that EGPT appears to have less affinity for PLP than EGOT and is, consequently, a more sensitive measure of B-6 status (86).

Another method for determining B-6 status biochemically can be achieved by measuring plasma PLP concentrations, since PLP is the active form of B-6. The level of PLP in the plasma reflects tissue levels of B-6 (89). Several investigators have found a significant decrease in the level of plasma PLP with increasing age (13, 87, 90). This decrease has been found in persons not taking a B-6 supplement (13). Plasma PLP increases significantly with pyridoxine supplementation (87, 90).

Using a protozoological organism with an absolute requirement for B-6, Baker et al. (91) found 31.3% of elderly persons tested had blood B-6 concentrations below a 95% confidence interval of

blood B-6 levels in young, healthy adults. Of this combined total, 36.9% of institutionalized elderly and 18.4% of non-institutionalized elderly were below these levels or at risk for B-6 deficiency. In a second study which used the same subjects, Baker, Frank and Jaslow (92) compared the effect of supplementary B-6, given orally or by intramuscular injection. They found that a single injection improved status as long as 3 months after the treatment, whereas daily oral supplementation left a significant portion of elderly with low plasma PLP levels.

Tryptophan requires PLP for its metabolism. A person at risk for B-6 deficiency will excrete elevated amounts of certain tryptophan metabolites in urine. By measuring one or more of these metabolites in the urine after a loading dose of tryptophan, an indication of B-6 status can be established. Hamfelt (87) gave 11 of 21 older persons a tryptophan load test before and after a B-6 supplement to observe whether low plasma PLP had any effect on its metabolism. Before B-6 supplementation the tryptophan load test revealed high level of xanthurenic acid in the urine, indicative of poor vitamin B-6 nutriture. Levels of tryptophan metabolites returned to normal after pyridoxine supplementation of 80 mg per day for 7 days. This test indicated that a relationship did exist between reduced plasma PLP and tryptophan metabolism in the elderly.

Another study related two biochemical status assessment

methods (12). In this experiment, Chen and Fau-Chiang related EGPT stimulation to the determination of the major urinary metabolite of B-6, 4-pyridoxic acid (4-PA). Their results showed a significant ($p < 0.05$) negative correlation between EGPT activation coefficient and 4-PA in the urine. The measure of 4-PA alone showed 20.8% of the total subjects below the critical level of 4-PA excretion (500 $\mu\text{g/g}$ creatinine) (12).

Several studies have attempted to relate B-6 consumption with biochemical B-6 status (7, 84, 93). The results revealed a great deal of variation. Not everyone who had a low dietary intake of B-6 showed biochemical deficiency. The majority of those with biochemical deficiency had low intakes ($< 2/3$ RDA) of B-6, but some elderly persons consuming multivitamin supplements showed biochemical deficiency (11,84). These discrepancies may reflect individual variation in B-6 requirements or variation in the ability of individuals to adapt to low dietary levels of B-6. Another possibility to explain the differences in intake and biochemical measures of B-6 status might be that the metabolism of B-6 changes in the aging process, but to varying degrees among aging persons. Aging is not a uniform process and in that sense one might expect increasing variation in a number of physiological functions with increasing age.

Despite the fact that B-6 consumed in the diet may not always correlate with biochemical indices, the results of the studies

reviewed here indicate that a significant portion of the elderly population is at risk for B-6 deficiency. This risk is confirmed particularly by the studies which show a significant relationship between 2 biochemical measures of vitamin B-6 status (12, 13, 87).

MATERIALS AND METHODS

Subjects

Subjects were recruited from the Corvallis, OR area residential centers, various senior citizen groups, retired OSU faculty and by personal contacts. Volunteers were interviewed and completed a health questionnaire to provide information on their medical history, use of medications, and nutritional supplements. Following an explanation of the study protocol, each participant signed an informed consent form. This study was approved by the OSU Committee for Protection of Human Subjects.

All subjects were 65 or more years of age. They were free from any known immunodeficiency, liver, kidney, or metabolic disorder. They were living independently and were eating self-selected diets. None of the subjects were taking drugs that are known to be immunosuppressive or known to affect vitamin B-6 metabolism (94). None were regularly taking vitamin or mineral supplements which might have had any known effect on the study. Information collected from the 15 participants (14 women and 1 man) is presented in Tables 1 and 2.

Experimental Treatment

The subjects were randomly divided into 2 groups. The B-6 group (n=11) took 50mg of pyridoxine hydrochloride (PN) (McKesson

Table 1

General information on subjects

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

* Nutritional supplements taken as reported by the subjects.

† Treatment group. The B-6 group received 50mg of PN-HCl daily, the placebo group received a placebo daily for 2 months during the investigation.

Table 2

Prescription medications taken by and medical conditions of subjects

Subject	Medication(s)	Medical Condition(s)
1	propranolol hydrochloride	hypertension
2	hydralazine hydrochloride metoprolol tartrate	hypertension
3	--	--
4	triamterene hydrochlorothiazide levothyroxine sodium	hypertension thyroid insufficiency
5	phenobarbital hydroscyaminosulfate atropine sulfate scopolamine hydrobromide	diverticulitis flare-ups
6	chlorothiazide digoxin allopurinol	hypertension heart rhythm regulation gout
7	furosemide nifedipine gemfibrozil hydroxyzine pamoate	heart rhythm regulation angina blood lipid regulation anxiety relief
8	--	--
9	--	--
10	triamterene hydrochlorothiazide quinidine	hypertension (pacemaker) heart rhythm regulation
11	phenytoin trifluoperazine- hydrochloride	epilepsy
12	--	--
13	--	--
14	--	--
15	cimetidine hydrochloride propranolol hydrochloride digoxin	ulcer hypertension

Laboratories, Dublin, CA) while the placebo or control group (n=4) received a placebo (Stayner Corporation, Berkeley, CA or Eli Lilly and Co., Indianapolis, IN) daily for the 8 week period. The subjects were not aware of which preparation they received. Compliance was verified by providing 2 weeks supply of tablets at a time, counting any remaining tablets every 2 weeks, and by personal visits and verbal reminders every 1 to 2 weeks throughout the 2 month period. With the exception of 1 to 3 days, the subjects took the PN supplement or the placebo daily.

