Original Article

Vitamin B6 Modifies the Immune Cross-Talk between Mononuclear and Colon Carcinoma Cells

(vitamin B6 / PBMC / cytokines / colon cancer cells / cross-talk / cell viability)

H. BESSLER, M. DJALDETTI

Laboratory for Immunology and Hematology Research, Rabin Medical Center, Hasharon Hospital, Petah Tiqva, and the Sackler School of Medicine, Tel Aviv University, Ramat Aviv, Israel

Abstract. The role of vitamin B6 as a key component in a number of biological events has been well established. Based on the relationship between chronic inflammation and carcinogenesis on the one hand, and the interaction between immune and cancer cells expressed by modulated cytokine production on the other hand, the aim of the present work was to examine the possibility that vitamin B6 affects cancer development by an interference in the cross-talk between human peripheral blood mononuclear cells (PBMC) and those from two colon carcinoma cell lines. Both non-stimulated PBMC and mononuclear cells induced for cytokine production by HT-29 and RKO cells from human colon carcinoma lines were incubated without and with 4, 20 and 100 μg/ml of pyridoxal hydrochloride (vitamin B6) and secretion of TNF-α, IL-1β, IL-6, IFN-γ, IL-10, and IL-1ra was examined. Vit B6 caused a dose-dependent decrease in production of all cytokines examined, except for that of IL-1ra. The results indicate that vitamin B6 exerts an immunomodulatory effect on human PBMC. The finding that production of inflammatory cytokines is more pronounced when PBMC are in contact with malignant cells and markedly inhibited by the vitamin suggests an additional way by which vitamin B6 may exert its carcinopreventive effect.

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Corresponding author: Hanna Bessler, Laboratory for Immunology and Hematology Research, Rabin Medical Center, Hasharon Hospital, 7 Keren Kayemet St, Petah Tiqva, Israel. Phone: (+972) 3-9372480; Fax: (+972) 3-9372388; e-mail: hannab@clalit.org.il

Abbreviations: CM – complete medium, FCS – foetal calf serum, HT-29 and RKO – human colon cancer cell lines, IFN-γ – interferon γ, IL – interleukin, MEM – modified Eagle's medium, PBMC – peripheral blood mononuclear cells, PBS – phosphate-buffered saline, PLP – plasma pyridoxal 5'-phosphate, TNF-α – tumour necrosis factor α, vit B6 – vitamin B6, XTT kit – cell proliferation assay kit.

Introduction

Pyridoxal 5'-phosphate, the biologically active form of vitamin B6 (vit B6), is a coenzyme that takes an essential part in one-carbon metabolism and in a number of enzymatic activities associated with synthesis and degradation of amino acids, biogenic amines, lipids and carbohydrates, properties thoroughly reviewed by Wu and Lu (2012) and Cellini et al. (2014). Vit B6 is actively integrated with the function of the nervous, immune and endocrine systems and it is not unexpected that its deficiency may be associated with a number of pathological conditions, including epileptic encephalopathy, cardiovascular diseases, rheumatoid arthritis, diabetes, as well as a rather long list of rare inherited diseases (Sakakeeny et al., 2012; Ahmad et al., 2013; Galluzzi et al., 2013; Cellini et al., 2014). A substantial number of studies have shown that vitamins in general, and B6 in particular, are closely connected with prevention and even amelioration of infections. Utilizing the C-reactive protein level and applying precise measurements of pyridoxal-5'-phosphate, Moris et al. (2010) have shown that higher vit B6 intake exerts a marked protective effect against infections. Similar results were obtained by Sakakeeny et al. (2012) in humans and by Selhub et al. (2013), who have reported that in mice with inflammatory bowel disease, vit B6 restriction has been associated with increased severity of their disease. According to the authors, B6 supplementation may alleviate the inflammatory process in the colon, observations that have been supported by other studies (Morris et al., 2010; Benight et al., 2011).

