New therapies

Summary

Pyridoxine deficiency leads to impairment of immune responses. It appears that the basic derangement is the decreased rate of production of one-carbon units necessary for the synthesis of nucleic acids. The key factor is a pyridoxine enzyme, serine hydroxymethyltransferase. This enzyme is very low in resting lymphocytes but increases significantly under the influence of antigenic or mitogenic stimuli, thus supplying the increased demand for nucleic acid synthesis during an immune response. Serine hydroxymethyltransferase activity is depressed by deoxypyridoxine, a potent antagonist of pyridoxal phosphate, and also by known immunosuppressive or antiproliferative agents. The combination of these agents is additive.

Our results lead us to suggest the following medical applications: (a) combination of deoxypyridoxine with immunosuppressive or chemotherapeutic drugs may be effective in cases of immunosuppressive therapy or organ transplantation, (b) the development of special agents directed against the serine hydroxymethyltransferase apoprotein may prove to be a valuable medical tool, since this enzyme presents an excellent target for chemotherapy, (c) lymphocytes of individual patients could be used to design tailor-made specific immunosuppressive or chemotherapeutic treatment, and (d) the serine hydroxymethyltransferase activity of lymphocyte culture presents an excellent indicator for the evaluation of potency of immunosuppressive, chemotherapeutic or genotoxic compounds in a simple and rapid test.

Keywords: deoxypyridoxine, serine hydroxymethyltransferase, immunosuppression, pyridoxine deficiency

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Pyridoxine deficiency: new approaches in immunosuppression and chemotherapy

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Pyridoxine (vitamin B₆)

Pyridoxine (vitamin B₆) was first isolated in 1938. Subsequent studies carried out by Snell and collaborators have demonstrated that the active coenzymes of pyridoxine are pyridoxal phosphate and pyridoxamine phosphate (figure 1). These coenzymes participate together with a large number of apoenzymes, mainly in conversion reactions of amino acids such as transaminations, deaminations, decarboxylations, racemisations, dehydrations, etc, and are considered the most versatile biocatalysts.

Many drugs and poisons antagonise the pyridoxal phosphate enzymes. One of them 4-deoxypyridoxine (dB₆) (figure 1), is phosphorylated by pyridoxine kinase to form 4-deoxypyridoxal phosphate, an antagonist of pyridoxal phosphate which competes for the active site of various B₆ apoenzymes.

The pyridoxine deficiency state in experimental animals is produced by feeding them diets devoid of vitamin B₆.

EFFECT OF VITAMIN B₆ ON HUMORAL IMMUNE RESPONSES

Numerous investigators, utilising a variety of antigens and experimental animals, have reaffirmed the nutritional requirement for pyridoxine in antibody production first established in 1946 by Stoerk and Eisen.¹ In all of these studies pyridoxine deficiency was consistently accompanied by an impaired antibody response to various antigenic stimuli.^{2–9} The reduction of circulating antibodies was accompanied by a decrease of antibody-forming cells in the spleen of pyridoxine-deficient animals.¹⁰ The effect of pyridoxine deficiency upon the anamnestic response to diphtheria toxoid was particularly striking, being diminished to a great extent than was the primary response to this antigen (figure 2).^{3,11}

Recent studies in our laboratory have demonstrated that, in the vitamin B₆ deficiency state, there is also a considerable delay in switching from IgM class antibodies to IgG class antibodies.

Figure 1 Structural formulae of pyridoxine (vitamin B₆), deoxypyridine (dB₆), pyridoxal phosphate and pyridoxamine phosphate

Figure 2 The effect of pyridoxine deficiency on primary and secondary responses to diphtheria toxin. The time of antigen injections is indicated by the unlabelled arrows

Figure 3 Survival of grafts in CBA/J mice treated with C3H/HeJ cells while in a state of pyridoxine deficiency and grafted with C3H/HeJ skin (group B₁ of table 1)

Vitamin B₆ deficiency leads to:

- low antibody response to various antigens
- impairment of delayed hypersensitivity
- prolonged survival of skin homografts
- facilitation of immune tolerance induction

Box

DELAYED HYPERSENSITIVITY AND VITAMIN B₆ DEFICIENCY

Delayed hypersensitivity is also affected by lack of vitamin B₆. Pyridoxine-deficient guinea pigs inoculated with *Mycobacterium tuberculosis* BCG, exhibited depressed delayed-hypersensitivity skin reactions to purified protein derivative.^{11,12} Deoxypyridoxine treatment of BCG-immunised animals sensitive to purified protein derivative also depressed previously manifested skin reactivity to the allergen.

