AN ABSTRACT OF THE THESIS OF

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The effect of vitamin B-6 (VB6) deficiency in mice on host susceptibility to primary and secondary Moloney-sarcoma virus (MSV)-induced tumor growth, cytotoxic activities of T cells, antibodies and natural killer (NK) cells, and phagocytosis by macrophages was examined. Five- to six-week old female C57Bl/6 mice were fed 20% casein diets with pyridoxine (PN) added at 7 (PN-7), 1 (PN-1), 0.5 (PN-0.5), 0.1 (PN-0.1), or 0 (PN-0) mg per kg diet, which represent 700, 100, 50, 10, and 0% of the VB6 requirement of mice adequate for both growth and reproduction, for 4-5 weeks prior to MSV challenge and throughout the period of tumor development or immunologic testing. Animals fed PN-0.1 and -0 diets developed deficiency signs including significantly lower body weight, denuding of the snout, skin irritation and elevated excretion of xanthurenic

acid before as well as after tryptophan loading. VB6 deficiency resulted in significant enhancement of tumor susceptibility. Following MSV/MSB challenge, total incidence of MSV/MSB/splenic tumors was 2/11, 1/11, 4/10, and 8/11 in mice fed PN-1, -0.5, -0.1, and -0 diets, respectively. In response to challenge with P815 mastocytoma cells, primary splenic and peritoneal T cell-mediated cytotoxicity (CMC) was significantly reduced in animals fed PN-0 or -0.1 diet. Mice fed PN-0 diet also showed significantly suppressed secondary T CMC of splenic and peritoneal lymphocytes against P815 tumor cells. Complement-dependent antibody-mediated cytotoxicity against P815 tumor cells, phagocytosis of sheep red blood cells by macrophages, and native and interferon-induced NK cell cytotoxicity against YAC tumor cells were not affected by lack of VB6. The percentage of macrophages present in the peritoneal exudate cells was increased in animals fed PN-0 diet. Immune responses were not enhanced or altered by the excess intake of VB6 (PN-7).

The present studies which showed compromised host resistance to MSV oncogenesis and altered T cell cytotoxicity in VB6 deficiency provided practical information on the impaired host defense mechanism by inadequacy resulting from VB6.

The Effect of Vitamin B-6 Deficiency on Antitumor Cytotoxic Immune Reactivity in Mice

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CONTRIBUTION OF AUTHORS

The research reported in Chapter 3 was conducted under the joint direction of Dr. Nancy I. Kerkvliet, Dept. of Veterinary Medicine, and Dr. Lorraine T. Miller, Dept. of Foods and Nutrition. Dr. Ha, the student, wrote both papers. Dr. Kerkvliet was in charge of the immunological assays, which were conducted in her laboratory, and Dr. Miller was responsible for the vitamin B-6 part.

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The Effect of Vitamin B-6 Deficiency on Antitumor Cytotoxic Immune Reactivity in Mice

Chapter 1

INTRODUCTION

It is well recognized that the lymphoid system and the immune response may be impaired by the metabolic disturbance due to a deficiency or an excess of a certain nutrient or nutrients. Since depressed antibody formation was reported in pyridoxine (PN)-deficient rats in the 1940's (Stoerk and Eisen, 1946; Stoerk, Eisen and John, 1947), the influence of vitamin B-6 (VB6) deficiency on immune responses has been studied extensively. VB6 deficiency is accompanied by changes in the structure of lymphoid organs and impairment of both humoral and cell-mediated immune responses.

Several excellent reviews on the role of nutrients in immunity are available (Beisel, 1982; Gross and Newberne, 1980, Axelrod, 1971). Since the roles of a wide range of nutrients on immunity were reviewed, each was, by necessity, not comprehensive. Thus, the purpose of this review is to give a more complete survey of the influence of VB6 on immune function.

Chemistry of Vitamin B-6

The essentiality of VB6 has been recognized since 1934 when it was identified by Gyorgy (1971). The term VB6 is used as the generic

descriptor for all 3-hydroxy-2-methylpyridine derivatives exhibiting qualitatively the biological activity of pyridoxine in rats (IUPAC-IUB Commission on Biochemical Nomenclature, 1973). VB6 in nature exists as 6 vitamers: three non-phosphorylated vitamers pyridoxal (PAL), pyridoxine (PN), pyridoxamine (PM); and their respective phosphorylated forms pyridoxal-5'-phosphate (PLP), pyridoxine-5'-phosphate (PNP), and pyridoxamine-5'-phosphate (PMP). PAL and PM are found largely in animal products, while PN is the more prevalent form in vegetable products (Rabinowitz and Snell, 1948). Deoxypyridoxine, a VB6 antagonist, does not occur naturally.

Figure 1.1 shows the interconversion of the B6 vitamers. The 3 free vitamers are phosphorylated by pyridoxal kinase which is widely distributed in mammalian tissues (McCormick, Gregory and Snell, 1961). PNP and PMP are oxidized to PLP by PNP oxidase. PMP and PLP are interconverted by transamination. The level of PLP is controlled principally by its hydrolysis by a cellular phosphatase and protein binding of PLP (Lumeng and Li, 1975). The phosphorylated vitamers are convertible to their nonphosphorylated forms by various phosphatases, primarily plasma membrane-bound alkaline phosphatase. Binding of PLP to protein protects it from the action of these enzymes (Anderson et al., 1974). No mechanism for the conversion of PN and PM to PAL is known in mammalian tissue (Shane and Snell, 1972). PAL is oxidized to 4-pyridoxic acid (4-PA) by aldehyde oxidase and aldehyde dehydrogenase (Stanulovic et al., 1976). Four-pyridoxic acid is not metabolized further and is the major excretory product of VB6 (Reddy, Reynolds and Price, 1958). Deoxypyridoxine alone or in combination with a VB6

deficient diet has been used to initiate VB6 deficiency in animals and human subjects.

Function of Vitamin B-6

VB6 functions as a coenzyme in the forms of PLP and PMP. Functions of PLP include coenzymatic roles in over 60 enzymes, a structural role in glycogen phosphorylase, and a secondary role in fat metabolism.

VB6 plays a central role in biochemical reactions involving amino acid metabolism such as transamination, deamination, decarboxylation, racemization, dehydration, and desulfhydration (Sauberlich and Canham, 1980). PLP functions in the formation of nonessential amino acids, niacin, various amines including histamine, the porphyrin ring in hemoglobin, nucleic acids, and substrates for gluconeogenesis (Sauberlich, 1968). PLP is also a coenzyme for the formation of neurotransmitters such as serotonin, dopamine, norepinephrine, and gamma-aminobutyric acid (GABA) and for the degradation of GABA (Dakshinamurti, 1982).

PLP has a role in carbohydrate metabolism through glycogen phosphorylase, which may be a storage site for VB6 (Black, Guirard and Snell, 1978). A recent paper reported a structural role of PLP in this enzyme by demonstrating conformational changes in the enzyme due to ionic interactions of the phosphate group of PLP with the active site in glycogen phosphorylase (Withers, Madsen and Sykes, 1981).

VB6 appears to affect hormonal action. Dopamine and serotonin, whose synthesis is VB6 dependent, regulate hypothalamic

hormones or releasing factors, which in turn regulate various endocrine activities including growth, thyroid stimulating, gonadotropic, and adrenal hormones (Krieger, 1980, Sauberlich, 1968). Litwack (1979) reported that PLP competes with a glucocorticoid complex for a DNA binding site. Thus, PLP may be able to inhibit or decrease glucocorticoid-induced protein synthesis in the liver.

In addition, VB6 exerts an indirect role in lipid metabolism, although this is a secondary function acting through hormonal mediation or changes in the metabolism of protein and carbohydrate (Sauberlich, 1968). DeLorme and Lupien (1976) reported that VB6 deficiency caused a general decrease in the proportion of arachidonic acid in the different phospholipids of the rat, and an increased proportion of linoleic acid, indicating a role of VB6 in the conversion of linoleate to arachidonate. VB6 functions in nerve myelination: PLP plays a role in one of the steps in the synthesis of sphingosine (Dakshinamurti,1982; Brady and Koval, 1958).

Evidence suggests that VB6 plays an important role in the development and maintenance of immunity, although the exact mechanism responsible for impairment of immunologic function in VB6 deficiency is not fully understood. A suggested mechanism is the critical role that PLP plays in the production of active formaldehyde, a nucleic acid precursor, via serine-glycine conversion by serine hydroxymethyltransferase, which is PLP-dependent (Axelrod and Trakatellis, 1964a). This one-carbon unit participates in biosynthesis of purine bases and of thymidylic acid from deoxyuridine-5'-phosphate (Erbe, 1975). Clark and Stoerk (1956)

reported decreased incorporation of radioactive phosphorus into thymic and splenic DNA in VB6 deficiency. Trakatellis and Axelrod (1965) observed that spleens from VB6-deficient rats had fewer cells and less DNA/mg of tissue than did controls. In studies on the incorporation of labeled precursors into nucleic acids, Trakatellis and Axelrod (1965) reported that incorporation of 14-C from DL-(3-14C)-serine into DNA and RNA of livers and spleens from VB6-deficient rats was decreased. This decreased incorporation could suggest a role of PLP in the production of one-carbon units via conversion of serine to glycine. The incorporation of 8-14C-adenine and Me-3H-thymidine was decreased, which may have reflected the overall lowered rate of nucleic acid synthesis. The incorporation of 2-14C-deoxyuridine was also decreased, which could be linked to the requirement of active formaldehyde for the biosynthesis of thymidylic acid from deoxyuridine-5'-phosphate. The specific defect for one-carbon production was substantiated further by the fact that VB6 deficiency had no effect on the incorporation of L-(Me-14C)-methionine into RNA. The biosynthesis of methylated derivatives of RNA is known to involve transmethylation with S-adenosylmethionine, and not active formaldehyde with PLP. Montjar et al. (1965) investigated the effect of VB6 deficiency on the number of polysomes in liver and spleen, and the ability of these tissues to incorporate amino acids into RNA in vitro. They found a decreased incorporation of labeled 6-14C-orotic acid into the ribosomal and messenger RNA in livers of VB6-deficient rats. The decreased synthesis of mRNA resulted in a decreased number of polysomes per unit weight of liver and spleen in VB6 deficiency,

and the resultant reduced ability of rRNA from these tissues of VB6-deficient rats to incorporate labeled leucine and valine in vitro. Thus, these deleterious effects of VB6 deficiency suggested a role for VB6 in protein synthesis, since mRNA is involved both in the translation of genetic information to polypeptide synthesis and in the formation of polysomal aggregates.

The above observations on the mode of action of VB6 in nucleic acid synthesis with subsequent protein biosynthesis formed the basis for an explanation of the adverse effects of VB6 deficiency on the development of immune phenomena. The deleterious effects of VB6 deficiency on immune response could be visualized at the stages of cellular proliferation as well as synthetic capacity of the cell.

Vitamin B-6 Deficiency and Lymphoid Organs

Of the lymphoid tissues, the thymus is most severely affected by VB6 deficiency, followed by the spleen and lymph nodes. Peripheral blood lymphopenia was consistently found in VB6 deficiency. Stoerk et al.(1947) reported severe atrophy (about 90% decrease in thymus weight) and a marked loss of cellularity of the thymus in VB6-deficient rats. The thymuses were totally depleted of lymphocytes, the cortexes had disappeared, and the glands consisted exclusively of epithelial cells and stroma. Hawkins and Evans (1952) demonstrated that rats fed a VB6 deficient diet and the VB6 antagonist, deoxypyridoxine, exhibited abnormalities in the thymus which showed, on histological examination, atrophy of lymphoid elements, disrupted architecture with loss of cortico-medullary

differentiation and fatty infiltration. Clark and Stoerk (1956) reported thymus, spleen and mesenteric node atrophy in VB6-deficient rats. They found thymic and splenic atrophy due to inhibition of cellular proliferation, and atrophy in lymph nodes due to either increased rate of cell destruction or to loss from migration without suppression of cell proliferation. Davis, Nelson and Shepard (1970) reported that induced prenatal VB6 deficiency also produced selectively adverse effects on lymphoid tissues. Severe thymic and splenic hypoplasia was observed in fetuses of dams fed a VB6 deficient diet plus deoxypyridoxine. Moon and Kirksey (1973) observed that a maternal VB6 deficiency in rats altered cellular growth and development in the organs of progeny during the prenatal period, and that the effects became more adverse when VB6 deficiency was extended into the early postnatal period. The most severe effects of VB6 deficiency occurred in thymic tissue.