Since the link between chronic bowel inflammation and colon cancer seems to be well established, studies have been conducted to elucidate the role of vit B6 in reducing the risk of cancer. In a meta-analysis of prospective studies, Larsson et al. (2010) have shown that for every 100-pmol/ml increase in the blood pyridoxal 5'-phosphate level, the risk of colorectal cancer decreased by 49 %. Komatsu et al. (2003) reported that mice fed with different amounts of pyridoxine showed a dose-dependent decrease in the appearance and number of colon tumours with an increase of vit B6 intake.

Previous studies from our laboratory have shown that peripheral blood mononuclear cells (PBMC), by their ability to produce inflammatory cytokines, may affect development and proliferation of malignant cells from colon carcinoma lines and that this capacity proceeds most probably by interfering with the cross-talk between these two types of cells (Bessler and Djaldetti, 2010). Considering the link between chronic inflammation and carcinogenesis, the question is posed whether vit B6 may modulate tumour development by affecting the cross-talk between immune and cancer cells. The present study was designed to examine the effect of vit B6 on inflammatory cytokine production by human PBMC and the possibility that modulation of the cross-talk between immune and colon cancer cells may reveal the mechanism by which vit B6 could interfere with carcinogenesis.

Material and Methods

Ethics statement

The protocol of this study was approved by the ethics committee of the Rabin Medical Center. Informed consent was signed by the blood donors.

Cell preparation

PBMC were separated from venous blood obtained from 12 adult blood donors by Lymphoprep-1077 (Axis-Shield PoC AS, Oslo, Norway) gradient centrifugation. The cells were washed twice in phosphate-buffered saline (PBS) and suspended in RPMI-1640 medium (Biological Industries, Beit Haemek, Israel) containing 1% penicillin, streptomycin and nystatin, 10% foetal calf serum (FCS, Biological Industries), and was designated as complete medium (CM).

Colon cancer cell lines

HT-29 and RKO human colon cancer cell lines were obtained from the American Type Cultural Collection, Rockville, MD. The cells were maintained in CM containing Mc-COY'S 5A medium (Sigma, Rehovot, Israel) and modified Eagle's medium (MEM, Biological Industries) respectively, supplemented with 10% FCS, 2 mM L-glutamine and antibiotics (penicillin, streptomycin and nystatin, Biological Industries). The cells were grown in T-75 culture flasks at 37 °C in a humidified atmosphere containing 5% CO_2.

Vitamin B_6 preparation

A stock solution of 10 mg/ml pyridoxal hydrochloride (vitamin B6, Sigma) was freshly prepared in RPMI-1640 medium and pH was adjusted to 7.0 using 1 N NaOH. Further dilutions were made in RPMI CM. The final concentrations of B6 used were between 1 and 100 µg/ml (equal to 5 µM and 0.5 mM, respectively).

Effect of vit B6 on cytokine production

The amount of 0.5 ml PBMC (4×10^6/ml of CM) was incubated with an equal volume of CM or one type of the colon cancer cells (4×10^5/ml of CM) suspended in appropriate CM layered in each of 24-well plates. B6 was added at the onset of cultures at concentrations as described. Control cultures contained CM. The cultures were maintained for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2. At the end of the incubation period, the cells were removed by centrifugation at 700 *g* for 10 min, the supernatants were collected and kept at –70 °C until assayed for the cytokine content.

Cytokine content in the supernatants

The concentration of the following cytokines: TNF-α, IL-1β, IL-6, IFN-γ, IL-10, and IL-1ra in the supernatants was tested using ELISA kits specific for these cytokines (Biosource International, Camarillo, CA), as detailed in the guideline provided by the manufacturer. The detection levels of these kits were: 15 pg/ml for IL-6 and 30 pg/ml for the remaining cytokines.

Effect of vit B6 on cell viability

Aliquots of 0.1 ml HT-29 or RKO cell suspension 2×10^5/ml of appropriate CM were added to each one of 96-well plates and incubated for 24 h in the absence or presence of vit B6 at concentrations as indicated. At the end of the incubation period, the cells were stained for viability using XTT proliferation assay kit (Biological Industries), according to the manufacturer's instructions. The plates were incubated in a CO_2 incubator at 37 °C for 2–4 h, and the absorbance was measured at 450 nm using an ELISA reader.