Experimental Design

The study consisted of 3 testing periods: pre-supplementation (baseline), post 1 month of PN or placebo supplementation and post 2 months of supplementation. At each of the three testing periods the following assays were performed: lymphocyte proliferation response to mitogens, lymphocyte subset analysis, plasma pyridoxal 5'-phosphate (PLP) concentration, hemoglobin (Hb), hematocrit (Hct), and white blood cell (WBC) and differential counts. Due to anticipated intra-person variability, the lymphocyte proliferation assay was conducted 1 week prior to and again on each full test day for each period. In total, blood was drawn 6 times from each subject. Subjects were tested on 2 days within the same week to facilitate efficient handling of samples. On each test day, 2 placebo and 5 or 6 PN treated

individuals were tested.

Blood Drawing

Between 7 and 9 am on each test day, blood was obtained from the forearm of fasting subjects by a registered medical technologist in each subject's home. The blood was collected into evacuated tubes containing heparin and held on ice up to 3 hours until the assays were performed. When the blastogenesis assay was conducted alone, 10 ml of blood were obtained. On the days when all tests were performed, 30 ml of blood were drawn. Of the 30 ml, approximately 4-5 ml of whole blood were used for Hb and Hct, plasma PLP, WBC counts and differential blood smears. The remaining 21-24 ml were separated on a density gradient in order to obtain mononuclear cells (monocytes and lymphocytes) for blastogenesis and lymphocyte subset analysis. The lymphocyte proliferation, lymphocyte subset, hematocrit, hemoglobin and WBC determinations were performed on the same day as the blood collection. Plasma was stored at -40 degrees C until analyzed for PLP concentration.

Isolation of Mononuclear Cells

Whole blood was diluted 1:1.5 with RPMI 1640 (GIBCO, Grand Island, NY) in 50ml centrifuge tubes and 18-20ml of Ficoll-Paque (Pharmacia, Piscataway, NJ) was layered under the diluted blood

using 6 inch blunt needles (Popper and Son, New Hyde Park, NY). Blood was centrifuged at 400 x g for 30 minutes at 4 degrees C. The mononuclear cells (MNCs) were removed from the interface, placed in 15ml tubes, and washed twice in RPMI 1640.

Blood Cell Counts

White blood cells were counted using a Coulter Counter, Model ZBI (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 Cowain I (SAC).

Appropriate dilutions of each mitogen were made and plated in triplicate, 0.1ml per well, into 96-well, flat-bottomed microtiter plates (Linbro, McLean, VA). All dilutions were made in RPMI 1640 supplemented with 10% fetal calf serum (10% RPMI). PHA (Wellcome, Beckenham, England) was diluted to concentrations of 10, 2.5, and 0.625µg/ml. Con A (Calbiochem, La Jolla, CA) was diluted to 40, 10, and 2.5µg/ml. PWM (Sigma, St. Louis, MO) was diluted to 2.0

and 0.5 μ g/ml. Staphylococcus aureus Cowain I (SAC) (Calbiochem, La Jolla, CA) killed bacteria were diluted to 3.2×10^8 , 8×10^7 , and 2×10^7 SAC/ml. PHA, Con A, and PWM were plated and frozen at -70 degrees C until the day before testing when SAC was plated. Wells containing 10% RPMI alone were included to determine background responses.

Separated MNCs were resuspended in 10% RPMI, the cell concentration was adjusted to 2×10^6 cells/ml, and 0.1ml was plated into microtiter plates. All plates were incubated 96 hours at 37 degrees C, in an atmosphere of 5% CO₂, and 95% humidity. Twenty-four hours prior to termination of the incubation, 0.5 μ Ci 3H-thymidine (New England Nuclear, Boston, MA) was added to each well in a volume of 20 μ l. The plates were frozen at -70 degrees C until harvested.

Cells were harvested onto glass microfiber filter paper (Whatman, Madestone, England) using an ADAPS (ADAPS, Dedham, MA) cell harvester. Filter disks were placed in pre-labeled minivials (Wheaton, Millville, NJ) and allowed to dry. Two milliliters of liquid scintillation fluid, Instagel (Packard, Downers Grove, IL), were added to each vial and each vial was counted in a β -scintillation counter (Packard Tri-Carb, Model 3255, Downers Grove, IL). Data were recorded as counts per minute (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, control cultures of each subject was subtracted from each of the respective mitogen responses. The means of the 2 blastogenesis responses done for each mitogen at each of the 3 time periods (baseline, 1 month and 2 months post supplementation) were used to represent response at a particular point in testing.

Lymphocyte Subpopulations

Separated MNCs were resuspended in RPMI 1640 and adjusted to a concentration of $5-10 \times 10^6$ cells/ml. A volume of 0.1ml of the cell suspension was pipeted into 12mm x 75mm plastic tubes. The appropriate mouse anti-human monoclonal antibody was added (0.105ml) to the MNCs. The tubes were lightly vortexed, and incubated for 30 minutes at 4 degrees C. The cells were washed twice in 1ml of cold 2% RPMI 1640 at 4 degrees C. and resuspended in 0.1ml of cold RPMI 1640. Five microliters of fluorescein conjugated (FITC) goat anti-mouse F(ab') antibody (New England Nuclear, Boston, MA) was added, and the lymphocytes were incubated and washed as before. The cells were resuspended in 0.9ml of phosphate buffered saline (PBS) and fixed with 0.1ml 10% formaldehyde solution. The monoclonal antibodies used were T3, T4, T8, and B1 (Coulter Immunology, Hialeah, FL). Purified mouse immunoglobulin (Coulter Immunology, Hialeah, FL) served as a control for non-specific fluorescent staining. Lymphocytes were analyzed using a Coulter Epics V flow cytometer (Hialeah, FL).

The laser light was adjusted to a wavelength of 488 nm. The lymphocyte population was determined by analyzing the log of the 90 degree light scatter of incident light (proportional to the degree of heterogeneity or granularity of the cell) versus 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 apo-tyrosine decarboxylase. The method used was that of Chabner and Livingston (95) with modifications made in our laboratory (96). 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% (SD= +/-6.9%).

Collection of Dietary Information

Prior to the initiation of testing, each subject completed a 3-day dietary history which included 2 week days and 1 weekend day. The investigator explained how to keep a 3-day dietary history by giving both verbal and detailed written instructions to each subject. The diet histories were reviewed with each subject to verify completeness and accuracy.

Foods were coded for computer analysis for general nutrient intake with special emphasis on vitamin B-6. The nutrient data base used was the 1981 version of the Ohio State University nutrient data base (97) with additional data for incomplete nutrient information or new foods by the OSU Department of Foods and Nutrition (98-105).