Severe lymphoid atropy in VB6 deficiency was reflected by consistent peripheral blood lymphopenia. Hawkins and Evans (1952) reported a marked lymphopenia which was reflected in decreased total white cell counts and lowered lymphocyte/neutrophil ratios in VB6-deficient rats. Robson and Schwarz (1975a) showed a significant reduction in thoracic duct lymphocytes in VB6 deficient-rats. Willis-Carr and St. Pierre (1978) confirmed that dietary VB6 deficiency causes a severe depletion of lymphocytes, which is the most dramatic in the thymic cortex. Debes and Kirksey (1979) demonstrated that the pups of VB6-deficient rat dams had decreased numbers of spleen cells and splenic antibody-forming cells as well as circulating

antibody level. Cheslock and McCully (1960) also observed lymphopenia in 5 of the 8 human subjects who were maintained for 52 days on a low-VB6 diet.

Vitamin B-6 Deficiency and Humoral Immunity

VB6 deficiency in animals interferes with antibody responses to various antigens. One of the most consistent findings in VB6-deficient animals was the impaired production of antibodies. Early experiments demonstrated that VB6-deficient rats produced significantly fewer antibodies in response to sheep red blood cells (SRBC) than did pair-fed controls; VB6-deficient rats showed no measurable titers of agglutinin or hemolysin (Stoerk and Eisen, 1946). A subsequent study also showed that the serum hemagglutinin titer in VB6-deficient animals was 23% of the control levels (Stoerk et al., 1947). Other workers reported similar findings and confirmed further that antibody production in response to a variety of antigens in experimental animals was markedly impaired in VB6 deficiency: VB6-deficient rats showed diminished levels of circulating antibodies following immunization with diphtheria toxoid (Axelrod, 1958; Pruzansky and Axelrod, 1955) and influenza virus (Axelrod and Hopper, 1960). VB6-deficient guinea pigs also showed the deleterious effects of VB6 deficiency on circulating antibody formation in response to the antigenic stimulus of diphtheria toxoid (Axelrod, Hopper and Long, 1961). Secondary anamnestic responses were inhibited to a greater extent than primary ones, even though adequate VB6 is required during both the primary and seondary phases of the antibody response to

diphtheria toxoid (Axelrod, 1958). Nutritional supplementation during the secondary immunization phase did not correct impaired antibody production which resulted from VB6 deficiency during the primary response (Axelrod, 1958). In further studies with diphtheria toxoid, Axelrod et al.(1961) reported that VB6 deficiency resulted in a reduced formation of specific antibodies as well as a defect (low avidity for antigen) in the antibody produced.

Kumar and Axelrod (1968) reported impaired cellular antibody synthesis in VB6-deficient rats by using the hemolytic plaque technique. They found a severe reduction in the number of antibody-forming cells in spleens from VB6-deficient animals immunized with the SRBC. This decreased immune response was independent of the inanition associated with the deficiency and was restored to normal by the administration of VB6 shortly before immunization. They noted that clearance of 51-Cr-labeled SRBC from the blood, which may be a measure of phagocytosis, and accumulation in the spleen were normal, despite the decreased splenic size in this deficiency, suggesting that antigen processing was intact. The decreased formation of antibody-forming cells observed in VB6-deficient rats was not simply a manifestation of altered kinetics, and was not overcome by increasing antigen dose 10-fold: it was the result of a profound disturbance in the basic mechanisms involved in the formation of these cells. Koros et al. (1976) reported that numbers of background PFC from unimmunized VB6-deficient animals to SRBC, syngeneic rat RBC and human RBC were increased, whereas postimmunization PFC were markedly depressed in VB6-deficient rats. This increase in background PFC was apparent,

that is, total background numbers per spleen increased despite a marked decrease in whole body weight as well as in spleen weight in VB6-deficient rats. Even though the significance of background PFC is unknown, the finding of increased backgound PFC against syngeneic rat RBC may reflect an increased tendency to autoimmunity or be the result of a possible loss in suppressor cells. This suggests that these background PFC are a parameter of immunoregulatory defect in VB6-deficient animals. The above observations raise the possibility that specific functional subpopulations of lymphocytes may be differentially affected in VB6 deficiency. Thus alterations in immunologic parameters may reflect changes in effector, suppressor, helper and memory cells.

In a small clinical study of VB6 deficiency induced in human volunteers by a low VB6 formula diet or deoxypyridoxine, Hodges et al. (1962a) found that antibody formation against tetanus and typhoid was only slightly impaired in four subjects, although they developed clinical signs of VB6 deficiency. In a subsequent study in which a simultaneous deficiency of VB6 and pantothenic acid was induced in other human subjects, impairment was more marked: antibody response to tetanus toxoid and typhoid antigens was almost completely diminished and hypogammaglobulinemia developed. Antibody response to polio virus immunization, however, was normal (Hodges et al., 1962b).

Vitamin B-6 Deficiency and Cell-Mediated Immunity

Cell-mediated immunity (CMI) is impaired by VB6 deficiency. A prolongation of allograft survival was a consistent finding in

VB6-deficient animals. Using two strains of rats, Axelrod et al. (1958) showed that dietary VB6 deficiency plus deoxypyridoxine increased the long-term survival of grafts (3-12 weeks) from control levels of 4-0% to 63-92%. Many grafts were still in good condition after 6 months. Herr and Coursin (1966) noted that VB6-deficient mice chronically rejected homografts, showing a delayed onset of cellular infiltration and increased total rejection time. In certain strains of VB6-deficient mice, the induction of specific tolerance to donor histocompatibility antigens enhanced consequent acceptance of a skin graft. Axelrod and Trakatellis (1964b) demonstrated that tolerance to skin grafts was achieved successfully in VB6-deficient mature mice by the prior administration of splenic cells from the skin donor. Since the recipient mice were injected with donor spleen cells while VB6 deficient, survival of subsequent skin graft was significantly prolonged, even though the recipient returned to a normal diet. In a sex-linked histocompatibility system, i.e., male skin isografts to a female, Trakatellis and Axelrod (1969) showed that a very high degree of immune tolerance of female mice to skin grafts from male mice was achieved by injection of splenic cells of donors into VB6-deficient recipients. The dose of spleen cells required to induce tolerance was also markedly reduced in VB6-deficient mice compared with controls.

The fact that VB6 deficiency induced by dietary manipulation or by the use of deoxypyridoxine can impair CMI was strengthened by studies showing marked impairment of skin responses against antigens. Axelrod et al. (1961) demonstrated that VB6 deficiency resulted in depressed early Arthus-type skin hypersensitivity reaction to

intradermal diphtheria toxoid in guinea pigs previously sensitized to the same antigen. VB6-deficient guinea pigs also showed a diminished delayed-hypersensitivity skin reaction to purified protein derivative after previous immunization with Mycobacterium tuberculosis intraperitoneally (Axelrod et al., 1963). However, guinea pigs sensitized with BCG while VB6 deficient regained normal delayed-hypersensitivity when supplied with VB6. In addition, thymocytes from VB6-deficient animals were capable of transferring sensitivity to normal recipients, suggesting that cellular sensitivity was achieved and the sensitization mechanism was not affected in VB6-deficient animals (Axelrod et al., 1963). Thus altered skin sensitivity was explained not by a lack of cellular sensitivity, but by a depressed ability of sensitized cells to react with the allergen.

Robson and Schwarz (1975a) reported quantitative and qualitative alterations in function of thoracic duct lymphocytes (TDL) from rats in which VB6 deficiency was induced by a combination of dietary deficiency and treatment with deoxypyridoxine. They found a 50% reduction in peripheral blood lymphocyte count, a marked reduction in TDL and a significantly reduced capacity (55% less 3H-thymidine incorporation) to respond in vitro in a mixed lymphocyte reaction (MLR) in VB6-deficient rats. They also observed an impaired ability of TDL from VB6-deficient animals to induce a localized graft-vs-host reactivity after intradermal injection of TDL into the abdominal wall and to incorporate 3H-uridine in vitro, which possibly reflected a change in distribution of T and B cells in the TDL. Thus, the defects represented both marked depletion of T cells in the TDL, either by

absolute loss or a shift in proportion of T and B cells, and impaired function of the remaining T cells. Further study demonstrated that VB6 deficiency in utero was associated with a higher mortality in the litter, and TDL of progeny of VB6-deficient mothers had a significantly reduced ability to respond in the MLR and a localized graft-vs-host reaction, while the numbers of cells in the TDL were only slightly reduced (Robson and Schwarz, 1975b). Willis-Carr and St. Pierre (1978) confirmed the effect of VB6 deficiency on cellular immune capabilities and identified a defect in thymic epithelial (TE) cell function. They observed that TE monolayers from VB6-deficient rats were unable to effect a maturation of T lymphocytes, suggesting that the defect in cellular immunocompetence following VB6 deficiency is due, at least in part, to the inability of TE cells to effect the differentiation of incompetent T lymphocyte precursors to immunocompetent functional T lymphocytes. The dietary VB6 deficiency did not, however, impair lymphoid precursors, which could be stimulated to further differentiation by exposure to normal TE cells. Therefore, it would appear that the adverse effect of VB6 deficiency on immunocompetence may be mediated by an impaired capacity of the thymus to convert the progenitors of T lymphocytes and a consequent reduction in number and function of immunologically competent lymphoid cells (Robson and Schwarz, 1975a, Willis-Carr and St. Pierre, 1978).

Sergeev et al. (1978) reported the effect of VB6 deficiency on the proliferation and cytotoxicity of mouse lymphocytes stimulated in vitro with irradiated allogeneic spleen cells. Significant impairment of lymphocyte proliferation and cytotoxicity were found in mice fed

the VB6-deficient diet for 5-6 weeks; however, neither proliferation of lymphocytes in vitro nor their cytotoxicity was impaired in mice maintained on the VB6 deficient diet for 3 weeks, despite the fact that the PLP content in the spleen was reduced nearly 30%. PLP, but not pyridoxal, added directly to the medium in vitro partially restored the impaired functions of T lymphocytes.

In contrast to the above studies, which demonstrated disturbances in CMI during VB6 deficiency, no differences were found in systemic reactivity as well as in in vitro cellular reactivity of splenic lymphocytes of VB6-deficient guinea pigs to E. coli endotoxin (Stinebring, Trakatellis and Axelrod, 1963). Other mitogens have not been employed to test spleen cells of VB6-deficient animals and no studies have been reported which investigate the functional competence of subpopulations of T lymphocytes and other effector cells. This requires further study.

There is a concordance of clinical symptoms and biochemical alterations between uremia and VB6 deficiency (Stone, Warnock and Wagner, 1975), suggesting that some symptoms of uremia may be the result of, or be at least intensified by, VB6 deficiency. Dobbelstein et al. (1974) reported that inhibition of pyridoxal kinase activity rather than insufficient VB6 supply is the most likely explanation for the depletion of PLP in uremia. The diminution of reactivity in mixed lymphocyte cultures from patients with uremia could be reversed by treating with VB6 orally, suggesting that suppression of CMI in uremia is probably due, in part, to VB6 depletion.

Vitamin B-6 Deficiency in Cancer Patients

Studies have shown the possibility that disturbances in VB6 nutrition might be related to clinical manifestions of certain cancers. Bell (1980) reported that patients with breast cancer who excreted subnormal amounts of a VB6 metabolite, 4-PA, had a significantly increased probability of recurrence of early breast cancer. The physiological significance of the relationship between low excretion of 4-PA and rapid recurrence of cancer is unknown. If low urinary amounts of 4-PA reflect VB6 deficiency, then it might be suggested that these patients have an impaired immune response. Potera, Rose and Brown (1977) reported plasma PLP was normal in early breast cancer, but was significantly reduced both in cases of local recurrence and systemic metastases. However, urinary 4-PA, which decreases promptly in experimental VB6 deficiency, was normal in breast cancer patients with reduced plasma PLP levels.

Abnormal tryptophan metabolism, a characteristic feature of VB6 deficiency, with or without low plasma PLP, has been reported in patients with carcinoma of the urinary bladder (Brown and Price, 1956, 1969) and breast (Rose, 1967ab) as well as in advanced Hodgkin's disease (Chabner et al., 1970). The possible role of urinary tryptophan metabolites in bladder carcinogenesis was suggested by the finding that diets supplemented with this amino acid enhanced the production of bladder cancer in rats fed 2-acetylaminofluorene (Dunning, Curtis and Maun, 1950), and by the fact that tryptophan is a precursor of several aromatic amines chemically similar to those known to produce bladder cancer (Price et al., 1960).

Rose (1967ab) reported that an abnormal excretion of tryptophan metabolites was found in over half of the breast cancer patients who had had a mastectomy as well as in women with untreated carcinoma of the breast. He suggested that the abnormal tryptophan metabolism might be related to the endocrine status along with a lack of PLP in some patients with breast cancer. In contrast, Bell et al. (1972) reported that pre-menopausal women with breast cancer who excreted a small amount of tryptophan metabolites after a 5g loading dose of the amino acid had a higher rate of recurrence of the disease after mastectomy than those with elevated metabolite levels.