Statistics

Data was analysed using analysis of variance (ANOVA) with repeated measures for each cytokine, and paired *t*-test to compare the difference between the level of cytokine produced with various concentrations of vit B6 and that found in control cultures (incubated without the vitamin). Probability values of $P < 0.05$ were considered as significant. The results are expressed as mean ± SEM.

Results

Effect of vit B6 on cell viability

Twenty-four hours of incubation of either HT-29 or RKO cells with increased concentrations of vit B6 between 1–100 µg/ml had no significant effect on the cell viability measured by XTT assay ($F_{5,40} = 0.135$, $P = 0.98$, for both cell lines, Table 1).

Effect of vit B6 on TNF-α production

Dose-dependent inhibition of TNF-α secretion by both non-stimulated and HT-29- or RKO-stimulated PBMC was found at vit B6 concentrations between 4 and 100 µg/ml ($F_{3,47} = 2.74$, $P = 0.055$; $F_{3,47} = 4.015$, $P =$

Table 1. Effect of vit B6 on colon cancer cell viability

	XTT cell proliferation assay Absorbance at 450 nm	
Vit B6 concentration	**HT-29 cells**	**RKO cells**
0	1625 ± 94	1722 ± 150
1 µg/ml	1647 ± 120	1688 ± 170
4 µg/ml	1621 ± 244	1881 ± 265
10 µg/ml	1565 ± 157	1688 ± 236
20 µg/ml	1680 ± 232	1816 ± 326
100 µg/ml	1641 ± 152	1690 ± 230

HT-29 or RKO cells were incubated for 24 h with vit B6 at doses as indicated. At the end of the incubation, a mixture of freshly prepared XTT reagent with XTT activator was added for additional 2–4 h of incubation and O.D absorbance was estimated at 450 nm. The results are expressed as mean ± SEM of eight experiments.

0.013; $F_{3,47}$ = 3.572, P = 0.02, respectively). At vit B6 concentrations of 4, 20 and 100 µg/ml, production of TNF-α by non-stimulated cells was inhibited by 16 %, 38 % and 63 %, respectively (P = 0.28, P = 0.03 and P = 0.015, respectively), whereas at the same doses of the drug, secretion of TNF-α by HT-29-stimulated PBMC was reduced by 2 %, 21 % and 53 %, respectively, (P = 0.62, P = 0.001 and P = 0.0002, respectively) and that by RKO-stimulated PBMC by 9 %, 24 % and 47 %, respectively (P = 0.007, P = 0.004 and P < 0.0001, respectively, Table 2).

Effect of vit B6 on IL-1β production

IL-1β secretion by non-stimulated or HT-29-stimulated PBMC was dose-dependently reduced when vit B6 was added at concentrations between 4 and 100 µg/ml ($F_{3,47}$ = 2.48, P = 0.07, $F_{3,4\,7}$ = 4.27, P = 0.0098, respectively), whereas that induced by RKO cells was not affected significantly ($F_{3,47}$ = 1.62, P = 0.198), although at 100 µg/ml of vit B6, secretion of IL-1β was reduced by 40 % (P = 0.0021). With 4, 20 and 100 µg/ml concentrations of vit B6, spontaneous secretion of IL-1β was lowered by 21 %, 32 % and 55 %, respectively (P < 0.05) and that by HT-29 stimulated cells by 13 % (P = 0.08), 24 % (P = 0.01) and 49 % (P = 0.0001), respectively (Table 2).

Effect of vit B6 on IL-6 secretion

Production of IL-6 by non-stimulated PBMC was not significantly affected by incubation with vit B6 ($F_{3,47}$ = 0.72, P = 0.54). However, when the vitamin was added to both HT-29 or RKO-induced cells, a dose-dependent inhibition of IL-6 secretion was observed ($F_{3,47}$ = 3.03, P = 0.039, or $F_{3,47}$ = 3.7, P = 0.017, respectively). At vit B6 concentrations of 4, 20 and 100 µg/ml, secretion of IL-6 by HT-29 stimulated cells was reduced by 14 %, 20 % and 30 %, respectively (P < 0.001) and that induced by RKO was lowered by 10 % (P = 0.1), 24.5 % (P = 0.015) and 48.5 % (P = 0.001), respectively (Table 2).

