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Dietary vitamin B₆ intake modulates colonic inflammation in the IL10^{-/-} model of inflammatory bowel disease☆,☆☆

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Abstract

Pyridoxal-5-phosphate, the biologically active form of vitamin B₆, is a cofactor for over 140 biochemical reactions. Although severe vitamin B₆ deficiency is rare, mild inadequacy [plasma pyridoxal 5'-phosphate (PLP) <20 nmol/L] is observed in 19–27% of the US population. Plasma PLP concentrations are inversely related to markers of inflammation such as C-reactive protein. Furthermore, plasma PLP is diminished in those with inflammatory conditions and, in the case of inflammatory bowel disease (IBD), more so in those with active versus quiescent disease. Restricting B₆ intake attenuates IBD pathology in mice; however, the effects of supplementation are unclear. We therefore sought to determine the effects of mild inadequacy and moderate supplementation of B₆ on the severity of colonic inflammation. Weanling IL-10^{-/-} (positive for *Helicobacter hepaticus*) mice were fed diets containing 0.5 (deficient), 6.0 (replete) or 24 (supplemented) mg/kg pyridoxine HCl for 12 weeks and then assessed for histological and molecular markers of colonic inflammation. Both low and high plasma PLP were associated with a significant suppression of molecular (TNFα, IL-6, IFN-γ, COX-2 and iNOS expression) and histological markers of inflammation in the colon. PLP is required for the breakdown of sphingosine 1-phosphate (S1P), a chemotactic lipid, by S1P lyase. Colonic concentrations of S1P and PLP were significantly and inversely correlated. If confirmed, vitamin B₆ supplementation may offer an additional tool for the management of IBD. Although B₆ is required in dozens of reactions, its role in the breakdown of S1P may explain the biphasic relationship observed between PLP and inflammation.

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Keywords

Pyridoxal 5' phosphate; Shingosine 1 phosphate; Inflammation; Colitis; Colon

1. Introduction

Vitamin B₆ is a water soluble vitamin that exists in several forms including pyridoxal, pyridoxine and pyridoxamine. All three forms may be phosphorylated; however, pyridoxal 5'-phosphate (PLP) is the biologically active form, serving as a cofactor for over 140 distinct enzyme reactions that are involved in the metabolism of proteins, lipids and carbohydrates, the synthesis or metabolism of hemoglobin, neurotransmitters, nucleic acids, one carbon units, immune modulatory metabolites and others [1]. The current RDA for B₆ is 1.3 mg/day for men and 1.1 mg/day for women. Plasma concentrations of 40 nmol/L total B₆ or 30 nmol/L PLP [2] are considered to reflect adequacy, and insufficiency is variously defined as either <30- or <20-nmol/L PLP. Although severe vitamin B₆ deficiency is rare, low plasma PLP (<20 nmol/L) occurs in 10–16% of the adult US population, depending on age, sex and ethnicity [3].

Over the last decade, it has become quite apparent that a robust relationship exists between vitamin B₆ and inflammation, but the mechanistic nature of that relationship remains ill defined. Investigations by our group began with the observation that patients with rheumatoid arthritis (RA) displayed reduced plasma PLP concentrations compared to healthy controls and that, amongst RA patients, PLP was inversely related to the severity of disease and markers of inflammation [4]. Later, within the Framingham Study, we found that plasma PLP was approximately 35% lower in those with elevated C-reactive protein (CRP ≥ 6 mg/L), an acute-phase reactant, than those with normal CRP (<6 mg/L)[5]. Our analysis of the National Health and Nutrition Examination Survey dataset similarly revealed an inverse relationship between blood levels of B₆ and inflammation and, interestingly, also suggested a potential two-way relationship between the two. Overall, the likelihood of having elevated CRP decreased with increasing plasma PLP; however, those with elevated CRP also had lower plasma PLP for a specific B₆ intake than those with low CRP, that is, a higher dietary intake of vitamin B₆ is required to achieve a set plasma PLP concentration when inflammatory markers are elevated, possibly because PLP is drawn out of the plasma into sites of active inflammation [6]. Using a more comprehensive inflammatory score (consisting of CRP, fibrinogen, IL-6, TNFα, TNFR-2, osteoprotegerin, P-selectin, CD40L, ICAM-1, MCP-1, myeloperoxidase, LPL-A2 mass, LPL-A2 activity and isoprostanes indexed to creatinine.) applied to the Framingham Offspring Cohort, we confirmed that those with elevated inflammatory markers require a higher dietary B₆ intake to achieve any given plasma PLP concentration than those with low/normal inflammation [7].