VITAMIN B₆ DEFICIENCY AND HOMOGRAFT REJECTION

It is generally agreed that the rejection of an homologous transplant is due to a cellular immune response of the recipient to antigens of donor tissue. A successful transplant can be established if the host immune response is blocked or suppressed. Immunosuppression is present in the vitamin B₆ deficiency state and, as a consequence, a high proportion of successful homotransplants in rats of certain strains has been achieved.^{11,13,14}

VITAMIN B₆ DEFICIENCY AND THE INDUCTION OF IMMUNE TOLERANCE

Induction of immune tolerance to tissue homografts can be achieved in adult mice through parabiotic union or by administration of appropriate viable splenic cells or cellular extracts. Induction of such tolerance to skin homografts^{11,15} and isografts^{11,16,17} has even been achieved by an otherwise ineffective dose of splenic cells derived from the skin of donor animals when the recipient animals were in a vitamin B₆ deficiency state, ie, vitamin B₆ deficiency facilitates the induction of immune tolerance. This facilitation is shown in table 1. All groups of animals received the same dose of splenic cells, which did not induce tolerance in control animals (group A₁); the same dose was rendered effective when administered to vitamin B₆-deficient recipients (group B₁, figure 3). Normal animals (ie, without B₆ deficiency and not injected with splenic cells, group A₂) did not accept any grafts, as one might have expected. Also, the mere existence of B₆ deficiency (group B₂) cannot induce tolerance and therefore the grafts were also rejected in these animals. Finally, the specificity of the process of immune tolerance is displayed by the vitamin B₆-deficient animals which received splenic cells from a different strain of mice to that providing the grafts (group B₃); in this case immune tolerance was not induced.

MODE OF ACTION OF VITAMIN B₆ IN IMMUNE RESPONSES

To explain the effects of pyridoxine deficiency on immune responses (box), Axelrod and Trakatellis postulated that the vitamin B₆-dependent enzyme serine hydroxymethyltransferase (L-serine:tetrahydrofolate-5,10-serine-hydroxymethyltransferase, SHMT) plays a key role in the phenomena observed.¹¹ This enzyme is extremely important in the production of one-carbon units used in the synthesis of nucleotides, as the C₂ and C₈ of the purine ring (donor = formyltetrahydrofolate) and the methyl group of deoxythymidylate (donor = N⁵, N¹⁰-methylene-tetrahydrofolate) are derived from these one-carbon units. Advancing their hypothesis, the authors demonstrated that vitamin B₆-deficient animals exhibited a decreased rate of production of one-carbon units and a decreased capability to synthesize nucleic acids and proteins.¹⁸⁻²⁰ These investigations demonstrated that lack of vitamin B₆ or blockade of SHMT with deoxypyridoxine, leads to a severely decreased production of one-carbon units, affecting DNA synthesis, especially in rapidly proliferating cells, and mRNA synthesis, especially for non-constitutive proteins coded by mRNAs with fast turnovers. This is precisely the case when an immune response is initiated. Therefore, according to these authors, this metabolic derangement caused by vitamin B₆ deficiency appears to constitute the underlying basic mechanism responsible for the impairment of humoral and cellular responses in this deficiency state (figure 4).

Table 1 Production of tolerance in CBA/J adult mice to skin homografts of C3H/HeJ mice

Subgroup	Type of recipient	Source of splenic cells	Number of mice grafted	Number of tolerant mice
A ₁	control	C3H/HeJ	17	0
A ₂	control	none	21	0
B ₂	intervening B ₆ deficiency	none	22	0
B ₃	intervening B ₆ deficiency	A/HeJ	6	0
B ₁	intervening B ₆ deficiency	C3H/HeJ	33	19

Figure 4 Mechanism of action of vitamin B₆ deficiency, acting via SHMT, on humoral and cellular immune response

Studies in human lymphocyte cultures

The *in vitro* responses of human lymphocytes to certain mitogenic factors in the presence or absence of dB₆, a potent vitamin B₆ antagonist, were studied in our laboratory.^{21,22} The results indicated that DNA synthesis and subsequent lymphocyte multiplication under the influence of mitogenic factors were dramatically reduced in the presence of dB₆; this effect was fully reversible by addition of vitamin B₆. Titration studies of deoxypyridoxine showed that a direct relation existed between the concentration of deoxypyridoxine and the degree of inhibition of DNA synthesis and lymphocyte proliferation.^{21,22}