Effect of vit B6 on IFN-γ secretion

Secretion of IFN-γ by non-stimulated PBMC was not affected at vit B6 concentrations between 4 and 100 µg/ml ($F_{3,47}$ = 0.32, P = 0.8). At the same concentrations, vit B6 caused dose-dependent inhibition of IFN-γ production by HT-29-stimulated or RKO-stimulated PBMC ($F_{3,47}$ = 2.77, P = 0.052; $F_{3,47}$ = 4.01, P = 0.013, respectively). Vit B6 added to HT-29 or RKO-stimulated PBMC at 4, 20 and 100 µg/ml inhibited secretion of IFN-γ by 39 %, 40 % and 61 %, respectively (P < 0.05)

Table 2. Effect of vit B6 on pro-inflammatory cytokine secretion

Vit B6	0	4 µg/ml	20 µg/ml	100 µg/ml
TNF-α, pg/ml				
Non-stimulated	398 ± 89	335 ± 75	247 ± 54*	148 ± 27*
HT-29-induced	1064 ± 145	1044 ± 151	843 ± 135***	505 ± 70***
RKO-induced	1701 ± 165	1555 ± 208**	1287 ± 208**	905 ± 205***
IL-1β, ng/ml				
Non-stimulated	0.81 ± 0.16	0.64 ± 0.12*	0.55 ± 0.10*	0.37 ± 0.05**
HT-29-induced	4.59 ± 0.49	3.97 ± 0.49	3.50 ± 0.38**	2.35 ± 0.26***
RKO-induced	4.57 ± 0.83	5.16 ± 0.97	4.47 ± 0.84	2.76 ± 0.55**
IL-6, ng/ml				
Non-stimulated	3.67 ± 0.72	3.53 ± 0.71	3.16 ± 0.71	2.35 ± 0.63*
HT-29-induced	27.5 ± 1.9	23.7 ± 1.8**	22.0 ± 2.0***	19.2 ± 2.2***
RKO-induced	30.9 ± 2.1	27.8 ± 2.5	23.3 ± 2.9**	19.0 ± 3.2***
IFN-γ, ng/ml				
Non-stimulated	0.73 ± 0.19	0.55 ± 0.15*	0.53 ± 0.14*	0.54 ± 0.14
HT-29-induced	1.70 ± 0.40	1.20 ± 0.26*	1.03 ± 0.17*	0.66 ± 0.12*
RKO-induced	3.43 ± 0.82	3.02 ± 0.66	2.37 ± 0.63	0.64 ± 0.12**

PBMC were incubated without or with colon cancer cells (HT-29 or RKO) in the absence or presence of vit B6 at concentrations as indicated. The level of cytokines in the supernatants was tested by ELISA. The results are expressed as mean ± SEM of 12 experiments. Asterisks represent a statistically significant difference from PBMC incubated without vit B6 (*P < 0.05; **P < 0.01; ***P < 0.001).

Table 3. Effect of vit B6 on anti-inflammatory cytokine secretion

Vit B6	0	4 μg/ml	20 μg/ml	100 μg/ml
IL-10, ng/ml				
Non-stimulated	0.47 ± 0.08	0.40 ± 0.07	0.35 ± 0.06**	0.22 ± 0.05***
HT-29-induced	1.15 ± 0.15	1.03 ± 0.13	0.95 ± 0.13**	0.46 ± 0.08***
RKO-induced	1.03 ± 0.13	0.83 ± 0.14**	0.76 ± 0.13***	0.48 ± 0.10***
IL-1ra, ng/ml				
Non-stimulated	0.45 ± 0.06	0.46 ± 0.06	0.42 ± 0.05	0.30 ± 0.04***
HT-29-induced	1.15 ± 0.12	1.14 ± 0.13	1.03 ± 0.1**	0.80 ± 0.11***
RKO-induced	1.09 ± 0.14	1.08 ± 0.15	0.98 ± 0.14**	0.75 ± 0.13***

PBMC were incubated without or with colon cancer cells (HT-29 or RKO) in the absence or presence of vit B6 at concentrations as indicated. The level of cytokines in the supernatants was tested by ELISA. The results are expressed as mean ± SEM of 12 experiments. Asterisks represent a statistically significant difference from PBMC incubated without vit B6 (*P < 0.05; **P < 0.01; ***P < 0.001).

or by 12 % (P = 0.2), 31 % (P = 0.073 and 81 % (P = 0.005), respectively (Table 2).