Similar to earlier observations in RA, Saibeni *et al.* [8] report that plasma PLP concentrations were significantly lower in patients with inflammatory bowel disease (IBD) than controls and that the prevalence of low PLP (<20 nmol/L) was significantly higher amongst patients with active compared to quiescent disease (26.9%, vs. 2.9%; *P* .001).

In addition to data showing that inflammation can deplete plasma vitamin B₆, Benight *et al.* report that B₆ inadequacy can attenuate inflammation [9]. Using the dextran sodium sulfate (DSS) model of acute and severe colitis in mice, they demonstrated that the consumption of a diet lacking vitamin B₆ (and B₁₂) reduced the risk of mortality as well as disease activity score, weight loss and mucosal expression of iNOS, TNF- α and IL-10 compared to mice fed with adequate B₆. It remains to be determined what effect, if any, vitamin B₆ supplementation has on disease severity in IBD patients and rodent models. Furthermore, it is not known whether these effects of short-term B₆ restriction in an acute colitis model can be recapitulated with long-term inadequacy in chronic IBD models.

Therefore, we sought to test the effect of both vitamin B₆ inadequacy and supplementation on the severity of the inflammatory phenotype in a rodent IBD model.

2. Methods

All animal procedures were approved by the institutional review board of the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University.

IL10 knockout mice (C3Bir.129P2(B6)-Il10^{tm1Cgn}/Lt) were purchased from Jackson laboratory (Bar Harbor, ME, USA). The supplier's colony is maintained in isolators "under conditions which preserve the presence of potentially strain-unique enteric flora required for the development of IBD." Analysis of fecal samples collected from random mice within our own colony confirmed the presence of *Helicobacter hepaticus* but not *Helicobacter bilis*, *ganmani*, *rodentium*, *trogontum*, *typhlonius* or sp. (Radil, Columbia, MO, and Charles River, Wilmington, MA).

Homozygous mice were bred, and male and female weanling offspring were randomly assigned to consume one of three amino acid-defined diets containing 0.5-, 6.0- or 24-mg/kg Pyridoxine HCl (Dyets, Bethlehem, PA, USA). Diets were provided in a group pair-fed manner (ensuring that all three groups received equal amounts of chow), but water was freely available. Mice were housed on a 12-h light-dark cycle. After 12 weeks on diet (age=15 weeks) mice were euthanized by CO₂ asphyxiation followed by cervical dislocation and exsanguination by cardiac puncture. Blood was collected into lithium heparinized tubes, spun within 2 h (800 g, 10 min) and plasma removed to a new tube for storage at -80°C.

The abdomen of mice was opened and the colon removed, rinsed through with PBS, opened longitudinally and then rinsed again with PBS then PBS with protease inhibitors (Roche, Indianapolis, IN, USA). The colon was placed luminal side face up on an ice-cold glass plate, and a 15-mm section was cut from the center of the colon and was fixed in formalin for 24 h before being embedded in paraffin, sectioned and mounted on microscope slides. The remainder of the colon was gently scraped with glass microscope slide and the liberated mucosa frozen in liquid nitrogen and stored at -80°C.

H&E-stained slides of the colonic epithelium were graded for the degree of inflammation in a blinded fashion by an expert rodent pathologist (R.B.). Tissues were graded from 0 (*healthy*) to 3 (*very inflamed*) based on the presence of infiltrating lymphocytes, absence of goblet cells and hyperproliferation of crypt cells (See Fig. 1).