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. Comparisons between means were based on Neuman-Keuls' Multiple Range Test, Student's t test, and paired t tests (106). Correlation coefficients were calculated to determine relationships between immune parameters and plasma PLP concentrations, between dietary intake of vitamin B-6 or B-6/protein ratio and plasma PLP, and between lymphocyte subsets and mitogen responses (106).

RESULTS

Dietary vitamin B-6 intake and nutritional status

The means of the subjects' dietary intake of kilocalories (kcal), protein (pro), and vitamin B-6 (B-6) and the ratio of mg B-6 to g protein as well as the subjects' mean initial plasma PLP concentration, Hb and Hct are presented in Table 3. The mean caloric intake of the 15 subjects fell within the RDA ranges given for females between 51-71 years of age (1400-2200 kcal) and 76+ years of age (1200-2000 kcal) (80). The mean caloric intake for the male subject (1877 kcal, aged 74 years) was below the RDA range for males aged 51-75 years (2000-2800 kcal). The mean protein intake of the group (1.01g protein/kg of body weight, 66.1g protein total) was above the RDA level (0.8g protein/kg of body weight). The mean vitamin B-6 intake of the group (1.51mg) was about 75% of the RDA although the average ratio of mg of vitamin B-6/g protein (0.022mg B-6/g protein) met the recommended 0.02mg B-6/g protein (80). One-third of the subjects had intakes below 0.02mg B-6/g protein.

The initial average Hb concentrations for the female subjects (15.1g/dl) and the male subject (16.7g/dl) were above the mean levels for females and males over 40 years of age (13.9 and 15.6g/dl, respectively) (107). The pre-supplementation mean Hct

Table 3

Mean dietary intake, PLP concentration, Hb, Hct of subjects before supplementation.*

Mean kcal intake	Mean Pro. intake (g) (g/kg)	Mean B-6 intake (mg)	Mean B-6/ Pro. (mg/g)	Mean Plasma PLP (nM)	Mean Hb (g/dl)	Mean Hct (%)
1518 +/-434	66.1 +/-17.9 1.01 +/-0.26	1.51 +/-0.70	0.022 +/-0.006	31.7 +/-14.1	15.1+ +/-0.9	43.5+ +/-2.3

* Dietary information provided by a 3-day dietary history and analyzed using the Ohio State U. data base (97). Blood values determined using blood obtained at the first drawing, prior to PN supplementation. Data are expressed +/-SD.

+ female subjects only.

level for the females was 43.5% which is slightly less than the mean for women (44.3%) (107). The male subject had a Hct of 51.0% which is above the mean for males, 46.3% (107). The Hb and Hct levels did not change significantly during the 2 month supplementation period (data not shown).

Plasma PLP

The initial mean plasma PLP (31.7nM) was marginal to normal (108) (Table 3). The subjects' plasma PLP concentration before PN supplementation is presented in Table 4. Five of the subjects receiving PN had plasma PLP concentrations of less than 32nM (Low B-6 group) and 3 of the subjects receiving a placebo had plasma PLP concentrations of less than 32nM (Placebo group) which, according to guidelines proposed by Schultz and Leklem (108) suggests marginal B-6 status. The remaining subjects had plasma PLP concentrations higher than 32nM (Normal B-6 group) (Table 4). This division provided a means for comparing the Low B-6, Normal B-6 and Placebo groups separately in terms of lymphocyte proliferation.

Plasma PLP concentrations increased significantly ($p < 0.001$) with PN supplementation (Fig.2). After rising to a mean increase of 195 ± 88 nM following one month of PN supplementation, little further increase in plasma PLP occurred. After 2 months of PN supplementation, the increase in PLP concentration from baseline

Table 4

Initial plasma PLP concentration in nM of experimental subjects*

PN-Supplemented				Placebo	
Low B-6		Normal B-6			
Subject	Plasma PLP	Subject	Plasma PLP	Subject	Plasma PLP
6	18.3	2	46.1	1	23.8
8	19.1	3	53.3	4	30.0
9	22.3	5	48.8	11	16.7
10	13.5	7	50.6	14	42.1
12	16.7	13	39.0	-	-
-	-	15	36.0	-	-

* In blood drawn before supplementation, 5 of the PN-supplemented and 3 of the placebo treated (control) subjects had plasma PLP concentrations of less than 32nM, suggesting marginal B-6 status (108). This criteria was used to divide the PN-supplemented subjects into Low B-6 and Normal B-6 groups.

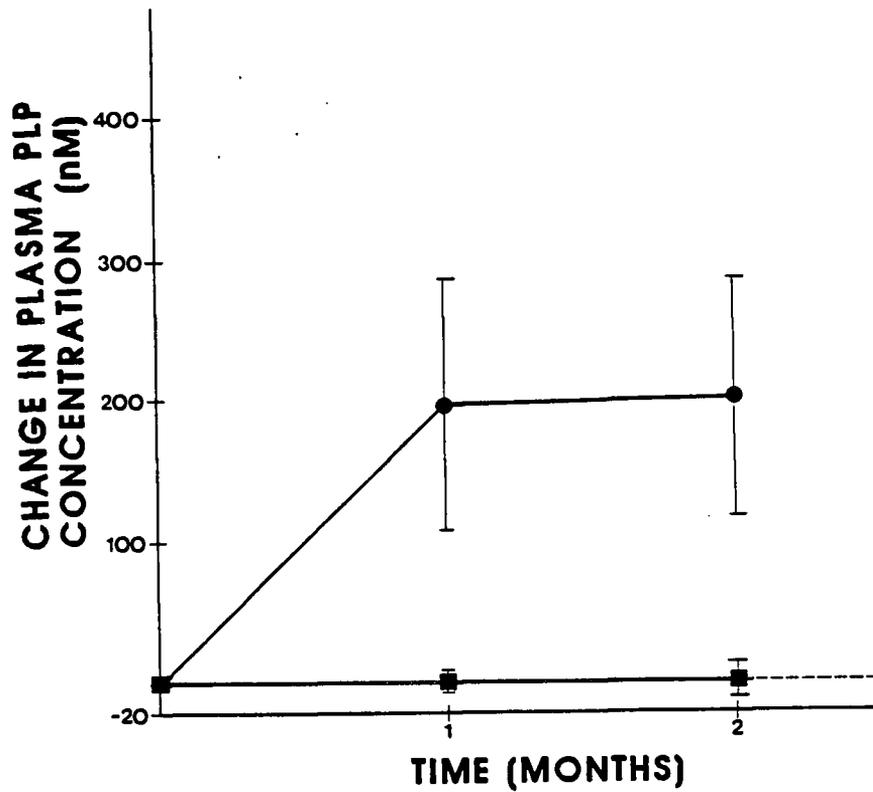


Fig. 2. Change in plasma PLP concentration during 2 months of 50mg PN-HCl or placebo supplementation. Each point represents the mean \pm SD at 1 and 2 months. ●, PN-treated group; ■, placebo group. **, $p < 0.001$ that mean PN group equal to mean placebo group.

was 201 +/- 84nM. Plasma PLP concentrations did not change in the subjects receiving a placebo.