Chabner et al.(1970) studied plasma levels of PLP and the metabolism of tryptophan in patients with Hodgkin's disease. Both tests gave abnormal results most frequently in patients with advanced or symptomatic disease. In contrast, both tests were within normal limits in patients in complete remission after chemotherapy for advanced disease. Other clinical features of Hodgkin's disease, such as lymphopenia and anemia, occurred predominantly in patients with low plasma PLP. The strong correlation between decreased plasma PLP in untreated patients and unresponsiveness to skin testing indicated the immunosuppressive effects of VB6 deficiency.

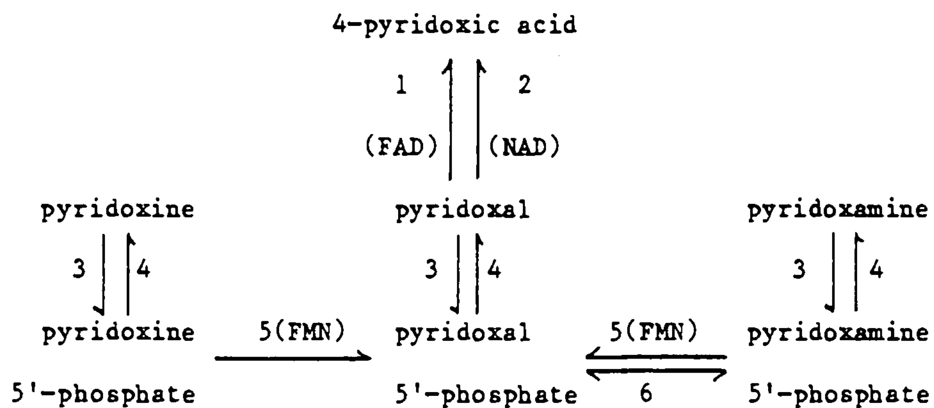

Figure 1.1. Interconversion of B-6 vitamers.

1. aldehyde oxidase (FAD dependent), 2. aldehyde dehydrogenase (NAD dependent), 3. pyridoxal kinase, 4. phophatases, 5. PNP oxidase (FMN dependent), 6. transaminases.

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CHAPTER 2

THE EFFECT OF VITAMIN B-6 DEFICIENCY ON HOST SUSCEPTIBILITY TO MOLONEY SARCOMA VIRUS-INDUCED TUMOR GROWTH

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Vitamin B-6 & Host Susceptibility to MSV/MSB

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ABSTRACT

The effect of vitamin B-6 deficiency on in vivo host susceptibility to primary Moloney sarcoma virus (MSV)-induced tumor growth and to secondary challenge with MSB sarcoma cells was examined in mice. Female C57Bl/6 mice, 6 weeks of age, were fed 20% casein diets with pyridoxine (PN) added at 1, 0.5, 0.1, or 0 mg/kg diet for 21 weeks. After 4 weeks of dietary treatment the mice were challenged with MSV. Vitamin B-6 deficiency resulted in an enhancement of tumor susceptibility as well as an increase in tumor size and regression time. The animals resistant to both MSV and MSB challenge showed splenic tumor development upon necropsy 51 days after MSB challenge. Total incidence of MSV/MSB/splenic tumors was 2/11, 2/11, 4/10, and 8/11 in animals fed PN-1, -0.5, -0.1, and -0 diets, respectively. Since MSV-induced tumors regressed spontaneously in immunocompetent hosts, the increased susceptibility to MSV oncogenesis in vitamin B-6 deficient animals suggests that reactivity of T-cells and/or other effector cells are impaired in vitamin B-6 inadequacy.

INDEXING KEY WORDS vitamin B-6. pyridoxine. Moloney sarcoma virus. MSB.

INTRODUCTION

Vitamins have been recognized for a long time for their importance in host resistance against infections and were shown to influence host immunocompetence (1). Vitamin B-6 as its biologically active form pyridoxal 5'-phosphate (PLP) plays a crucial role as a coenzyme in numerous metabolic processes required for normal nucleic acid synthesis, protein metabolism, and cellular proliferation (2,3). Deficiency of vitamin B-6 has also been shown to alter many aspects of immunocompetence in both experimental animals and humans, including changes in the structure and loss of cellularity of lymphoid organs (4), and significant impairment of both humoral (1,5) and cell-mediated (3,6,7) immune responses.

A controlled host susceptibility study provides a valuable measure of overall immunocompetence and a prediction of immunomodulation by nutritional factors. The Moloney sarcoma virus (MSV)-induced tumor is a sensitive and reproducible model for assessing immunocompetence and antitumor responses in inbred mice. It has a predictable and short induction time of strongly antigenic tumors which spontaneously regress unless the host is immunosuppressed (8-10). Tumors induced in mice by MSV appear in 5 to 10 days at the site of virus injection, reach a peak size around day 14 and then regress spontaneously. MSV tumor regression has been shown to be immunologically-mediated and dependent primarily on an intact cytotoxic T-lymphocyte response which is capable of tumor-cell lysis

(9). However, cytotoxic antibodies (9,11), macrophages (12-14), and killer cells (8,15,16) are also involved in the immune response to MSV. Animals which undergo primary MSV tumor growth and regression have persisting cytolytic memory T cells (10,17) and are immune to secondary tumor challenge with MSV-transformed tumor cells (MSB) at a dose that produces a 100% incidence of progressive tumors in nonimmune animals. Mice with depressed memory immunity develop progressive MSB tumors that kill the host (18). Thus the MSV-MSB tumor model is applicable to the in vivo study of both primary and secondary antitumor immune responses.

Susceptibility to tumor growth and impaired immunocompetence in relation to vitamin B-6 deficiency is an important issue. Marginal vitamin B-6 intake may be prevalent among children and the elderly (19). The 1977-78 Nationwide Food Consumption Survey indicated that vitamin B-6 intakes of females over 14 years and males over 64 years were below 2/3 the recommended daily allowance (20).

No reports have been published concerning the effects of vitamin B-6 deficiency on host susceptibility to tumor growth. However, studies have shown the possibility that alterations in vitamin B-6 nutrition might be responsible for clinical manifestations of certain cancers. Patients with breast cancer who excreted subnormal amounts of the vitamin B-6 metabolite, 4-pyridoxic acid (4-PA), had a significantly high probability of cancer recurrence after mastectomy (21). Plasma PLP was normal in early breast cancer, but was significantly reduced both in cases of local recurrence and systemic metastases, whereas urinary excretion of 4-PA was normal

(22). Abnormal tryptophan metabolism, after a tryptophan load, with or without low plasma PLP level has been reported in patients with carcinoma of the urinary bladder (23) and breast (24) as well as in advanced Hodgkin's disease (25). Cancer patients with metabolic disturbances are of interest in view of the immunosuppressive effects of experimental pyridoxine deficiency.

The present study examined the effect of vitamin B-6 deficiency on in vivo virus-induced tumor growth in mice which were challenged with MSV and MSB. We observed that MSV oncogenesis was modulated by vitamin B-6 depletion.

MATERIALS AND METHODS

Animals and care

Female C57Bl/6 mice, 6 weeks of age, were purchased from the Jackson Laboratory, Bar Harbor, ME. The animals were marked individually and housed in groups of 3 or 4 in suspended cages with wire mesh bottoms in a room controlled for temperature, humidity and light. Each dietary level of vitamin B-6 was fed to a group of 15 mice. Food and water were available to the animals ad libitum, and food consumption was recorded twice a week. Body weights and signs of vitamin B-6 deficiency were recorded weekly.

Diets

Animal diets containing 20% casein (Vitafree, United States Biochemical Co., Cleveland, OH) were prepared according to the AIN-76 formula (26), except that pyridoxine (PN) was added at 1, 0.5, 0.1, and 0 mg/kg diet, hereafter called PN-1, -0.5, -0.1, and -0 diets. These levels represent 100, 50, 10, and 0% of the pyridoxine requirement of mice adequate for both growth and reproduction (27). The vitamin B-6 content of casein, as analyzed microbiologically using _Saccharomyces uvarum_ (28), was 0.5 mg/kg. The animals were fed their respective diets throughout the experiment.

Tryptophan load test

Vitamin B-6 deficiency in the animals was checked by a

tryptophan load test prior to inoculation of MSV and MSB. Tryptophan, diluted in distilled water and phosphate buffered saline, was given by intraperitoneal injection at a dose of 1 mg and 0.5 mg tryptophan/g body weight 18 days and 88 days, respectively, after dietary treatment was initiated (23). Twenty four-hour pre- and post-tryptophan urines were analyzed for xanthurenic acid (29).

Tumors

Stocks of Moloney murine sarcoma virus (MSV), prepared from a homogenate of MSV tumors induced in C57Bl/6 weanling mice, were maintained frozen at -70° C. MSB is a MSV-producing cell line syngeneic to C57Bl/6 mice, tumorigenic in vivo and releases MSV (30). MSB was maintained in a monolayer culture in Eagle's minimum essential medium (GIBCO, Grand Island, NY), supplemented with 10% fetal calf serum (Sterile Systems, Logan, UT), 1% nonessential amino acids (GIBCO, Grand Island, NY), 1% vitamin mix (GIBCO, Grand Island, NY), 1mM sodium pyruvate (GIBCO, Grand Island, NY), 2mM L-glutamine (Flow Lab., Ingle Wood, CA), 50 μg/ml gentamicin (Schering Corp., Kenilworth, NJ), and 10mM HEPES buffer (Sigma Chemical, St. Louis, MO).

MSV-induced tumors were produced in C57Bl/6 mice by intramuscular inoculation of 0.1 ml of 1:6 dilution of the stock virus preparation in the right hindleg 4 weeks after starting the dietary treatment. At this time animals fed PN-0.1 or -0 diets began to show differences in weight gain and mild vitamin B-6 deficiency as indicated by increased xanthurenic acid excretion following the

tryptophan load test. Animals were examined daily for appearance of tumors. Tumor growth rates were estimated by visually grading the tumor on a scale of 0 to +5, as previously reported (18).

Sixty-seven days after MSV inoculation, animals were inoculated in the left hindleg with 1 x 10^6 MSB sarcoma cells to assess memory immunity in MSV-regressor animals. The animals were examined twice a week for appearances of secondary tumors. Mice were sacrificed when moribund.

Histopathology

On the 51st day after MSB injection, all surviving animals were necropsied to examine for metastases. Selected tissues of spleen, thymus, liver, and MSV and MSB tumors for histopathological examination were fixed in 10% neutral-buffered formalin.

Statistical analysis

ANOVA and Chi square analyses were used for statistical evaluation of tumor growth and progressive tumor incidence, respectively. When ANOVA indicated significant F values ($P<0.05$ or $P<0.01$), significant differences among means were determined by the LSD.

RESULTS

Growth and food intake

Animal growth reflected the level of dietary vitamin B-6 (figure 2.1). Mean weight gains during the 21-week experiment in animals fed PN-1, -0.5, -0.1, and -0 diets were 12.1, 12.1, 6.3, and 3.7 g, respectively. Growth patterns were similar for all groups for the first 4 weeks of the dietary treatment. Mice fed the PN-0.1 and -0 diets ceased to gain or lost weight at about week 10-11, and at 21 weeks their growth was significantly ($P<0.01$) impaired, compared to animals fed the PN-1 and -0.5 diets. Mice fed PN-0 diet gained significantly ($P<0.05$) less weight than did mice fed PN-0.1 diet. Mice fed PN-1 and -0.5 diets showed increasing body weight throughout the experiment and their body weights were not significantly different.

During the first 6 weeks of dietary treatment food intakes were not different among the four dietary treatment groups, and averaged between 2.8 and 3.0 g per mouse per day. Thereafter, food consumption by animals fed PN-0.1 and -0 diets was 2.7 g per mouse per day, while that by animals fed PN-1 and -0.5 was increased to 3.2-3.4 g.

After 21 weeks most of the mice fed the PN-0 diet and some mice on PN-0.1 diet had rough fur, denuding of the snout and skin irritation. No such lesions were observed in the mice fed the PN-1 or -0.5 diet. Upon necropsy at 21 weeks, mice fed PN-0 and -0.1 showed

severe thymic atrophy, compared to mice fed PN-1, as had been reported earlier (4).

Tryptophan load test

Vitamin B-6 deficiency in the animals was determined by a tryptophan load test (intraperitoneal injection of 1 mg L-tryptophan/g body weight) 18 days after dietary treatment. Animals fed PN-1 and -0.5 diets excreted 0.8 and 3.4% of tryptophan load as xanthurenic acid, whereas the animals fed PN-0.1 and -0 diets excreted 9.8 and 11%, respectively, indicating development of vitamin B-6 inadequacy (table 2.1). After 88 days of dietary treatment the tryptophan load test was administered again, but with a reduced dose (0.5 mg L-tryptophan/g body weight) to minimize stress to the animals. At this time the animals fed PN-0.1 and -0 diets excreted 18.8 and 22.8%, respectively, of the administered amino acid as xanthurenic acid, as compared to animals fed PN-1 and -0.5 diets which excreted less than 1% and 2%, respectively. In addition, animals fed PN-0.1 and -0 diets excreted more xanthurenic acid in their basal (pre-tryptophan) urine than did mice maintained on PN-1 and -0.5 diets. This spontaneous excretion of xanthurenic acid is also an indication of vitamin B-6 deficiency (31).