Plasma and colonic PLP were determined enzymatically using tyrosine decarboxylase based on the principles described by Shin-Buehring *et al.* [10]. In this method, PLP activity in the plasma sample is determined on the basis of release of tritiated tyramine following the incubation of tyrosine decarboxylase apoenzyme with the supernatant fraction of TCA-precipitated serum samples and tritium-labeled tyrosine.

Tryptophan, kynurenine and kynurenic acid were measured in plasma using the LC/MS/MS method of Middtun *et al.* [11]. Sphingosine-1-phosphate was measured in colon scrapings by ELISA. Briefly, approximately one quarter of the colonic scraping was transferred to 300 μ L of homogenization buffer (20-mM Tris-HCl; 20% glycerol; 1-mM B-mercaptoethanol; 1-mM EDTA; 1-mM Na orthovanadate, 15-mM NaF; 1-mM PMSF; protease inhibitor cocktail; 0.5-mM deoxypyridoxine; and 40-mM B-glycerophosphate [all from Sigma, St Louis, MO, USA]) and homogenized with motorized homogenizer. The samples were then sonicated for four cycles of 30 s, returning samples to ice between cycles. The samples were spun at 10,000 g for 10 min (4°C) and sphingosine 1-phosphate (S1P) was measured in the supernatant according to manufacturer's instructions (Echelon Biosciences, Salt Lake City, UT, USA).

Colonic PLP and S1P concentrations were corrected for protein content, which was measured by the Bradford Assay (Bio-Rad, Hercules, CA, USA).

Total RNA was extracted from colonic mucosal scrapings using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and 0.5 μ g subjected to reverse transcription using Superscript II enzyme and Oligo dT primers (Invitrogen). The relative expression of a panel of genes was then studied by real-time PCR using SYBR green master mix and an ABI7300 thermocycler (Applied Biosystems, Foster City, CA, USA). Key genes involved in inflammation [TNF- α , IL-6, IFN- γ , i-NOS, Ptges2 (Cox-2), IL1- β , NF-kB, Vegf α], antiinflammation (Tgf β 1) and tryptophan metabolism [indoleamine 2,3-dioxygenase (IDO), kynureninase (KYNU)] were selected for analysis. GAPDH was used as the control gene. Primer sequences specific to each of these genes of interest were retrieved from the qPrimer database (<http://mouseprimerdepot.nci.nih.gov>) and were synthesized by Invitrogen. Relative expression, compared to the control (replete) diet, was calculated using the formula 2^{-Ct} . Data analysis was performed on delta Ct values; however, for ease of understanding, relative expression values are shown in Fig. 3B.

Statistical analyses were performed using Microsoft Excel 2007 and SYSTAT v11 (Systat Software, Chicago, IL, USA). Significance was accepted when $P < .05$. All data are reported as mean $\pm$ S.E.M.

3. Results

After 12 weeks of dietary intervention, plasma PLP concentrations were significantly different between dietary groups ($P < .0001$). Compared to mice in the control (replete) group, plasma PLP was 85% lower in the deficient mice and 30% higher in supplemented mice, respectively ($P < .05$). Similarly, colonic PLP content declined in a stepwise fashion

with decreasing dietary intake (Fig. 2A). PLP concentrations in plasma and colon were significantly correlated ($R=0.47$, $P=.009$).

The body weight of mice steadily increased over the 12-week diet intervention (Fig. 2B). For females, the weights of the three different dietary groups were superimposable. For males, however, there was a separation of growth curves that became apparent by the end of Week 3. After this time, males in the deficient group were consistently lighter than the other two groups by approximately 10%. Separation of weights was the highest from Weeks 4–7, although these differences failed to reach significance when considering each week separately ($P=.06$ – 0.1). When considering these 4 weeks together, the weights of the deficient males were significantly lower (repeated measures, $P=.045$).

A bell-shaped relationship was observed between colonic inflammatory score and dietary B₆ content ($P=.05$). When stratifying mice according to their tertile of plasma PLP, this bell-shaped relationship was strengthened: mice in both the lowest and highest tertiles of plasma PLP displayed an approximately 60% reduction in inflammatory score compared to the midtertile ($P<.05$) (Fig. 3A). Although not powered to detect an effect of gender on inflammation, there did not appear to be an appreciable effect of gender in this study [two-factor analysis of variance (ANOVA): PLP tertile, $P=.02$; gender, $P=.23$].