Lymphocyte Proliferation

In the blastogenesis analyses, individual subjects showed peak responses at different mitogen concentrations. For this reason the peak response was used to compare lymphocyte proliferation between groups. Peak levels of 3H-thymidine incorporation in response to T and B cell mitogens are presented in Table 5. In the PN-treated group, the PHA response increased significantly after both 1 and 2 months of PN supplementation, while in the placebo group no significant increase in PHA response was observed over the 2 months of treatment. The response to Con A in both the PN and the placebo treated groups did not increase significantly over time with supplementation. The response to SAC increased significantly in the PN group at the end of 2 months of supplementation while the SAC response in the placebo group remained constant over the treatment period. The response to PWM increased over time in PN-treated group, after both 1 and 2 months of supplementation, whereas, the PWM response of the placebo-treated group did not change significantly over the 2 months of treatment.

Since baseline responses of placebo and PN-treated groups differed, it was not appropriate to compare them or subsequent

Table 5

Peak lymphocyte incorporation of 3H-thymidine in response to mitogens in PN and placebo-treated subjects.*

Mito- gen	Group	Before Supplement (cpm)	1 Month Supplement (cpm)		2 Months Supplement (cpm)	
PHA	B-6 (n=11)	50038 +/-3172	57406 +/-1893	p <0.01+	57843 +/-2616	p <0.01\$
	P (n=4)	60923 +/-5164	53714 +/-3572	NS	59285 +/-3521	NS
Con A	B-6 (n=11)	43222 +/-3484	47589 +/-3301	NS	48910 +/-2949	NS
	P (n=4)	54820 +/-6533	52516 +/-5326	NS	51415 +/-8220	NS
SAC	B-6 (n=11)	16833 +/-3481	15551 +/-2812	NS	23174 +/-3334	p <0.01#
	P (n=4)	30928 +/-1608	26454 +/-6219	NS	29290 +/-2022	NS
PWM	B-6 (n=11)	35022 +/-2362	40883 +/-2102	p <0.01	40055 +/-1526	p <0.01
	P (n=4)	45495 +/-5488	42242 +/-416	NS	38992 +/-3710	NS

* B-6 is the PN treated group; P is the placebo group. Data are expressed +/- SEM.

+ Probability that mean 1 month response equal to baseline response.

\$ Probability that mean 2 month response equal to baseline response.

Probability that 2 month response equals 1 month response and 2 month response equal to baseline response.

responses directly. However, we followed individual responses over time with each individual serving as his/her own control. Thus, variation in response between individuals could be corrected by subtracting each person's baseline response from itself and subsequent responses to the same mitogen and we could compare the changes in mitogen response between PN and placebo treated groups. (Fig. 3a, 3b, 3c, 3d). Since an initially low or high vitamin B-6 status may have influenced the subsequent responses in the PN supplemented subjects, we also analyzed the mitogen data by separating it according to the initially Low B-6 and Normal B-6 groups (Table 4, Fig. 4, 5). The response of MNCs to PHA from subjects receiving PN increased significantly (Fig. 3a) after 1 month of supplementation as compared to the placebo-treated subjects ($p < 0.01$). After 2 months of PN supplementation, the PN and placebo treated groups were not significantly different ($p < 0.08$). Being low or normal with respect to initial plasma PLP levels made no significant difference in response changes to PHA over time and PN treatment. In response to Con A (Fig. 3b), changes in MNC responses from the PN-treated group did not significantly increase over time in comparison to the placebo group. However, subjects with initially low plasma PLP concentrations (Low B-6 group) showed significantly increased proliferation over the 2 months of PN supplementation as compared to both the Normal B-6 and placebo groups (Fig. 4).