Host susceptibility to primary MSV-induced tumor growth and secondary challenge with MSB sarcoma cells

Following MSV inoculation intramuscularly in the right hindleg, all animals developed primary tumors at the site of the virus

injection. Tumors grew to a larger peak size in animals fed PN-0 (+3.23) and PN-0.1 (+3.14) diets than in mice that had received PN-0.5 (+3.00) and PN-1 (+2.93) diets. Tumor development was similar in all animals with peak tumor size observed on day 10. However, the rate of tumor regression was inversely related to vitamin B-6 intake. Tumors in mice fed PN-0 diet regressed significantly ($P<0.05$) more slowly, requiring more than 28 days while other groups required less time (figure 2.2). Tumor growth recurred in 2 of 11 animals fed the PN-0 diet (one on about day 59, the other on about day 69); complete primary tumor regression was observed in all other groups.

When tumor regressor animals were challenged in the left hindleg with 1×10^6 MSB sarcoma cells, the incidence of progressive secondary tumor growth was 2/11, 0/11, 1/10, and 2/9 in animals fed PN-1, -0.5, -0.1, and -0 diets, respectively, and apparently was not altered by vitamin B-6 status (table 2.2).

On the 51st day after MSB challenge, surviving animals were necropsied and the incidence of splenic tumor development was recorded. The incidence of splenic tumors in animals that rejected the MSV/MSB challenge was 0/9, 2/11, 3/9, and 4/7 in animals fed PN-1, -0.5, -0.1, and -0 diets, respectively. Splenic tumor formation was also observed in all of the MSB tumor-bearing animals. Thus the combined MSV/MSB/splenic tumor incidence was significantly ($P<0.01$) enhanced from 2/11 and 2/11 in animals fed PN-1, and -0.5 diets to 4/10 and 8/11 in animals maintained on PN-0.1 and -0 diets, respectively (table 2.2). Histologically, the splenic tumors resembled lymphosarcomas and fibrosarcomas, whereas MSV tumors were

fibrosarcomas. Although tumor development was not observed generally in other organs, tumors in thymus and ovary along with recurrence of MSV tumor developed in one animal fed PN-0 diet. No significant pathological lesions were observed in kidney or liver, but hepatic necrosis was observed in one animal maintained on PN-0 diet.

DISCUSSION

Deficiency of vitamin B-6 in animals generally induces growth retardation, an acrodynia-like syndrome, tremors, convulsive seizures, and atrophy of lymphoid organs (4). In the present study we designated animals fed PN-0.1 and -0 diets as vitamin B-6 deficient animals based on their poor growth (figure 2.1) and elevated xanthurenic acid excretion (table 2.1) in response to tryptophan. Although 0.5 mg PN/kg diet is not adequate according to NRC (27), mice fed that level had body weights comparable to and excreted only slightly more xanthurenic acid following tryptophan loading than mice fed PN-1 diet.

Animals fed PN-0.1 and -0 diets developed deficiency signs including significantly lower body weight (figure 2.1), denuding of the snout, skin irritation, and thymic atrophy. In addition, elevated excretion of xanthurenic acid by animals fed PN-0.1 and -0 diets before as well as after a test dose of tryptophan indicated an altered metabolism of this amino acid due to depletion of vitamin B-6. Abnormal tryptophan metabolism occurred before appreciable growth depression. This indicates that xanthurenic acid excretion after tryptophan loading is a sensitive indicator of vitamin B-6 deficiency.

In studying the effect of vitamin B-6 deficiency on immune function and host susceptibility, monitoring food intake is important to determine if alterations in the immunologic response are due to inanition or vitamin B-6 deficiency. Vitamin B-6 inadequacy is known

to cause some degree of inanition. Furthermore, decreased food intake or energy restriction alone has been shown to cause chronic infections, inhibit tumor growth and alter tumor incidence and kinetics (32). The food intake (2.8-2.9 g/day) of animals fed PN-0.5, -0.1, or -0 diet for the first 6 weeks of our study was not markedly reduced, compared to that (3.0 g/day) of animals fed PN-1 diet. This may indicate that the amount of vitamin B-6 in the PN-0 diet supplied by vitamin-free casein may have been sufficient to prevent severe loss of appetite of adult mice, at least at the beginning of experiment. In addition, mice may be less susceptible to vitamin B-6 deficiency than other species, as reported earlier (33). Our results are in good agreement with the previous report by Beck et al. (33) in which 8-16 week-old C57 mice were maintained on a PN-deficient diet containing 25% casein for 4 months. During the early period of PN deficiency their animals showed weight loss and mild deficiency symptoms such as increased excitability without marked reduction of food consumption. Ideally, in vitamin B-6 studies the effect of reduced food intake should be taken into consideration. Pair-feeding would have been difficult in this experiment. However, it should be noted that tumor growth in the vitamin B-6 deficient animals, despite their poor growth, was greater than that in the animals receiving the higher levels of vitamin B-6 (figures 2.1 & 2.2).

The impact of vitamin B-6 deficiency on in vivo tumor growth was explored. Primary MSV-induced tumors exhibited a larger peak size in vitamin B-6 deficient mice despite poor body growth than in those maintained on PN-1 and -0.5 diets (figure 2.2). Although regression

of the primary MSV-induced tumors was not prevented by vitamin B-6 deficiency, animals fed the PN-0 diet showed increased regression time (>28 days) and susceptibility (2/11) to the recurrence of primary tumors, and their tumors had a greater tendency to metastasize following MSB tumor inoculation, compared to those fed PN-1 or -0.5 diet. Whereas none of the 9 tumor regressors fed PN-1 diet developed splenic tumors, 2 of 11, 3 of 9, and 4 of 7 mice fed PN-0.5, -0.1, and -0 diets, respectively, showed tumor dissemination in the spleen (table 2.2). Spleen metastasis was noted in MSV-injected neonatal mice (34) and MSV-induced disseminations in various organs were seen in adult mice which were pretreated with sublethal X-irradiation or thymectomy (35). Since immunologic events are decisive factors in MSV-induced tumor induction, growth and dissemination, enhanced susceptibility to MSV/MSB challenge observed in vitamin B-6 deficient mice appears to be due to host immunosuppression, that is, a decrease in activity of effector cells and/or an increase in suppressor cells.

In contrast to the results of our studies, Tryfiates et al. (36) reported impaired growth of the transplantable minimum deviation Morris hepatomas in female Buffalo rats under conditions of limited availability of vitamin B-6. This discrepancy may be due to different mechanisms by which vitamin B-6 deficiency inhibits growth of Morris hepatoma, but stimulates MSV oncogenesis. The growth of Morris hepatoma was controlled by aberrant nucleic acid and/or protein biosynthesis due to altered enzyme activity under pyridoxine restriction. Thymidylate synthetase and serine hydroxymethyltransferase, which are key enzymes in the rate limiting

step in DNA synthesis, appear to be inactivated by lack of vitamin B-6, resulting in a decrease in cell proliferation and tumor growth (36). On the other hand, certain transformed cells could grow under conditions where a growth factor and/or nutrient was inadequate to support the growth of normal cells (37). This suggests that such cells may be more efficient in acquiring or utilizing certain nutrients and growth factors or are less dependent on them. It is difficult to define the precise interaction of host vitamin B-6 status and MSV-induced tumor metabolism in the present experiment. The observed failure to inhibit tumor growth and metastases under vitmin B-6 deficiency might reflect an impaired immunological function of the host.

In conclusion, the present study suggests that the resistance of animals to MSV oncogenesis was compromised by vitamin B-6 deficiency. Vitamin B-6 deficient C57Bl/6 mice exhibited susceptibility to MSV/MSB induced tumor growth and splenic tumor development, suggesting an impaired immunological response, which is a major factor in control of MSV-induced tumor growth. The following are required for an accurate evaluation of in vivo tumor susceptibility with vitamin B-6 deficiency: 1) the study of cytotoxic T-lymphocytes in spleen and lymph nodes, and 2) the assessment of the contribution of other host defense mechanisms such as macrophages, antibodies, suppressor cells, and natural killer cells. These studies are in progress.

TABLE 2.1

Xanthurenic acid (XA) excretion before and after tryptophan (try) load test

Dietary group	n[1]	XA excretion Pre-try (μmol/24 hr)	XA excretion Post-try (μmol/24 hr)	Try dose (μmol)	Excretion as XA (%)
		Day 18[2]			
PN-1	15	0.05	0.9	100.9	0.8[3]
PN-0.5	15	0.05	3.1	90.6	3.4
PN-0.1	14	0.24	8.8	87.6	9.8
PN-0	15	0.18	9.0	80.3	11.0
		Day 88[4]			
PN-1	11	0.13	0.6	64.2	0.8[5]
PN-0.5	11	0.13	1.4	61.2	2.0
PN-0.1	10	0.82	11.5	56.8	18.8
PN-0	10	1.13	11.7	50.9	22.8

1. number of animals in pool.
2. i.p. injection of 1 mg tryptophan/g body weight, 11 days before MSV injection.
3. PN-0 and -0.1 groups were significantly different (χ^2, $p<0.01$) from PN-1 group, PN-0 group was significantly different (χ^2, $p<0.05$) from PN-0.5 group.
4. i.p. injection of 0.5 mg tryptophan/g body weight, 8 days before MSB injection.
5. PN-0 and -0.1 groups were significantly different (χ^2 , $p<0.01$) from PN-0.5 and -1 groups.

Table 2.2

Effect of vitamin B-6 in mice on susceptibility to progressive primary MSV-induced tumor growth, secondary MSB challenge and splenic tumor development[1]

Dietary group	Progressive tumor incidence		Splenic tumor incidence in regressor mice[2]	Total number of mice bearing MSV/MSB/Splenic tumor
	Primary MSV	Secondary MSB		
PN-1	0/11	2/11	0/9	2/11
PN-0.5	0/11	0/11	2/11	2/11
PN-0.1	0/10	1/10	3/9	4/10
PN-0	2/11	2/9	4/7	8/11 [3]

1. Mice were injected with MSV in the right hindleg after 4 weeks of dietary treatment. Tumor growth and regression/progression were monitored for 67 days. On day 67 regressor animals were inoculated in the left hindleg with 1×10^6 MSB cells. Secondary tumor growth was monitored for an additional 51 days. All surviving animals were necropsied.
2. All mice developing progressive MSB tumors also exhibited splenic tumors upon necropy, not included in total.
3. Significantly different from PN 0.5 and 1 groups, χ^2, $p<0.01$.

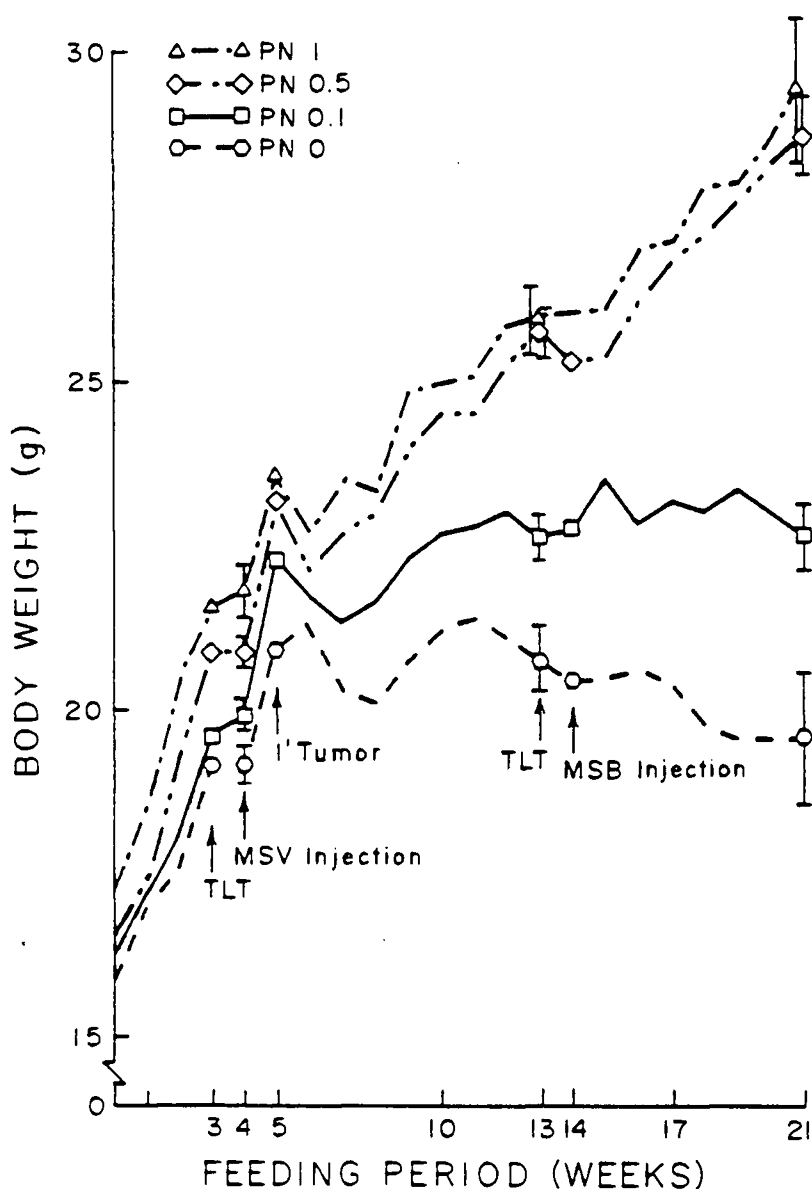

Figure 2.1. Effect of vitamin B-6 on growth of C57Bl/6 female mice. Each point represents mean of 7-15 mice. Standard error bars are shown for selected points. Body weights of MSB tumor-bearing mice were excluded. Body weights on weeks 4, 13, and 21 were significantly different among groups (P<0.01). MSV: Moloney sarcoma virus, TLT: tryptophan load test.