These data confirmed the previous reported findings, based on *in vivo* experiments in animals, and are in accordance with the postulated Axelrod and Trakatellis effect of pyridoxine deficiency on the production of one-carbon fragments with concomitant decrease of RNA and DNA synthesis. This production of one-carbon units, mentioned above, depends to a great extent on vitamin B₆ enzymes, especially SHMT. The effect of dB₆ can be exerted not only at the level of production of one-carbon units, but also at the level of SHMT biosynthesis.

Figure 5 Correlation of tritiated thymidine incorporation into lymphocyte DNA with SHMT activity

Figure 6 Effect of phytohaemagglutinin (PHA) and dB₆ on IL-1b production in human lymphocyte cultures measured 48 h after the initiation of incubation

SHMT LEVELS IN RESTING AND STIMULATED LYMPHOCYTES

The activity of SHMT in resting lymphocyte cultures is very low (figure 5).²² However, SHMT is induced by mitogenic stimuli and its activity increases significantly. This finding demonstrates the importance of this enzyme in cell multiplication. SHMT is an important cell regulator, functioning as a switch from a slow rate of nucleic acid synthesis to a high one. It can be postulated that, under circumstances which lead to cell multiplication, one or more signals trigger the synthesis of SHMT leading to increased production of one-carbon units. Under our experimental conditions the signal was a mitogen, such as phytohaemagglutinin or concanavalin A. This mitogenic stimulation is inhibited by dB₆ (figure 5). In other words, dB₆ is not only an inhibitor of the enzyme itself by antagonising the coenzyme but also inhibits the biosynthesis of its apoprotein.²²

We do not know the mechanism by which dB₆ inhibits SHMT induction. We can postulate, however, that synthesis of the specific mRNA coding for SHMT is restricted because of decreased production of one-carbon units. An alternative explanation is one which visualizes specific regulatory events at the transcription level of the SHMT gene. Thus, all indications show that dB₆, ie, pyridoxine deficiency, acts at two different levels: inhibition of the enzyme by coenzyme antagonism, and biosynthesis of the enzyme itself.

Influence of vitamin B₆ deficiency on interleukin production

The interpretation of data up to this point has been within a general framework relating, by inference, impaired immune or mitogenic response in the vitamin B₆ deficiency state to the decreased production of one-carbon units and consequent decrease of nucleic acid synthesis. The specificity for each kind of response is achieved by a combination of factors such as special messenger biomolecules (antigens, mitogens, interleukins) or membrane receptors. Experiments on various human lymphocyte subclasses clearly showed that the T-helper cell (T_H, T₄) is especially sensitive to vitamin B₆ deficiency and, as a result, the production of interleukin (IL)-1b (figure 6) and IL-2, as well as the IL-2 receptor are also depressed.²²

T-Cell activation is inhibited in the pyridoxine deficiency state

The immune response to an antigen proceeds with the activation of the corresponding clone of T_H lymphocytes. Since pyridoxine is required for normal nucleic acid and protein synthesis, as well as for cellular proliferation, pyridoxine deficiency would have a profound effect on T_H cell activation. Specifically, the stimulation of the culture cells by antigen or mitogen causes the production of the mRNA coding for IL-1b. This lymphokine triggers IL-2 production from T₄ lymphocytes which in turn causes IL-2 receptor synthesis. It should be noted that up to this point the synthesis of the specific mRNAs for IL-1b, IL-2, and IL-2R require minimum levels of SHMT activity and one-carbon units. The production, however, is decreased by inhibition of the reaction catalysed by SHMT. These data support the interpretation that the vitamin B₆ deficiency is responsible not only for inhibition of DNA synthesis but also for the inhibition of the synthesis of the mRNAs coding for IL-1b, IL-2 and IL-2 receptor proteins.²² On the contrary, these syntheses are increased as the induced enzyme feeds the biosynthetic processes with more one-carbon units. The interaction of IL-2 and IL-2R triggers the cells to enter the S phase. At that time the level of induced SHMT is high and therefore the greater quantities of one-carbon units are produced so that DNA synthesis may proceed uneventfully, leading to T_H cell clone expansion.²² This sequence of events is depicted in figure 7 and further illuminates the mechanisms by which pyridoxine deficiency causes significant reduction of immune responses.