Consistent with the observed changes in colonic inflammatory score, bell-shaped relationships were also noted for the relative expression of five key inflammatory mediators, TNF- α , IL-6, IFN- γ , i-NOS and Cox-2, when stratified by PLP tertile (Fig. 3B). Importantly,

Ct values (NB, higher Ct=lower expression) for each of these transcripts was significantly and negatively correlated with inflammatory score (Multiple $R=-0.41$ to -0.55 , $P<.05$). The expression of IL1- β , NF-kB, TgfB1, Vegfa, IDO and Kynu did not differ between PLP tertiles ($P>.05$) (data not shown).

Because two enzymes involved in tryptophan catabolism are B₆ dependent (KYNU and kynurenine-oxoglutarate transaminase) while at least one other (IDO) is sensitive to inflammatory mediators, we measured the concentration of tryptophan and its breakdown product kynurenine in the plasma of our mice. Absolute concentrations of these metabolites did not differ between tertiles of plasma PLP ($P>.05$); however, the ratio of kynurenine to tryptophan displayed the same bell-shaped relationship with PLP seen with inflammation score and the expression of inflammatory genes (Table 1). A higher K:T may be indicative of a greater conversion of tryptophan to kynurenine by IDO [12]. Elevated K:T may also reflect a slowed metabolism of kynurenine though to kynurinic acid, anthranilic acid and 3-hydroxykynurenine; the former two by B₆-dependent enzymes. If this were the case, differences in the ratio of kynurenine to kynurinic acid would be expected; however, we did not see any differences according to plasma PLP or groups ($P>.05$, data not shown).

Similarly, two enzymes involved in the metabolism of the signaling lipid, shingosine-1-phosphate (S1P), require PLP as a cofactor (serine C-palmitoyltransferase and S1P lyase). Colonic S1P did not differ between mice according to tertiles of plasma PLP ($P=.15$); however, a significant stepwise reduction of S1P was observed with increasing tertiles of

colonic PLP (ANOVA, $P=.01$). Moreover, as shown in Fig. 4, colonic PLP and S1P concentrations were significantly inversely correlated ($R=-0.52$, $P<.01$).

Although colon cancers have been previously reported in this model (14% of mice 9 weeks of age and 65% of 10–31-week-old mice) [13], we did not observe any tumors in our study.

4. Discussion

Our data clearly indicate that both moderate vitamin B₆ supplementation and mild depletion significantly attenuate histological and molecular features of colonic inflammation in the IL10^{-/-} mouse model of IBD (Fig. 3). The results of our deficiency arm are in good agreement with those reported by Benight et al., who show that removing B₆ from diets resulted in a significant reduction in mortality and disease activity index following exposure of mice to 3% DSS [9]. Although our observations constitute a unambiguous confirmation of the antiinflammatory effect of vitamin B₆ inadequacy, more novel and much more relevant to potential therapeutic applications is the fact that we show for the first time that moderate vitamin B₆ supplementation substantially (~60%) attenuates the inflammatory phenotype of this model.

IBD is a major public health issue in the United States with a prevalence of around 25–250 per 100,000 persons for both ulcerative colitis and Crohn's disease [14]. It is estimated that 40–60% of patients will not benefit from available IBD treatments [15]. Moreover, 60–80% of CD patients and up to 30% of UC patients eventually require surgical resection to remove some or all of the diseased intestinal tissue [14]. Annually, IBD is responsible for 2.3 million physician's visits and 180,000 hospitalizations at a cost of US\$6.3 billion [16]. Taken together, these figures clearly indicate that there is a compelling need to develop novel strategies to prevent and treat this disease.