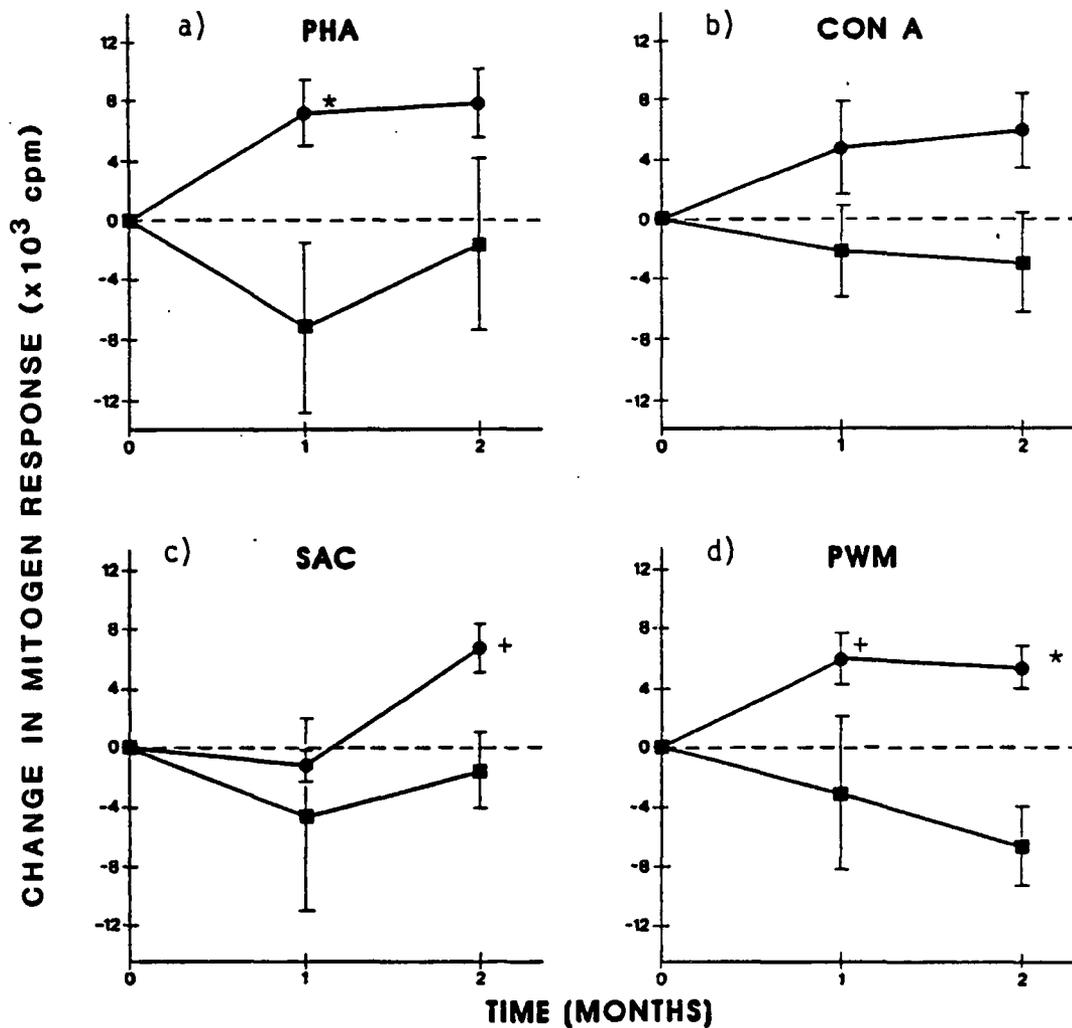


Fig. 3. Change in peak lymphocyte incorporation of ^3H -thymidine in response to mitogens during 2 months of PN-HCl or placebo supplementation. Each point represents the mean \pm SEM at 1 and 2 months. a) response to PHA; b) response to Con A; c) response to SAC; d) response to PWM. ●, PN group; ■, placebo group. *, $p < 0.01$ that means of PN and placebo groups equal. +, $p < 0.05$ that means of PN and placebo groups equal.

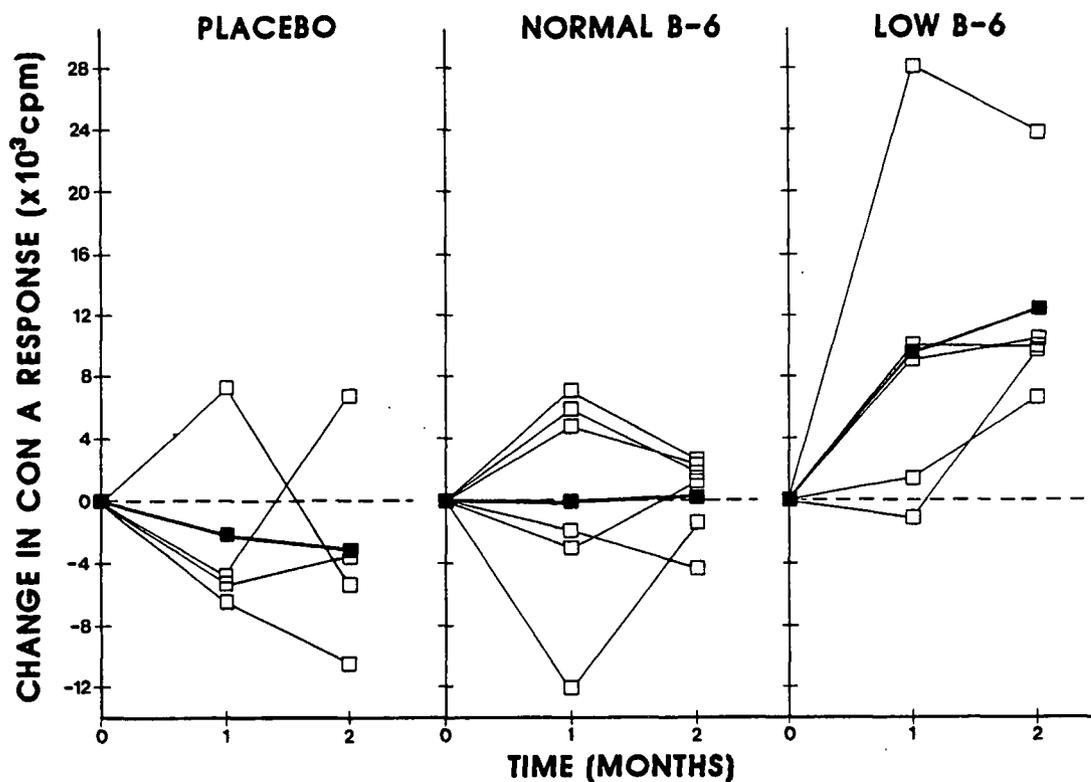


Fig.4. Influence of initial B-6 status on change in lymphocyte incorporation of ³H-thymidine in response to Con A. Low B-6 and Normal B-6 groups were treated with 50mg PN-HCl daily and the Placebo group was treated with a placebo daily for 2 months. Normal B-6 and Low B-6 groups were determined on the basis of initial B-6 status (Table 4). □, individual data; ■, mean data. *, p < 0.01 that means of Low B-6 and Normal B-6 groups equal and means on Low B-6 and Placebo groups equal.