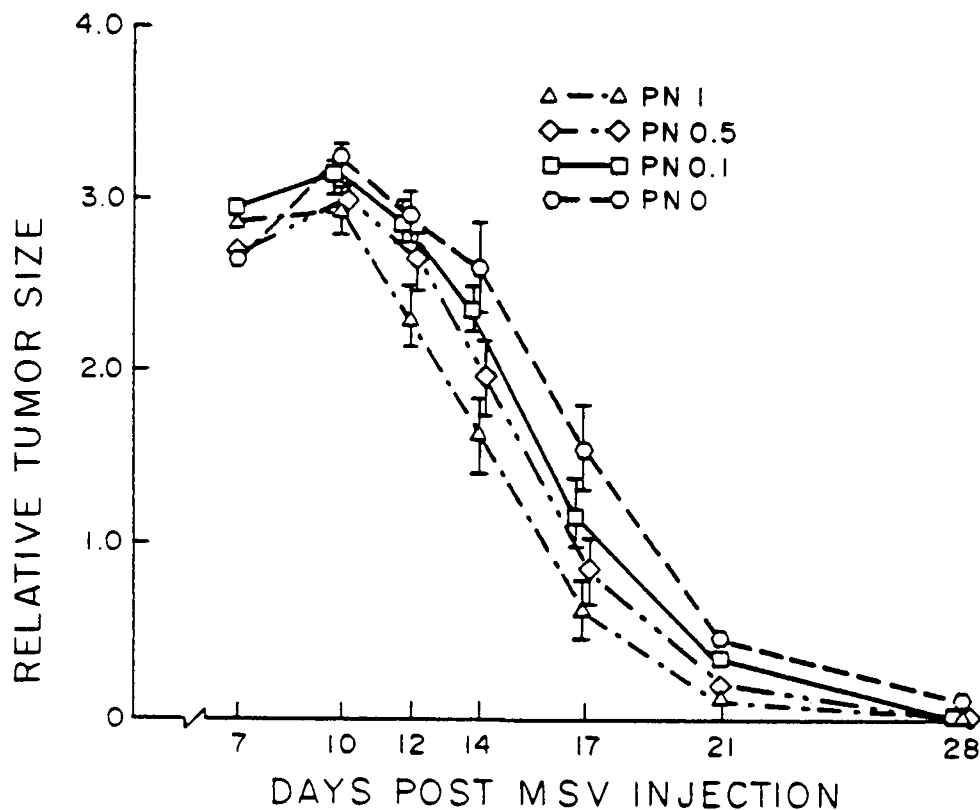

Figure 2.2. Effect of vitamin B-6 on MSV-induced primary tumor growth. MSV-induced tumor growth was initiated by im inoculation in right hindleg of a 1:6 dilution of a stock virus preparation after 29 days of dietary treatment. Tumor growth rate was estimated by visually grading the tumor on a scale 0 to +5. Each point represents mean of 14-15 animals. Standard error bars are included at selected points. Relative tumor sizes on days 12, 14, and 17 were significantly ($P<0.01$) different among groups.

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CHAPTER 3

THE EFFECT OF VITAMIN B-6 DEFICIENCY ON CYTOTOXIC IMMUNE RESPONSES OF T CELLS, ANTIBODIES AND NATURAL KILLER CELLS, AND PHAGOCYTOSIS BY MACROPHAGES

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ABSTRACT

The effect of vitamin B-6 on cytotoxic immune responses of T cells, natural killer (NK) cells, cytotoxic antibody production and macrophage phagocytosis was assessed in 5-week old female C57Bl/6 mice. Mice were fed 20% casein diets with pyridoxine (PN) added at 7, 1, 0.1, or 0 mg/kg diet which represent 700, 100, 10, and 0% of requirement. Compared to mice fed 7 or 1 mg PN diet, animals fed 0 or 0.1 mg PN diet showed significantly reduced primary splenic and peritoneal T cell-mediated cytotoxicity (CMC). Animals fed 0 mg PN diet also showed significantly depressed secondary T CMC of splenic and peritoneal lymphocytes against P815 tumor cells. Complement-dependent antibody-mediated cytotoxicity against P815 cells, phagocytosis of SRBC by macrophages, and native and interferon-induced NK cell activities against YAC cells were not affected by the level of vitamin B-6 intake. The percentage of macrophages present in the peritoneal exudate cells was increased in animals fed 0 mg PN diet. The immune responses were not enhanced or altered by the excess intake of vitamin B-6 (7 mg PN). It appears that vitamin B-6 is an essential nutrient for maintenance of normal T cell function in vivo.

INTRODUCTION

The effect of nutrition on immunity is manifold. Both under- and over-nutrition may cause metabolic abnormalities, which, in turn, can be associated with an increased incidence of infectious diseases and altered immune response (1,2). Thus nutritional manipulation of immunity could have wide-ranging clinical, biologic, and therapeutic implications.

It has been demonstrated that any single nutrient required for synthesis of DNA and protein, e.g., vitamin B-6, folic acid, zinc, has an effect on the immune response (1,3,4). Vitamin B-6 deficiency is accompanied by impairment of both humoral and cell-mediated immunity. Vitamin B-6 deficient animals have reduced delayed hypersensitivity responses (5,6) and prolonged survival time of skin allografts (7,8). Their humoral response to antigenic challenge is greatly diminished, as reflected by a reduction in antibody-forming cells (5,9,10). Vitamin B-6 deficiency has been associated with a dramatic depletion of thoracic duct lymphocytes and reduction in lymphocyte proliferation in vitro as measured by the mixed lymphocyte reaction (MLR) (11). Thymic epithelial (TE) cell function was also impaired in vitamin B-6 deficient animals resulting in the inability of TE cells to effect the differentiation of T lymphocyte precursors to functional T lymphocytes (12). The cytotoxicity of T lymphocytes from mice fed a vitamin B-6 deficient diet for 5-6 weeks was also significantly reduced (13).

We previously observed that vitamin B-6 deficient mice

exhibited enhanced susceptibility to Moloney sarcoma virus (MSV)-induced tumor growth and splenic tumor development (14). In this paper we report impaired production and reduced activity of cytotoxic T lymphocytes in vitamin B-6 deficient mice; cytotoxic antibody responses, NK cell cytotoxicity, and the phagocytic response of macrophages were normal in vitamin B-6 deficient mice.

MATERIALS AND METHODS

ANIMALS AND DIETS

Female C57Bl/6 mice, 5 weeks of age, were purchased from the Jackson Laboratory, Bar Harbor, ME. The animals were housed in groups of 3 in suspended cages with wire mesh bottoms in a room controlled for temperature, humidity and light. Food and water were available to the animals ad libitum. Body weights were recorded weekly.

Animal diets containing 20% vitamin-free casein were prepared according to the AIN-76 formula (15), except that pyridoxine (PN) was added at 7, 1, 0.1, and 0 mg/kg diet, hereafter called PN-7, -1, -0.1, and -0 diets, respectively. These levels represent 700, 100, 10, and 0% of the pyridoxine requirement of mice adequate for both growth and reproduction (16). Three separate experiments were conducted in which three or four levels of PN were fed. In each experiment groups of 12-13 animals were fed experimental diets for 4-5 weeks prior to immunologic testing and remained on these respective diets throughout the experiments.

The concentration of vitamin B-6 in mouse livers was determined microbiologically using *Saccharomyces uvarum* (17).

TUMORS

P815 mastocytoma: P815 tumor cells, originated from a methylcholanthrene-induced tumor in DBA/2 mice, were maintained in ascites form by weekly intraperitoneal passage of 5×10^6 tumor cells

in syngeneic mice.

YAC: YAC, a Moloney virus-induced lymphoma of A strain mice, was maintained in stationary suspension culture in RPMI 1640 (GIBCO, Grand Island, NY), supplemented with 10% fetal calf serum (FCS) (Sterile systems, Logan, UT) and gentamicin (Schering Corp., Kenilworth, NJ).

PRIMARY AND SECONDARY T CELL-MEDIATED CYTOTOXICITY

Allogeneic sensitization for induction of primary and secondary cytotoxic T cells in vivo: C57Bl/6 mice were injected ip once with 1 x 10^7 P815 tumor cells and sacrificed 10 days later for assessment of primary cytotoxic T cell activity. Mice which had been injected ip with 1 x 10^7 P815 tumor cells 24 days previously were rechallenged with 1 x 10^7 P815 cells and killed 5 days later for measurement of secondary T cell-mediated cytotoxicity (CMC).

Induction of cytotoxic T cells in vitro: Secondary T CMC was generated in vitro in mixed lymphocyte-tumor cell cultures (MLTC). Spleen cells from animals which had been sensitized with 1 x 10^7 P815 cells 24 days previously were co-cultured for 5 days with mitomycin C-treated P815 tumor cells at a 10:1 responder to stimulator ratio. Parallel cultures containing spleen cells alone, without tumor cells for restimulation, were included as controls. After 5 days in culture, effector cells were harvested and cytotoxicity was assayed.

Effector cell preparation: Splenic and peritoneal lymphocytes used as effector cells in the CMC assay were prepared as previously reported (18). The effector cells were confirmed to be T cells based

on the abrogation of cytolysis by treatment of effector cells with anti-θ serum and complement (19).

<u>51-Chromium</u> <u>(Cr)-release</u> <u>cytotoxicity</u> <u>assay</u>: The cytotoxic activities of splenic and peritoneal T lymphocytes were assayed using a 4-hour 51-Cr-release assay as previously described (18). Percentage of specific cytotoxicity was calculated for each lymphocyte-to-target cell ratio as follows: % specific cytotoxicity = [(cpm experimental - cpm SR) / (cpm MR - cpm SR)] x 100. The maximum release (MR) and spontaneous release (SR) were determined by incubation of tumor cells in 0.5 % sodium dodecyl sulfate and in media, respectively.

Cytotoxic T lymphocyte activity was also expressed as lytic units (LU), with one LU defined as the number of cells needed to achieve 30% specific lysis of target cells in the 4-hour 51-Cr-release assay.

COMPLEMENT-DEPENDENT ANTIBODY-MEDIATED CYTOTOXICITY

Blood samples were collected by heart puncture from animals which donated splenic and peritoneal lymphocytes for primary and secondary T CMC and for macrophage phagocytosis. Anti-P815 sera were stored at -20° C until assayed for antibody cytotoxicity. Complement was inactivated by heating the sera at 56° C for 30 minutes before assay.

For measuring primary and secondary antibody-mediated lysis, a 0.05 ml aliquot of serial twofold dilutions of sera in DMEM (GIBCO, Grand Island, NY) containing 5% FCS, 0.05 ml of a 1:5 dilution of rabbit complement (C') (Cedarlane Lab., Hornby, Ontario), and 1×10^4

51-Cr-labeled P815 tumor cells as target (0.1 ml) were mixed in round bottom microplates and were incubated for 2 hours at 37° C. At the end of incubation, the plates were centrifuged and 0.1 ml aliquots of the supernatants were counted in a gamma counter (Packard Instrument Co., Downers Grove, IL). The presence of 2-mercaptoethanol (2-ME) resistant antibody (IgG) was assayed by preincubation of sera with an equal volume of 0.1 M 2-ME at 37° C for 30 minutes before serial dilution. To assess the indirect response of IgG of the secondary sera, 25 µl of a 1:800 dilution of rabbit anti-mouse IgG (Cappel Lab., Cochranville, PA) was dispensed into plates containing 25 µl aliquots of diluted sera and incubated at 37° C before adding C' and labeled tumor target cells. Control tests consisted of replacing immune anti-P815 sera (IS) with non-immune serum (NS). Percentage of specific lysis was calculated as follows: % sp. cytotoxicity = [(cpm IS - cpm NS) / (cpm MR - cpm SR)] x 100. The titer was defined as the reciprocal of the highest dilution giving 50% or 30% specific cytotoxicity.