SHMT, chemotherapy and immune suppression

From *in vitro* studies in our laboratory in human lymphocyte cultures, the enzyme SHMT emerged as a key element in the processes of cell proliferation and immune responses. Based on this finding, two propositions were suggested for possible future medical application. Firstly, combination of dB₆ with immunosuppressive drugs in cases of immunosuppression for therapy or organ transplantation, and secondly, as the enzyme presents an excellent target for chemotherapy, the development of special agents directed against its apoprotein may prove to be a valuable approach.

Figure 8 depicts the metabolic pathway by which the one-carbon units produced by SHMT catalysis are transferred via N-5,10-tetrahydrofolic acid to

Figure 8 Metabolic pathway for the production of deoxythymidylate using one-carbon units produced by SHMT catalysis

Figure 7 Schematic representation of the mechanism by which pyridoxine deficiency reduces the immune response

Figure 9 Effect of actinomycin on SHMT activity in human lymphocyte culture with and without dB₆

deoxyuridylate to produce deoxythymidylate, a basic building block for DNA. The cycle is completed by the reaction of dihydrofolate reductase, regenerating tetrahydrofolic acid. The cycle is operated by the enzymes thymidylate synthetase, dihydrofolate reductase and SHMT. The first two enzymes are already targets of well-known chemotherapeutic agents (5-fluorouracil, methotrexate, etc), and our results suggest that SHMT may also be an excellent chemotherapeutic target.²³⁻²⁵ Indeed, in our laboratory, measurements of SHMT activity in lymphocyte cultures after the addition of certain antiproliferative or immunosuppressive agents, namely actinomycin, cytarabine, asparaginase and cyclosporin, led to the following observations²⁴:

- antiproliferative and immunosuppressive agents cause a decrease in the mitogen-induced activity of SHMT. The higher the concentration of the antiproliferative/immunosuppressive compound, the greater the decrease of enzymatic activity (figure 9)
- when an antiproliferative/immunosuppressive agent is combined with dB₆, its effect on SHMT is considerably greater (figure 9)
- ineffective concentrations of antiproliferative/immunosuppressive agents become effective when combined with dB₆ (figure 9)
- the observed changes in SHMT activity are not, as one would expect, the same in the case of all four drugs. So, when concentrations are ineffective, the combination with dB₆ gives varying results, ranging from a 10% reduction in the case of asparaginase, to 26% in the case of actinomycin, and 32% in the case of cytarabine and cyclosporin
- the combination makes it possible to use much smaller doses of these agents with much better results, at least as far as the decrease of SHMT activity is concerned.²⁴

The development of a simple screening test based on SHMT

The above observations are very important for two practical reasons.²⁵ Firstly, they show that combinations of known antiproliferative and immunosuppressive agents with dB₆ can be extremely effective with respect to the effect of these compounds on SHMT activity. These data are very promising for clinical use of antiproliferative agents in cancer chemotherapy and immunosuppressive agents in transplantation, because combining these drugs with dB₆ will make possible the use of smaller doses over a longer period of time with greater effectiveness.

The second practical application which derives from these results is the development of a simple test for rapidly assessing the antiproliferative or

Table 2 The SHMT-test: relative effectiveness of antiproliferative and immunosuppressive agents and effect of combination with dB₆

Compound	Relative effectiveness	dB ₆ multiplication effect
Actinomycin	1000	2.5–30
Asparaginase	300	1.3–10
Cyclosporin	280	1.2–25
Cytarabine	1.4	1.3–30

immunosuppressive or genotoxic potency of various compounds and their combinations, with or without dB₆ (table 2). This test would consist of measuring the SHMT activity in mitogen-stimulated human lymphocyte cultures after the addition of various compounds with, potentially, antiproliferative or immunosuppressive activity.

This test, being quite easy, fast and relatively cheap, would provide the means to screen and assess new substances for their ability to stop lymphocyte proliferation and, hence, act as immunosuppressives or chemotherapeutic drugs, at the same time providing preliminary data on their potency and synergism.²⁵ In addition, it could provide a patient-tailored assessment of candidate antiproliferative or immunosuppressive agents or combination of them, using the individual's lymphocytes *in vitro*. It could also be a valuable tool in the search for and estimation of mutagenic (genotoxic) activity of various compounds.

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