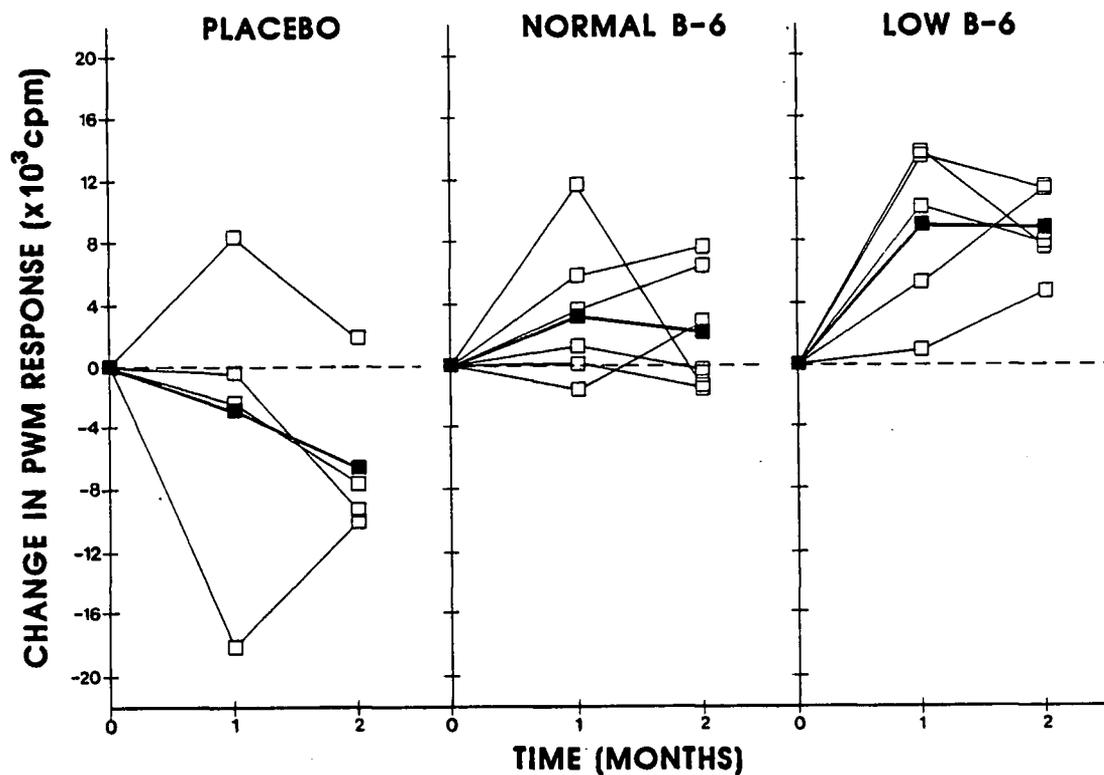


Fig. 5. Influence of initial B-6 status on change in lymphocyte incorporation of 3H-thymidine in response to PWM. Low B-6 and Normal B-6 groups were treated with 50mg of PN-HCl daily and the Placebo group was treated with a placebo daily for 2 months. Low B-6 and Normal B-6 groups were determined on the basis of initial B-6 status (Table 4). \square , individual data; \blacksquare , mean data. *, $p < 0.01$ that means of Low B-6 and Placebo groups equal. +, $p < 0.05$ that means of Low B-6 and Normal B-6 equal.

MNCs from the subjects receiving PN showed a significant increase in proliferation to SAC as compared to placebo controls after 2 months of PN supplementation (Fig.3c). No significant changes were observed when the PN-treated subjects were divided into the Low B-6 and Normal B-6 groups. In response to PWM, the PN-treated group increased and the placebo group decreased over time. These 2 groups were significantly different both at 1 and 2 months after initiation of PN supplementation (Fig.3d). When the data were analyzed by separating the responses according to initially low plasma PLP (Low B-6) and adequate plasma PLP (Normal B-6) concentrations, the Low B-6 group increased and the Normal B-6 group maintained their responses to PWM while the placebo group decreased its response to PWM over the PN supplementation period (Fig.5). Both the Low B-6 and the Normal B-6 groups were significantly different from the placebo group and from each other after 2 months of PN supplementation (Fig.5).

The significant correlation coefficients between plasma PLP concentrations and mitogen responses are as follows; after 1 month of PN treatment, PHA response was negatively correlated to initial plasma PLP levels ($r = -0.66$, $p = 0.03$), and the change in response to Con A after 2 months of PN supplementation was negatively correlated with initial plasma PLP concentration ($r = -0.72$, $p = 0.01$). These results imply that the lower the initial plasma PLP concentration, the greater the increase in proliferation.

Leukocyte Subpopulations

The total concentration of white blood cells in the subjects did not change significantly during the 2 months of supplementation (Table 6). The percentage of neutrophils increased significantly with PN supplementation while the percentage of neutrophils in placebo group was not significantly altered. However, when the concentration of neutrophils (cells/ml) was analyzed, the PN-treated and placebo group increased similarly and significantly (Table 6). The percentage of lymphocytes did not change significantly with PN treatment. The percentage of monocytes decreased during the PN supplementation program, however, similar decreases were observed in the placebo-treated group (Table 6).

Lymphocyte Subset Analyses

A relatively high level non-specific staining was observed with cells from several of the subjects in this study. Since this phenomenon resulted in a lack of separation between positively and negatively stained peaks in some subjects, it was not possible to reliably estimate the number or percent positive cells. For this reason, only data which showed a clear separation between background (negative) fluorescence and higher intensity (positive) fluorescence were included in the analyses (Table 7).

Table 6

Comparison of white blood cell and differential blood cell counts with 2 months of PN or placebo supplementation.*

Type Cell Count	Group	Before Supplement	1 Month Supplement		2 Months Supplement	
WBCs $\times 10^6$ /ml	B-6	4.8 +/-2.2	5.3 +/-1.0	NS+	6.4 +/-1.9	NS§
	Placebo	4.3 +/-0.5	5.2 +/-0.5	NS	6.2 +/-1.4	NS
% Neutrophils	B-6	47.0 +/-9.8	61.5 +/-7.3	p < 0.01	59.8 +/-12.7	p < 0.01
	Placebo	50.2 +/-9.9	58.3 +/-8.7	NS	55.2 +/-5.9	NS
Neutrophils $\times 10^6$ /ml	B-6	2.3 +/-1.4	3.3 +/-0.8	p < 0.05	3.9 +/-1.3	p < 0.01
	Placebo	2.1 +/-0.3	3.0 +/-0.5	NS	3.7 +/-1.1	p < 0.05
% Lymphocytes	B-6	22.5 +/-7.8	23.8 +/-6.0	NS	23.9 +/-10.9	NS
	Placebo	21.0 +/-8.6	29.9 +/-9.0	NS	33.9 +/-3.7	NS
% Monocytes	B-6	16.1 +/-6.0	6.8 +/-2.4	p < 0.01	8.2 +/-8.2	p < 0.01
	Placebo	12.4 +/-2.9	6.0 +/-1.6	p < 0.05	6.9 +/-2.9	p < 0.05

* B-6 is the PN treated group; P is the placebo group. Data are expressed as +/-SD.

+ probability that mean 1 month value equal to baseline value.

§ probability that mean 2 month value equal to baseline value.