PHAGOCYTOSIS BY MACROPHAGES

Mice were allosensitized by ip injection with 1×10^7 P815 tumor cells. After 9 days peritoneal exudate cells (PEC) were harvested from P815-sensitized mice for assessment of phagocytosis of 51-Cr-labeled sheep red blood cells (SRBC), which were opsonized and nonopsonized, in vitro (18). For morphological analysis, PEC were cytocentrifuged (Shandon Southern Instruments, Camberley, England) and the percentage of macrophages was determined by counting a total of

200 cells on each Wright-Giemsa stained slide (20). Phagocytic activity was expressed as the mean count incorporated by 2 x 10^6 macrophages.

NATURAL KILLER CELL-MEDIATED CYTOTOXICITY

Splenic lymphocytes were used as effector cells in the native and interferon (IF)-induced NK CMC assay. IF-boosted NK cells were obtained 24 hours following an ip injection of 100 μg polyinosinic acid-polycytidylic acid (poly I-poly C) (Calbiochem-Behring Corp., La Jolla, Ca). Spleen cell suspensions were prepared as described previously (18). The cytotoxic activity of NK cells was measured using a 4-hour 51-Cr-release assay with YAC tumor target cells. Percent cytotoxicity was calculated by the formula: % cytotoxicity = [(cpm experimental - cpm thymocytes) / (cpm MR - cpm SR)] x 100. Thymocytes with labeled tumor cells were used as a control of the non-NK cell population.

STATISTICAL ANALYSIS

Cytotoxicity and phagocytosis data were expressed as means and standard errors of 6 to 13 mice tested individually or of 3-4 pools of 3 mice each. Comparison among the means were made by ANOVA and LSD.

RESULTS

GROWTH AND LIVER VITAMIN B-6

Body weight was significantly (P<0.01) altered by different dietary levels of vitamin B-6. After 4-5 weeks of dietary treatment, mean weight gains in mice fed PN-7, -1, -0.1, and -0 diets were 5.8, 6.2, 5.0, and 4.3 g, respectively. In addition the concentration of vitamin B-6 in the livers was significantly (P<0.01) decreased by 50% in animals fed PN-0 and -0.1 diets, compared to mice which received the two higher levels of PN (Table 3.1). The duration of feeding, 5 vs 9 weeks, did not affect the level of vitamin B-6 stored in the whole liver in any group. We have previously observed an elevated excretion of xanthurenIc acid by mice fed PN-0 and -0.1 diets before and after a test dose of tryptophan indicating an altered metabolism of this amino acid due to depletion of vitamin B-6 (14).

PRIMARY T CELL-MEDIATED CYTOTOXICITY

Vitamin B-6 deficiency resulted in significantly (P<0.01) reduced primary cytotoxic response of splenic T lymphocytes (SL) to allogeneic P815 tumor cells in vitro (Figure 3.1). The reduction in the primary response of SL was dose-dependent in mice fed PN-0 and -0.1 diets. The similarity in the slopes of the curves (Figure 3.1) suggested a quantitative rather than a qualitative difference in the primary cytotoxic response of SL to vitamin B-6 deficiency. Thus, in terms of LU-30 the number of splenic lymphocytes required to induce

30% specific tumor lysis increased 43% and 83% in mice fed PN-0.1 and -0 diets, respectively, compared to mice fed the PN-1 diet (Table 3.2). A significantly ($P<0.05$) decreased relative ratio of spleen weight to body weight in mice fed PN-0 and -0.1 diets also indicated a decrease in the proliferative response to tumor cell challenge in vitamin B-6 deficient mice (data not shown).

The primary cytotoxic response of peritoneal T lymphocytes (PL) was also suppressed in mice fed PN-0 and -0.1 diets (Figure 3.2). The altered cytotoxic response of PL from mice fed PN-0 and -0.1 diets appeared to be qualitative in nature since the maximum cytotoxicity induced by PL of vitamin B-6 deficient mice was significantly lower than the maximum cytotoxicity by PL of mice fed PN-1 and -7 diets and was not improved by increasing the A/T ratio.

SECONDARY T CELL-MEDIATED CYTOTOXICITY

When P815-allogeneic sensitized mice were challenged in vivo with P815 mastocytoma cells 24 days after primary injection, the secondary cytotoxic response of SL and PL was significantly ($P<0.01$) reduced only in animals fed the PN-0 diet (Figures 3.3, 3.4). LU-30 values for SL and PL from mice fed the PN-0 diet were increased approximately 3 fold and 2 fold, respectively, compared to mice fed the PN-1 diet. There were no significant differences in cytotoxicity among the mice fed PN-7, -1, and -0.1 diets. A strong localized reactivity of PL to the injection site was clearly shown in the secondary cytotoxic responses with LU-30 values of PL 5-9 times less than the LU-30 of SL (Table 3.2).

When SL from mice which had been sensitized with P815 tumor cells 24 days previously were restimulated in vitro for 5 days, the secondary T CMC of SL from mice fed PN-0 diet was also significantly ($P<0.05$) reduced (Figure 3.5). Comparable to the in vivo secondary response, LU-30 values were increased by approximately 3 fold in SL from mice fed PN-0 diet, compared to the other dietary groups (Table 3.2). However, the cytotoxic activity generated in vitro was approximately 30 times higher than in vivo generated secondary responses regardless of vitamin B-6 status. Background cytolysis by non-restimulated spleen cells was less than 11% in all groups (Figure 3.5).

COMPLEMENT-DEPENDENT ANTIBODY MEDIATED CYTOTOXICITY

Cytotoxic antibody titers to P815 tumor cells were not affected by vitamin B-6 status. In all groups, primary anti-P815 antibody titers yielding 50% lysis showed wide variation, ranging from <1/200 to >1/6400. The absence of detectable cytotoxicity after 2-ME treatment indicated that the primary antibody response to a single injection of P815 tumor cells was derived from IgM (21).

Secondary serum titers of 30% lysis were also highly variable, ranging from 1/50 to 1/800 with no apparent relationship to vitamin B-6 deficient status. Secondary antibody titers were also consistently lower than primary titers in all groups. This may indicate that the secondary response, measured on the fifth day following a booster injection given 24 days after the initial injection of P815 tumor cells, had not reached its peak. Secondary

serum had substantial 2-ME sensitive and resistant antibodies. The 2-ME resistant antibody did not show increased lysis in the presence of facilitating anti-IgG.

MACROPHAGE PHAGOCYTOSIS IN VITRO

Macrophages from animals fed PN-0.1 and -0 diets showed a slightly increased uptake of opsonized- and nonopsonized-SRBC (Table 3.3), compared to mice fed PN-1 diet. The number of cells recovered from the peritoneal cavity was not different statistically among groups; however, macrophages recovered from mice fed PN-0 diet comprised a higher percentage of the PEC population than from other dietary groups (Table 3.3). This resulted in slightly increased phagocytosis of opsonized SRBC by macrophages recovered from animals fed PN-0 diet. In all 3 dietary groups, phagocytosis of opsonized SRBC was greater than that of unopsonized SRBC, indicating that the phagocytosis of opsonized SRBC by the macrophages is a more efficient mechanism of ingestion than phagocytosis of nonopsonized SRBC (22).

NK CELL MEDIATED CYTOTOXICITY

Vitamin B-6 deficiency (PN-0) produced no significant alteration in native or IF-induced NK CMC of splenic lymphocytes (Figure 3.6). Poly I-poly C injection raised responses about twofold in all groups (B curves in Figure 3.6) indicating an intact ability of NK cells respond to endogenous IF stimulation in vitamin B-6 deficiency.

DISCUSSION

The present studies confirmed that the immune system is compromised by severe vitamin B-6 deficiency. Changes in cell- and antibody-mediated cytotoxic activity were examined in mice after administration of vitamin B-6 deficient diet for 4-5 weeks. Vitamin B-6 deficiency produced a complex effect on cytotoxicity, which reflected the type of effector cell being measured.

The primary and secondary T cell-mediated cytotoxic responses of splenic T lymphocytes (SL) against P815 target cells were significantly ($P<0.01$) impaired under vitamin B-6 deficiency (Figures 3.1, 3.3, 3.5). Primary T CMC was dose-dependently suppressed in animals fed PN-0 and -0.1 diets, whereas secondary T CMC was depressed only in mice fed PN-0 diet. The similarity in the slopes of the primary T CMC of SL curves (Figure 3.1) indicated that the reduction in primary T cell cytolytic activity of SL appeared to be a quantitative change in vitamin B-6 inadequacy, whereas the decreased slope of secondary T CMC of SL from mice fed PN-0 diet suggested a qualitative as well as a quantitative change (Figure 3.3). Our data are consistent with a recent report by Sergeev et al. (13) of reduced proliferation and cytotoxicity of splenic lymphocytes in vitamin B-6 deficient mice.

The primary cytotoxic response of peritoneal T lymphocytes (PL) was suppressed to a similar degree in mice fed PN-0.1 and -0 diets (Figure 3.2), and the maximum level of cytolysis appeared to be

altered by vitamin B-6 deficiency. On the other hand, secondary T CMC of PL was depressed ($P<0.01$) only in mice fed PN-0 diet (Figure 3.4) and appeared to be strictly a quantitative change. The fact that in the mice fed PN-0.1 diet, secondary T CMC of SL as well as PL was equivalent to that in mice fed higher levels of vitamin B-6, indicates that the anamnestic T CMC response was intact with a suboptimal intake of vitamin B-6 and thus, is less sensitive to vitamin B-6 deficiency than the primary response.

It is of interest that the effect of vitamin B-6 deficiency on in vivo and in vitro generation of T CMC were parallel. Secondary T cells generated in vitro in MLTC did not recover the impaired cytotoxic response of T lymphocytes from vitamin B-6 deficient animals (PN-0) during a 5-day exposure to a medium which contained vitamin B-6 as PN and presumably small amounts of PLP. The failure of cells to recover the impaired function during a 5-day culture in vitro suggests that the precursors of memory cells were altered in severe vitamin B-6 inadequacy or PLP, the active form of vitamin B-6, is not present in sufficient quantity or could not be converted by lymphocytes in vitro in MLTC. Sergeev et al. (13) reported that the impaired function of cytotoxic T lymphocytes from vitamin B-6 deficient mice was restored partially by adding PLP directly to the medium in vitro.

In general, primary and secondary T CMC of SL and PL among mice fed PN-0.1 or -0 diet showed greater within-group variation, as compared to those on PN-7 or -1 diet (Figures 3.1-3.5). This may suggest that there is individual sensitivity even among inbred mice to vitamin B-6 deficiency. It is also an important finding that an

increasing level of vitamin B-6 (PN-7) above the 100% requirement did not enhance or alter immunocompetence.

The developement of cytotoxic antibody and macrophage activation for phagocytosis in alloantigen-stimulated mice as well as NK CMC were well preserved in vitamin B-6 deficiency (Table 3.3, Figure 3.6). There are no data available in the literature on the effect of vitamin B-6 deficiency on cytotoxic antitumor-antibody responses, phagocytosis by macrophages, or NK CMC. Earlier reports on vitamin B-6 deficiency in rats consistently demonstrated marked impairment of antibody response to SRBC (9, 23) as well as other antigens such as diphtheria toxoid (24, 25) and influenza virus (26). Vitamin B-6 deficient guinea pigs also showed diminished levels of circulating antibodies in response to diphtheria toxoid (27). These are not in agreement with our data showing normal cytotoxic antibody response to P815 tumor cells in vitamin B-6 deficient mice and may be due to antigen differences and/or the relative resistance of mice to vitamin B-6 deficiency, compared to other species (28).

Vitamin B-6 deficiency did not alter the phagocytic response of macrophages. Kumor and Axelrod (23) also reported that the clearance of 51-Cr-labeled SRBC from the blood, which may be a measure of phagocytosis, and accumulation in the spleen were normal in vitamin B-6 deficiency, suggesting that antigen processing was normal. Mice fed the PN-0 diet showed an increase in the proportion of macrophages present in the peritoneal cell population (Table 3.3). The significance of this increased number of macrophages and the mechanism whereby vitamin B-6 deficiency exerts this effect on macrophages are

not clear from these experiments and remain to be studied further.