Based on our results, we suggest that modulation of vitamin B₆ status should be considered for further investigation as a potential strategy in the management of IBD. Because vitamin B₆ is required in dozens of cellular reactions and is found together with other essential vitamins in food, strategies to antagonize B₆ or induce B₆ deficiency through dietary restriction are likely not practical or compatible with overall health. For example, in our study, male mice on the B₆ deficient diet displayed a retarded weight gain over the course of our experiment compared to those fed control and supplemented diets (Fig. 2B). On the other hand, vitamin B₆ supplementation represents an attractive therapeutic candidate for IBD because the vitamin is freely available, inexpensive and does not produce toxicity at moderate levels of supplementation, even for extended periods [17]. Although vitamin B₆ is required in many cellular reactions, we suggest that its involvement in the metabolism of sphingosine and tryptophan represents plausible mechanisms by which availability of this vitamin modulates inflammation [18].

Firstly, sphingolipid metabolism was considered because the breakdown of S1P, a potent chemotactic lipid, to hexadecenal and phosphoethanolamine is catalyzed by S1P lyase, a PLP-dependent enzyme. The importance of this pathway was demonstrated when

researchers testing the safety of caramel food colorants showed that the component 2-acetyl-4-tetrahydrobutylimidazole (THI) induced lymphopenia in rats [19] by inhibiting the egress of T cells from the thymus, thus trapping them in this organ [20]. Schwab *et al.* [21] recapitulated these findings and further demonstrated that THI is an inhibitor of S1P lyase. THI inhibition of S1P lyase led to an elevation of S1P in the thymus, as did inhibition of vitamin B₆ with 4-DPD and destroyed the concentration gradient (low in thymus to high in blood) required for the egress of T cells into blood. Importantly, simultaneous B₆ supplementation prevented the S1P lyase inhibition and S1P elevation in the thymus. These findings may, at least partially, explain our observations in the B₆-deficient mice — B₆ deficiency impairs S1P lyase activity in the thymus and traps T cells in the thymus thus precluding their migration to and infiltration into the colon. Similar to the inhibition of S1P lyase, stimulation of the S1P receptor by the drug FTY720 is also protective in IL10^{-/-} [22] and DSS [23] models of IBD by trapping T cells in secondary lymphoid organs and precluding the infiltration of CD4⁺ cells into the inflamed colonic mucosa. Pertinent to our supplemented arm, Snider *et al.* [24] showed that mice lacking shingosine kinase 1 (SK1), the enzyme that synthesizes S1P, are relatively protected against DSS-induced weight loss, colon shortening, colon histological damage and splenomegaly compared to wild-type mice. We explored the possibility that high B₆ might promote clearance of S1P from the colon and observed a highly significant inverse correlation between the two (Fig. 4). Thus, high B₆ may be achieving the same result as SK1 inhibition, reducing colonic S1P, except by maximizing S1P clearance through S1P lyase rather than impairing its synthesis.

A second pathway of potential relevance to our observations is that of tryptophan catabolism. In this pathway, tryptophan is converted to kynurenine via IDO. IDO is induced by a number of proinflammatory molecules including lipopolysaccharide, CD40 ligands and interferon gamma (IFN γ) [12,25–29]. Moreover, pharmacological inhibition of IDO promotes colitis [30] while induction of IDO expression with immunostimulatory DNA attenuates colitis in trinitrobenzene sulfonic acid-exposed mice. The product of IDO, kynurenine, is degraded by three enzymes, two of which are PLP dependent; KYNU and kynurenine-oxoglutarate transaminase. We measured tryptophan metabolites in the blood of our IL10^{-/-} mice but did not observe any differences in the absolute concentrations of tryptophan, kynurenine or kynurenic acid between dietary groups (Table 1). When considering the ratios of each of these metabolites as a proxy for enzyme activity, albeit a poor one, we observed a bell-shaped association between vitamin B₆ intake and kynurenine: tryptophan (K/T) ratio. Because a higher K/T is explained by greater conversion of tryptophan to kynurenine, the reaction catalyzed by IDO, these observations are consistent with previous studies showing that IDO is induced by IFN γ [27]. In contrast, the ratio of kynurenic acid:kynurenine (the reaction catalyzed by B₆-dependent KYNU) was not affected by blood PLP ($P>.05$). These observations suggest to us that altered tryptophan metabolism in our mice is likely an effect of, rather than a modulator of inflammation, and is associated with PLP because of the relationship between PLP and inflammation.