Table 7

Lymphocyte subsets as determined by monoclonal antibodies T3, T4, T8 before and after 2 months of PN or placebo treatment.*

Subset Marker	Group	Before Supplement (% positive cells)	2 Months Supplement (% positive cells)	
T3	B-6 (n=5)	43.4 +/-3.7	54.2 +/-7.4	p <0.05+
	P (n=2)	47.3 +/-9.4	52.1 +/-0.8	NS
T4	B-6 (n=5)	32.3 +/-8.3	44.4 +/-6.9	p <0.05
	P (n=2)	38.2 +/-12.4	42.6 +/-5.5	NS
T8	B-6 (n=3)	13.9 +/-6.3	11.8 +/-4.8	NS
	P (n=2)	8.7 +/-0.5	8.3 +/-3.2	NS

* B-6 is the PN treated group; P is the placebo treated group. The data are expressed as +/- SD.

+ Probability that mean proportion T cells after 2 months is equal to the mean proportion T cells at baseline.

Furthermore, only data from persons with interpretable data at both pre- and post-supplementation testing periods are presented in Table 7. This limited the data to 7 subjects at baseline and 14 subjects at 2 months. Few data from the monoclonal antibody B1 could be objectively analyzed due to the low specific staining in relation to high background fluorescence and, therefore, are not included.

No significant changes were seen in the percentage of T8+ cells in the PN or the placebo groups over 2 months of supplementation (Table 7). Significant increases in the proportions of T3+ and T4+ cells were seen within the PN-treated group. Similar, although not significant, increases were seen in the placebo controls.

At the 2 month post PN supplementation testing period, data for 14 of the subjects showed separation of peaks of negatively and positively stained cells, so these data were used to calculate correlation coefficients together with the pre-supplementation values (Table 8). Percentages of T3+ cells were negatively correlated with PHA response at baseline testing ($r = -0.77$, $p = 0.04$); however, this relationship was not seen after 2 months of PN treatment (Table 8). Percentages of T3+ cells were not significantly related to Con A response prior to PN supplementation, but were positively correlated to Con A response after 2 months of PN treatment ($r = 0.54$, $p = 0.05$). Proportions of

Table 8

Correlation coefficients between lymphocyte subsets and lymphocyte incorporation of 3H-thymidine in response to mitogens.

Relationship described	r	p*
Pre T3+ vs Pre PHA (n=7)	-0.77	0.04
Post 2 month T3+ vs Post 2 month PHA (n=14)	0.32	NS
Pre T3+ vs Pre Con A (n=7)	-0.57	NS
Post 2 month T3+ vs Post 2 month Con A (n=14)	0.54	0.05
Pre T4+ vs Pre PHA (n=7)	0.89	0.008
Post 2 month T4+ vs Post 2 month PHA (n=14)	0.56	0.04
Pre T4+ vs Pre Con A (n=7)	0.97	0.0005
Post 2 month T4+ vs Post 2 month Con A (n=14)	0.72	0.004
Pre T4+ vs Pre PWM (n=7)	0.82	0.03
Post 2 month T4+ vs Post 2 month PWM (n=14)	0.30	NS

* The p value indicates the significance of the analysis of variance on the regression and the probability that the slope of the regression line is 0.

T4+ cells correlated positively to PHA and Con A responses both before and after PN supplementation. Prior to PN supplementation the correlation between T4+ cells and PHA response was; $r=0.89$, $p=0.008$ and after supplementation, $r=0.56$, $p=0.04$. Before PN supplementation the relationship between T4+ cells and Con A response was; $r=0.97$, $p=0.0005$ and after 2 months of PN, $r=0.72$, $p=0.004$. At the initial test period, but not after PN treatment, the percentage T4+ cells were also positively correlated with PWM response ($r=0.52$, $p=0.03$).

DISCUSSION

Several studies in animals relating vitamin B-6 to immune function have found that B-6 deficiency results in reduced antibody production (21, 22, 109), mixed lymphocyte reaction (15, 18), DTH reaction (22) and response to T cell mitogens (18, 109). In humans, Hodges et al. (20) found reduced antibody production in B-6 deficiency. In the present study, we used a group of persons likely to be at risk for both vitamin B-6 deficiency and reduced immunocompetence, the elderly, to test whether PN supplementation would improve B-6 status and lymphocyte function. Each subject served as his/her own control enabling us to measure change in B-6 nutriture and immune responsiveness with PN supplementation while correcting for individual variation. Baseline data were collected for dietary and biochemical B-6 status indicators and for immune parameters, which were then compared with results using the same tests after 1 and 2 months of supplementation.

The subjects who participated in this study showed relatively good overall hematological status as judged by Hb, Hct (107) and WBCs (110,111). Most had adequate dietary intakes for protein, kilocalories, and vitamin B-6. However, 8 of the 15 subjects showed marginal vitamin B-6 status (108) using plasma PLP as an indicator. No direct correlation was found between plasma PLP and

dietary B-6 or vitamin B-6 to protein ratio although 5 of the 8 with low plasma PLP had a dietary vitamin B-6 to protein ratio of 0.02mg B-6/g protein or less. Of these 8, 5 had a B-6 to protein ratio of 0.02mg/g or greater which suggests that for some elderly the RDA recommendation of 0.02mg B-6 /g protein may not be adequate. The plasma PLP concentration of all subjects receiving PN increased dramatically with a mean increase of 20nm after 2 months of PN supplementation, well above the marginal vitamin B-6 status indicator of 32nm (108).

In vitro proliferation of lymphocytes in response to mitogen stimulation is a widely used model for assessing in vivo immune responsiveness to antigens (112). To assess immune function before and after PN supplementation, we used PHA and Con A to measure T cell responsiveness (112), PWM to measure T cell dependent B cell responsiveness (112) and SAC to measure T cell independent B cell responsiveness (113, 114).

Responses to PHA and Con A increased significantly with PN supplementation (Fig. 3a, 4). The increases in response to Con A and PHA differed in that only persons with low plasma PLP at baseline increased their response to Con A (Fig. 4) with PN supplementation, while all subjects increased their response to PHA with PN supplementation (Fig. 3a, Table 5). The possibility that PHA and Con A stimulate different subpopulations of T lymphocytes (112) and are affected differently by PN could explain

this difference in response to PHA and Con A. However, the increases in PHA and Con A response in lymphocytes from elderly persons demonstrates that PN supplementation increases in vitro T cell responsiveness in the elderly. B-6 deficiency has been shown to produce decreased proliferation of lymphocytes to PHA (15, 109) and Con A (15) in rats.