Our findings which showed quantitative as well as qualitative reduction in T CMC may be explained by the following facts. The mechanism of impairment of immunologic function in vitamin B-6 deficiency may have resulted from the critical role PLP plays in the production of one-carbon units from serine, which are required for the synthesis of nucleic acids (5). Insufficient PLP supply not only leads to a reduced DNA synthesis, with a consequent drop in mRNA, but also to a decrease in protein synthesis (5, 29, 30). When lymphocytes encounter antigens under these conditions, their subsequent immunologic response to these antigens, requiring increased synthesis of protein, RNA, and DNA, would be impaired. This could produce deleterious effects on acquisition of functional competence, cellular proliferation and biosynthesis of several factors by the activated lymphocytes. Thymic epithelial (TE) cell function was also reduced in vitamin B-6 deficient rats (12). Lymphoid precursors from neonatally thymectomized donors deficient in vitamin B-6 were converted to functional T lymphocytes when exposed to normal TE cells, indicating that vitamin B-6 deficiency did not impair lymphoid precursors. However, TE cells from vitamin B-6 deficient animals were unable to effect a maturation of T cell precursors, suggesting that the defect in cellular immunocompetence following vitamin B-6 deficiency is due, at least in part, to the inability of TE cells to differentiate immature T lymphocyte precursors to functional T lymphocytes. In our study, secondary cytotoxicity was less sensitive to vitamin B-6 deficiency than the primary cytotoxicity suggesting that precursors of

memory T cells may also be resistant to vitamin B-6 deficiency. A dramatic decrease in circulating lymphocyte numbers and depression of lymphocyte proliferation measured in MLR in vitro were also reported in vitamin B-6 deficient rats (11). Thus the suppression of T CMC against P815 tumor cells in our studies may be due not only to the impaired differentiation of CTL-precursor to functional CTL, but also to reduced proliferation as well as function of CTL resulting from inhibited DNA synthesis in vitamin B-6 deficiency. The fact that vitamin B-6 deficient animals exhibited generally normal responses requiring other effector cells may indicate that T CMC is the most sensitive parameter for detecting immunosuppression in vitamin B-6 depletion.

In conclusion, our observations raise the possibility that in vitamin B-6 deficiency, functional subpopulations of lymphocytes can be differentially and selectively affected. The reduction of the primary T CMC in SL appeared to be dose-dependent, whereas the secondary T CMC of SL, both in vivo and in vitro, and of PL in vivo seems to be more resistant to vitamin B-6 deficiency, altered only in severe vitamin B-6 deficiency. In vitamin B-6 deficiency, suppression of strong immune responses by cytotoxic T lymphocytes may be compensated to a certain degree by the intact function of macrophages and NK cells, and normal production of cytotoxic antibodies. This in fact may explain the ability of vitamin B-6 deficient mice to effect the regression of MSV-induced primary tumors that we observed previously (14).

Table 3.1

Weight and vitamin B-6 (VB6) concentrations in liver of mice fed test diets for 5 and 9 weeks

Dietary group	Week 5[1]		Week 9[2]	
	Liver Weight (LWI[3])	VB6/g liver	Liver Weight (LWI[3])	VB6/g liver
	g	μg	g	μg
PN-7	1.52[4] ±0.67 (7.71)	4.26^{A}_{a} ±0.86	1.15^{AC}_{b} ±0.02 (5.42)	5.09 a ±1.23
PN-1	1.49 ±0.10 (7.80)	3.69^{AB}_{a} ±0.47	1.37^{A}_{a} ±0.04 (5.65)	4.80 ab ±0.50
PN-0.1	1.40 ±0.09 (7.54)	2.19^{BC}_{b} ±0.21	1.29^{AB}_{a} ±0.07 (5.92)	2.55 c ±0.07
PN-0	1.36 ±0.05 (7.49)	2.09^{C}_{b} ±0.24	1.11^{C}_{b} ±0.04 (5.29)	2.71 bc ±0.90

1. Livers obtained from animals used as spleen donor for primary T CMC.
2. Livers obtained from animals used as spleen donor for secondary T CMC.
3. Liver weight index = (liver weight/body weight) x 100.
4. Mean ± SE, 6-12 animals tested individually. Means with different letters are significantly different from one another (ABC, $P<0.01$; abc, $P<0.05$).

Table 3.2

LU-30 values of splenic and peritoneal T lymphocytes from mice fed different levels of vitamin B-6

	LU-30[1]			
	PN-7	PN-1	PN-0.1	PN-0
Primary T CMC				
SL	10.9	10.4	14.9	19.0
Secondary T CMC				
SL (in vivo)	28.7	32.9	33.0	97.4
SL (in vitro)	1.6	0.8	1.2	3.7
PL	5.9	5.6	6.1	10.9

1. LU-30 = number of cells required to give 30% lysis of 1×10^4 P815 tumor cells from Y = a log X + b, Y = % cytotoxicy; X = number of lymphocytes ($\times 10^4$); a, b = constants. SL, splenic lymphocytes; PL, peritoneal lymphocytes.

Table 3.3

Effect of vitamin B-6 on phagocytosis of opsonized- and nonopsonized-SRBC by P815-activated macrophages

Dietary group	Total peritoneal exudate cells	(macrophages)	51-Cr-EA / 2 x 10^6 macrophages	51-Cr-E / 2 x 10^6 macrophages
	x 10^6	%	cpm x 10^4	cpm x 10^4
PN-1	44.91 ± 3.16[2]	(51.7 a ± 2.1)	6.92 ±0.37	4.52 ±0.38
PN-0.1	39.73 ± 5.72	(52.3 ab ± 2.9)	8.99 ±0.98	7.80 ±1.13
PN-0	43.51 ± 4.30	(59.0 b ± 2.4)	7.42 ±0.37	5.32 ±0.95

1. Macrophages obtained from P815-allogeneic sensitized mice fed experimental diets for 5 weeks. EA, opsonized SRBC; E, nonopsonized SRBC.
2. Mean ± SE, 9-12 animals tested individually. Means with different letters are significantly (P<0.05) different from one another.

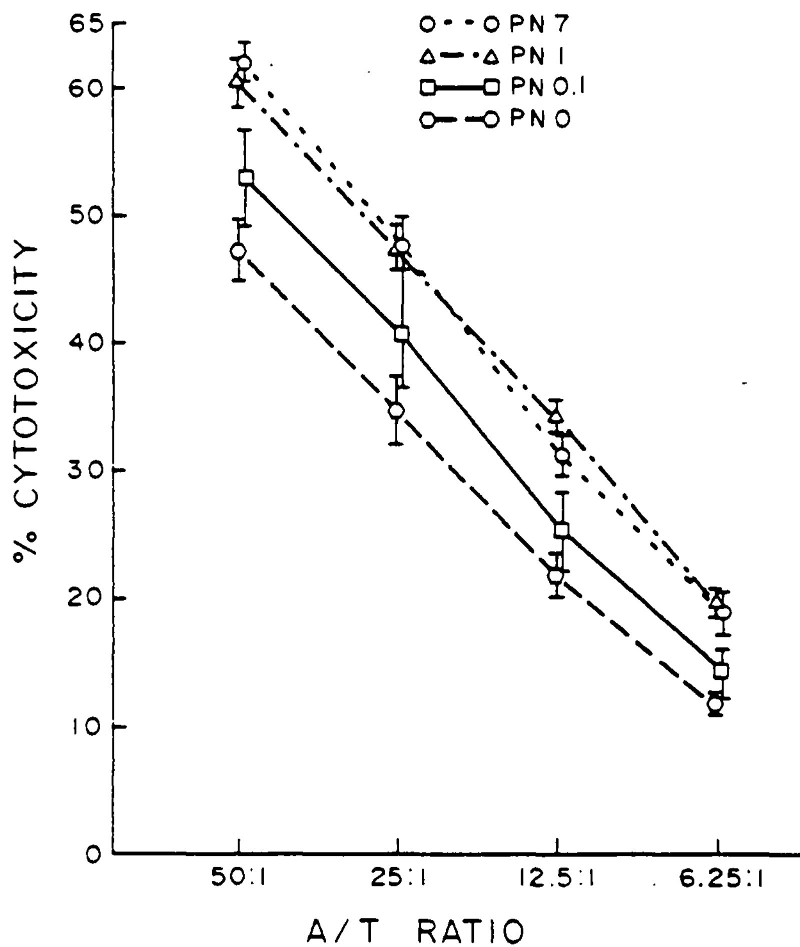

Fig. 3.1. Effect of vitamin B-6 on primary T CMC of splenic lymphocytes. Primary splenic T CMC from P815-allogeneic sensitized mice determined in a 4-hour 51-Cr-release assay using P815 cells as target. Mean of 7-12 mice tested individually.

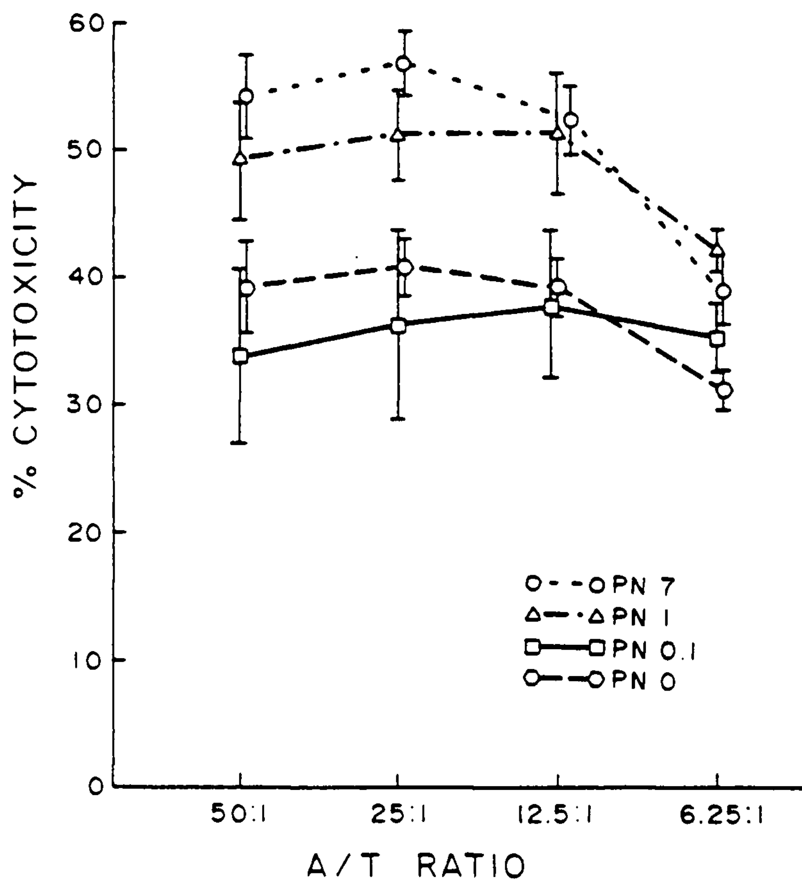

Fig. 3.2. Effect of vitamin B-6 on primary T CMC of peritoneal lymphocytes (PL). Primary T CMC of PL from P815-allogeneic sensitized mice determined in a 4-hour 51-Cr-release assay using P815 tumor cells as target. Mean of 6-12 mice tested individually.

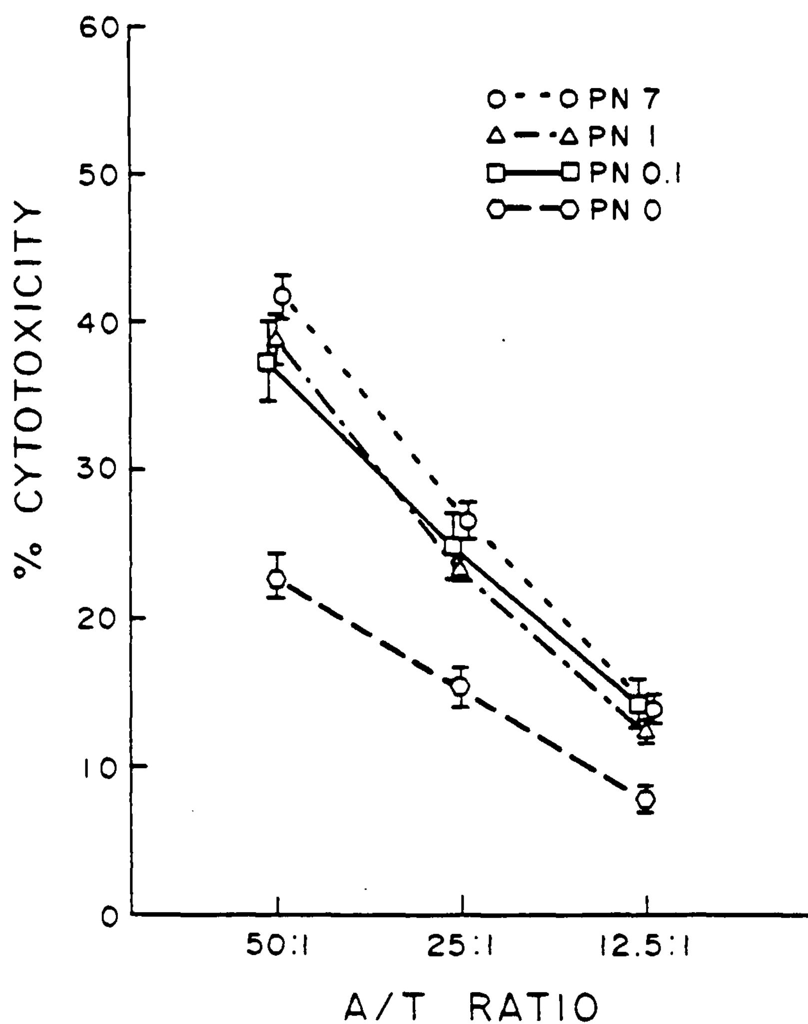

Fig. 3.3. Effect of vitamin B-6 on secondary T CMC of splenic lymphocytes. Secondary splenic T CMC from mice rechallenged with P815 tumor cells 24 days after primary injection with P815 cells determined in a 4-hour 51-Cr-release assay using P815 tumor cells as target. Mean of 11-13 animals tested individually.