In conclusion, we have demonstrated that histological and molecular features of colonic inflammation are significantly attenuated by both mild B₆ inadequacy and moderate B₆ supplementation in the IL10^{-/-} mouse model of IBD. The findings of our deficiency arm

parallel those of our colleagues [9], but to the best of our knowledge, this is the first report of the suppression of IBD with supplemental B₆. That colonic inflammation may be suppressed by modulating B₆ intake raises the possibility that this vitamin may be used in the therapy of IBD, a notion we are currently pursuing. A thorough understanding of the mechanisms involved is crucial here, and our preliminary analyses, coupled with the work of others [19–21], suggest that modulation of S1P concentrations in a tissue-specific fashion may be the foundation of this biphasic relationship.

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Abbreviations

PLP	Pyridoxal 5'-phosphate
S1P	Sphingosine 1-phosphate
IBD	Inflammatory bowel disease

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Fig. 1. Scoring of colonic inflammation in IL10^{-/-} mice. Photomicrographs of H&E-stained cross-sections of colons taken from IL10^{-/-} mice. Tissues were graded on a scale of 0–3 for degree of inflammation with 0 being normal healthy colon and 3 being highly inflamed colon or frank colitis. Above are representative images of tissues scored 0, 1, 2 and 3. Scoring is based on infiltration of immune cells, disappearance of goblet cells and hyperproliferation of crypt cells. Photographs are taken under magnification $\times 400$.

Fig. 2.

Impact of dietary vitamin B₆ intake on plasma and colonic PLP and body weight in IL10^{-/-} mice. Weanling IL10^{-/-} mice were euthanized ($n=9$) or randomly assigned to be fed a diet deficient, replete or supplemented with vitamin B₆ (pyridoxal Hydrochloride) for 12 weeks (10–11/gp). (A) Pyridoxal-5-phosphate concentration in plasma (columns) and colon mucosal scrapings (line). ANOVA $P<.0001$ (plasma) and $P=.002$ (colon). Different letters indicate significant differences by Tukey's posttest ($P<.05$). (B) Body weight of mice, according to diet and sex.

Fig. 3.

Impact of plasma PLP on colonic inflammatory score and gene expression in $IL0^{-/-}$ mice. Weanling $IL10^{-/-}$ mice were euthanized ($n=9$) or randomly assigned to be fed a diet deficient, replete or supplemented with vitamin B₆ (pyridoxal Hydrochloride) for 12 weeks (10–11/gp). (A) Colonic inflammation was graded from 0 (*healthy*) to 3 (*very inflamed*) based on the presence of infiltrating lymphocytes, absence of goblet cells and hyperproliferation of crypt cells; (B) Relative expression of key inflammatory genes in colonic mucosa (ANOVA P).

Fig. 4.

Pyridoxal phosphate and sphingosine-1-phosphate concentrations are significantly correlated in the colonic mucosa. Concentrations of S1P and PLP were measured in the colon of IL10^{-/-} mice fed differing amounts of vitamin B6. A significant inverse relationship was observed between these two variables ($R=0.52$, $P=.003$, $N=29$, 18 male, 11 female).

Table 1

Impact of dietary vitamin B6 intake on plasma concentrations of tryptophan and its metabolites

	DEF	CTRL	SUPP	ANOVA <i>P</i>
Tryptophan (nmol/L)	70681±6865	61301±6323	64991±3903	.53
Kynurenine (nmol/L)	2649±168	3335±386	3010±372	.18
Kynurenic acid (nmol/L)	112±24	115±15	129±21	.81
Kynurenine/Tryptophan	4.1±0.5a	6.9±1.2	4.3±0.5b	.04
Kynurenine/Kynurinic acid	4.6±1.3	3.8±0.5	4.3±0.4	.78

CTRL, control (6.0-mg/kg diet); DEF, deficient (0.5 mg/kg); SUPP, supplemented (24-mg/kg diet).

^a*P*=.06 vs. CTRL.^b*P*=.07 vs. CTRL.