Proliferation to the T cell independent B cell mitogen SAC increased significantly in the PN-treated group after 2 months of supplementation (Fig. 3c). This suggests that increasing vitamin B-6 intake in the elderly can also improve B cell responsiveness.

Response to the T cell dependent B cell mitogen PWM increased in the low plasma PLP group, was maintained in the high plasma PLP group and decreased in the placebo group (Fig. 5). The unexpected decline in the placebo group may be the result of the small sample size of 4 or may reflect natural variability in individual responses or variability in the cell culture conditions between testing periods. If we assume the latter, the maintenance of PWM response of the Normal B-6 group may be interpreted to represent an increase in response in comparison to the placebo group, and the increased response of the Low B-6 group may be considered a larger increase in response than the Normal B-6 group (Fig. 5). Most likely the increase in proliferation to PWM reflects an increase in both T and B cell responsiveness in response to PN supplementation in light of our findings that B cell and T cell

responses to mitogens improved with PN supplementation.

The pre-supplementation levels of plasma PLP were negatively correlated to PHA response after 1 month and negatively correlated to change in Con A response after 2 months of PN supplementation. These correlations suggest that T cell responses to mitogens may be related to vitamin B-6 status. The lymphocyte response to other mitogens did not correlate with plasma PLP concentrations which may indicate that other factors may influence blastogenesis and that a direct correlation may not be necessary to observe a PN effect on lymphocyte proliferation to mitogen stimulation.

No changes were noted in white blood cell counts nor in differential blood cell counts in response to 2 months of PN supplementation that were significantly different from the placebo controls. White blood cell counts and differential counts are general indicators of immune status, and one might not expect changes in these gross measures with a short PN supplementation period. Cheslock and McCully found a significant decrease in lymphocyte number in humans after 7 weeks of a vitamin B-6 deficient diet (115). In vitamin B-6 deficient rats, Robson and Schwarz (18) found a 25% reduction in WBCs, a 50% reduction of lymphocytes and a 2 fold increase in neutrophils. Comparing the results of these two studies with the results of this study indicates that although leukopenia and altered differential blood counts exist in B-6 deficiency, PN supplementation of a population

with relatively normal WBC and differential blood counts does not necessarily cause an increase in the WBC population.

After 2 months of PN supplementation we observed an increase in the percentages of T3+ and T4+ cells and no change in the percentage of T8+ cells or in the T4+/T8+ ratio of cells. The increases in lymphocyte subpopulations T3 and T4 with 2 months of PN supplementation may be real and may explain the increases in PHA and Con A responses, or may be an artifact due to difficulty in interpreting the flow cytometry data. If real, the increases in T3+ and T4+ cells indicate that PN supplementation may have influenced the differentiation of immature T cells to mature T cells. Further studies should be done to confirm any changes in lymphocyte subpopulations with PN supplementation.

We found a significant negative correlation between the percent of T3+ cells and PHA response before PN supplementation. However, this relationship was not significant after 2 months of PN supplementation (Table 7). A positive correlation was found between T3+ cells and Con A response after PN supplementation, but not before. These differences in relationships between T3+ cells and Con A and PHA may support the idea that PHA and Con A stimulate different subpopulations of T lymphocytes and that PN supplementation affected PHA response differently from Con A response. A significant positive correlation was observed between T4+ and PHA and Con A responses before and after PN

supplementation, indicating that the percentage of the helper/inducer population may be important to these T cell responses to mitogens. T4+ cells were positively correlated to PWM response before but not after PN supplementation which suggests that T helper/inducer cells may be stimulated to proliferate in response to PWM. No significant correlations between T8+ cells or the T4+/T8+ ratio of lymphocytes and mitogen responses were observed. In contrast to this study, Mascart-Lemone et al. (34) found significant negative relationships between T8+ cells and both PHA and Con A responses in older women and positive correlations between the T4+/T8+ ratio and proliferation to PHA and Con A in all subjects. They reported no relationship between T3+ and T4+ cells and mitogen responses. The basis for the differences between the 2 studies is unknown.

The results of this study indicate that PN supplementation improves lymphocyte function in healthy, independently living elderly persons and suggest that vitamin B-6 nutriture is important in maintaining 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. Although these results are encouraging, it would be

advisable to repeat the study with a larger population of elderly subjects and include more measures of immune function such as DTH reactions and antibody production to confirm and expand upon the results we have found. Since we used a pharmacological dose of PN (about 25 times the RDA), it would also be of interest to repeat the studies with a more physiological level of PN supplementation and over a longer period of time. Future studies should also consider the effect of varying the PLP concentration of the culture media. In this study a constant level of PLP was maintained by using a specific culture media (RPMI 1640). MNCs were washed thoroughly so plasma PLP would not influence the culture conditions. Sergeev et al. (17) found that the addition of PLP to the culture media of PHA-stimulated lymphocytes partially restored the response of T lymphocytes from vitamin B-6 deficient mice.

SUMMARY AND CONCLUSIONS

Immune and vitamin B-6 status were assessed in a group of 15 independently-living elderly volunteers (65-81 years old) by measuring lymphocyte proliferation to T and B cell mitogens, lymphocyte subsets with monoclonal antibodies (T3, T4, T8), plasma PLP concentration, Hb, Hct, WBC and differential blood counts at pre-supplementation and after 1 month and 2 months of PN or placebo supplementation. Eleven subjects received 50mg of PN-HCl and 4 received a placebo. Prior to treatment, dietary histories were collected. Mean dietary intake of vitamin B-6 was 1.51mg or 0.022mg B-6/g protein. Following 2 months of PN supplementation, plasma PLP levels increased 201 +/- 84nM from an initial mean concentration of 31.7nM. WBCs, differential counts, Hb and Hct were not significantly affected by PN treatment. Lymphocyte proliferation to PHA, PWM and SAC increased significantly ($p < 0.05$) with PN treatment. Only persons with initially low plasma PLP concentrations had a significantly increased response ($p < 0.01$) to Con A after PN treatment. Initial plasma PLP levels were negatively correlated ($r = -0.66$, $p = 0.03$) to PHA response after 1 month of PN supplementation and negatively correlated ($r = -0.72$, $p = 0.01$) to change in Con A response after 2 months of PN treatment. Percentages of T3+ and T4+ cells increased

significantly with PN treatment. T3+ and T4+ cells correlated significantly to PHA and Con A responses. These results demonstrate improved in vitro lymphocyte responsiveness with PN supplementation in elderly persons.

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