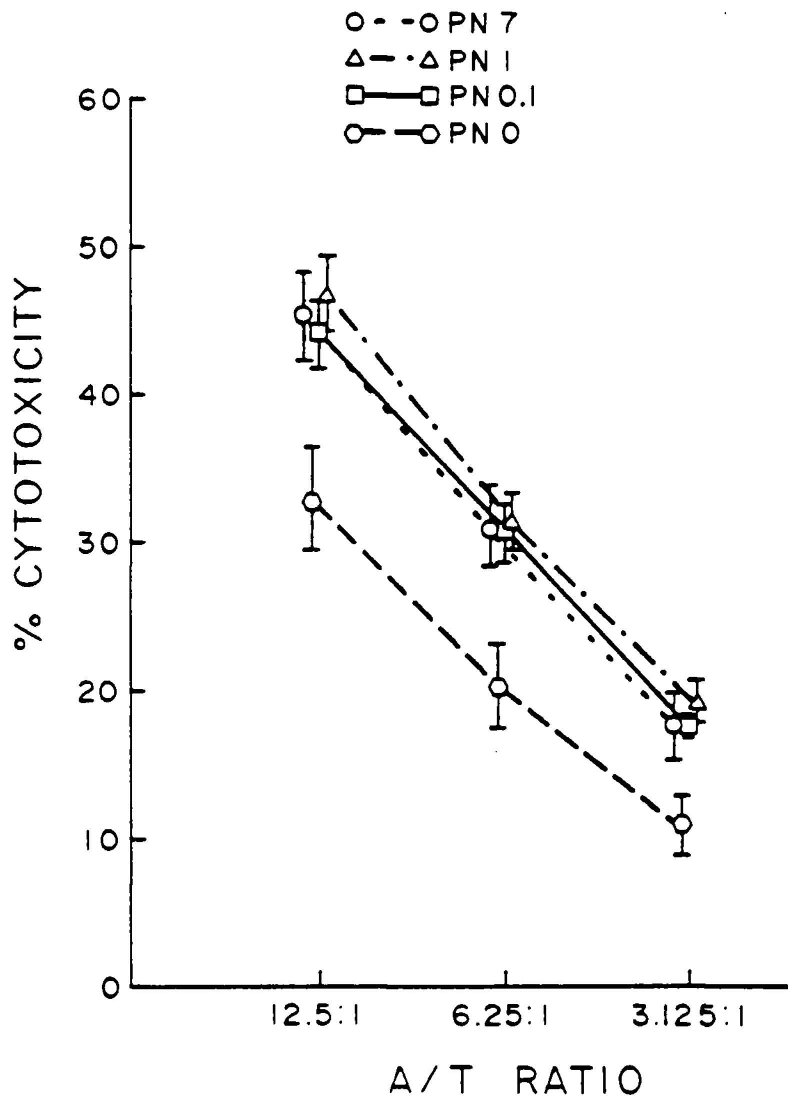

Fig. 3.4. Effect of vitamin B-6 on secondary T CMC of peritoneal lymphocytes (PL). Secondary T CMC of PL from mice rechallenged with P815 tumor cells 24 days after primary injection with P815 cells determined in a 4-hour 51-Cr-release assay using P815 cells as target. Mean of 12-13 mice tested individually.

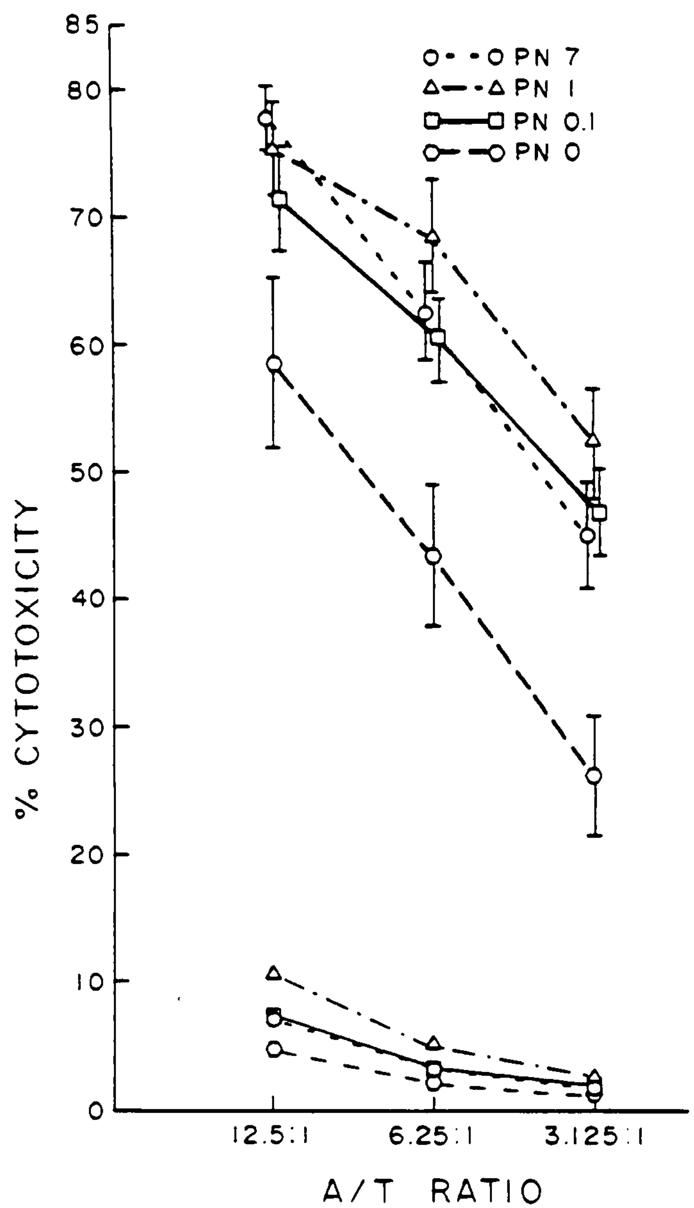

Fig. 3.5. Effect of vitamin B-6 on secondary T CMC generated in vitro in MLTC. Spleen cells from animals sensitized with P815 mastocytoma cells 24 days previously were cultured for 5 days with (solid lines) and without (dashed lines) P815 tumor cells as stimulators at R/S ratio of 10:1. CMC determined in a 4-hour 51-Cr-release using P815 tumor cells as target. Mean of 4 pools of 3 animals/pool.

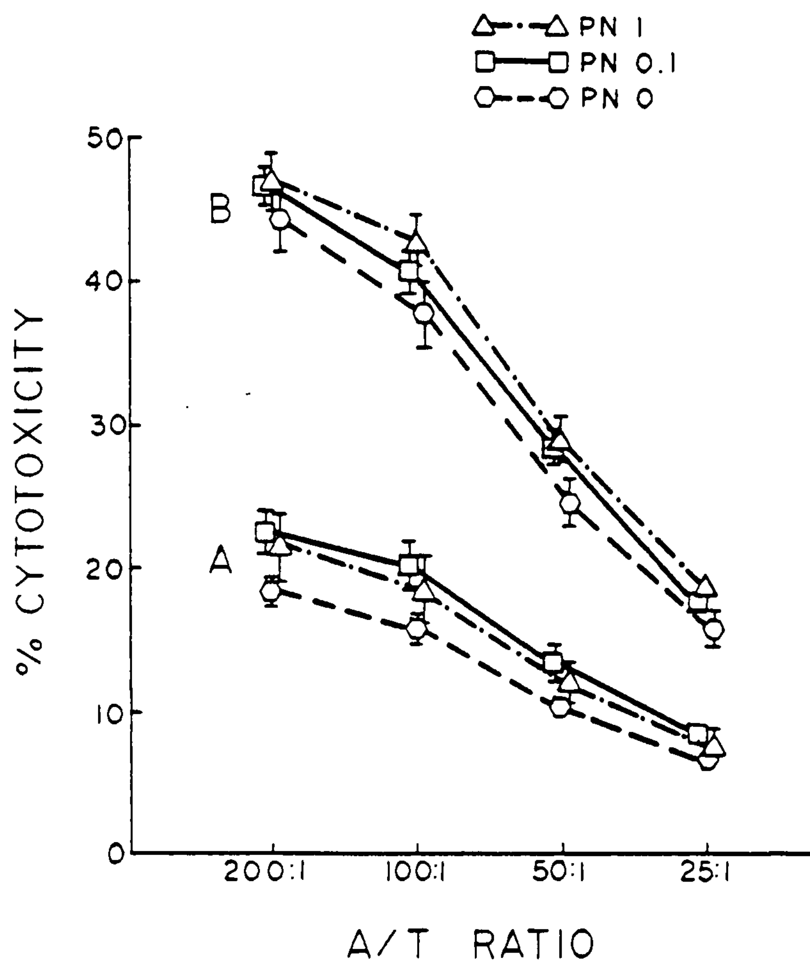

Fig. 3.6. Effect of vitamin B-6 on native (A) and IF-induced (B) NK CMC. NK CMC of spleen cells determined in a 4-hour 51-Cr-release assay using YAC tumor cells as targets. Mean of 12 animals tested individually.

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Chapter 4

CONCLUSION

According to the immune surveillance theory, tumors may arise because of a defect in the immune system. Indeed, immunological defects in cancer patients and tumor-bearing animals have been observed in a variety of tests for general immune competence. Since nutritioanl modulations may cause disturbances in the immune system, a relationship between nutritional deficiencies and susceptibility to tumor development may be anticipated.

In the study reported in the first paper, we observed that resistance of mice to MSV oncogenesis was compromised by VB6 deficiency. VB6-deficient mice fed PN-0.1 and -0 diets exhibited increased incidence of MSV/MSB-induced tumors and splenic tumor development as well as increased tumor size and regression time. This suggests an impaired immunological response. In the subsequent studies reported in the second paper, we confirmed suppression of cytotoxic T cell activity in VB6-deficient allogeneic tumor sensitized mice. Suppression of primary T cell-mediated cytotoxicity (CMC) was dose-dependent in mice fed PN-0 and -0.1 diets, whereas secondary T CMC was depressed only in mice fed PN-0 diet. The reduction in primary T CMC of splenic lymphocytes appeared to be a quantitative change in the number of cytotoxic effector cells, whereas secondary T CMC of splenic lymphocytes appeared to be a qualitative as well as a quantitative change, especially in severe VB6 deficiency. The primary

cytotoxic response of peritonel T lymphocytes was suppressed to a similar degree in VB6-deficient mice, and the maximum level of cytolysis appeared to be altered by vitamin B-6 deficiency. Secondary T CMC of peritoneal lymphocytes was also depressed only in mice fed PN-0 diet and appeared to be a quantitative change. Thus, the intact anamnestic T CMC of splenic and peritoneal lymphocytes from mice fed PN-0.1 diet suggested that memory T cell response is less sensitive to VB6 deficiency than the primary response. In contrast to the impaired T CMC, activities of the other effector cells such as NK cells and macrophages, and the production of cytotoxic antibody were generally normal, indicating functional subpopulations of lymphocytes were differentially and selectively affected in VB6 deficiency. This may also indicate T CMC is the most sensitive parameter for detecting immunosuppression in VB6 depletion. Immune responses were not enhanced or altered by the excess intake of VB6. In VB6 deficiency, suppression of strong immune response by cytotoxic T lymphocytes may be compensated to a certain degree by the intact function of macrophages and NK cells, and normal production of cytotoxic antibodies. This in fact may explain the ability of VB6-deficient mice to effect the regression of MSV-induced primary tumors. The present studies which showed increased susceptibility to MSV oncogenesis and compromised T cell cytotoxicity in VB6 deficiency provide practical information on the impaired host defense mechanism by inadequacy of VB6.

To understand the mechanism responsible for the altered T cell cytotoxicity we observed, comparisons of the functional

competence of T lymphocyte subpopulations are suggested to define if there is a defect in regulation involving helper and/or suppressor T cells or in maturation involving effector cells.

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