Effect of vit B6 on IL-1ra secretion

Incubation of PBMC with increased concentrations of vit B6 between 4 and 100 μg/ml had no significant effect on the production of IL-1ra by non-stimulated cells ($F_{3,47}$ = 1.27, P = 0.295), or by cells stimulated with either HT-29 ($F_{3,47}$ = 1.9, P = 0.14) or RKO cells ($F_{3,47}$ = 2.11, P = 0.11), although at 100 μg/ml of vit B6, secretion of the cytokine by non-stimulated and stimulated PBMC was reduced by 30 % (P < 0.001, Table 3).

Effect of vit B6 on IL-10 secretion

Dose-dependent inhibition of IL-10 production was found following incubation of non-stimulated PBMC or cells stimulated with either HT-29 or RKO colon cancer cells ($F_{3,47}$ = 2.65, P = 0.06; $F_{3,47}$ = 3.15, P = 0.034 and $F_{3,47}$ = 6.036, P = 0.0016, respectively). Spontaneous secretion of IL-10 was reduced by 15 % (P = 0.09), 25 % (P = 0.005) and 53 % (P = 0.0005) following incubation with 4, 20 or 100 μg/ml of vit B6, respectively. Similar amounts of the vitamin resulted in lower HT-29-induced IL-10 secretion by 10 % (P = 0.19), 17 % (P = 0.001) and 40 % (P < 0.0001), respectively, and reduction of RKO-induced production by 19 % (P = 0.0024), 26 % (P = 0.0001) and 53 % (P < 0.0001), respectively (Table 3).

Discussion

It is conceivable that the palliative effect of vit B6 on carcinogenesis proceeds by a number of intricate mechanisms, such as reducing oxidative stress and nitric oxide production, as well as inhibition of cell proliferation and angiogenesis as it has been shown in earlier publications (Komatsu et al., 2003; Matsubara et al., 2003). The vitamin augments immune responses; it is involved in the methyl donor cycle and exerts anti-apoptotic and anti-proliferation activities (Lee et al., 2009; Sujol et al., 2011; Maharath et al., 2014; Mollerreau and Ma, 2014). Studies on rats have shown that increased dietary B6 supplementation significantly reduced production of the toxic bile lithocholic acid, which is a risk factor for colon cancer, and increased the faecal mucin levels in a dose-dependent manner (Okazaki et al., 2012). Research with mice prompted to colon carcinogenesis by azoxymethane demonstrated that vit B6 down-regulates Cd8a and Ccl8 mRNA expression, genes with a critical role in colon cancer development (Toya et al., 2012). At the molecular level, it has been shown that the carcinopreventive effect of vit B6 may proceed by its enhancing activity on P53, which is an important suppressor of tumour formation, and mostly of gastro-intestinal cancers (Levine, 1989; Rokavec et al., 2014). Based on the fact that pyridoxal increases *p21* gene expression via p53 activation in an array of colon carcinoma cells from different lines, Zhang et al. (2014) suggest that this may be an additional way to explain the beneficial effect of vit B6 in preventing tumorigenesis.

Notably, vit B6, being closely related to prevention of chronic inflammation, should be able to affect inflammatory and tumour-advancing processes by its influence on inflammatory cytokine production. The level of plasma pyridoxal 5'-phosphate (PLP) was found to be inversely associated with inflammatory markers in mice with inflammatory bowel disease, such as C-reactive protein (Morris et al., 2010; Sakakeeny et al., 2012; Selhub et al., 2013). According to Selhub et al. (2013), low and high PLP levels were related to significant suppression of pro-inflammatory cytokines TNF-α, IL-6 and IFN-γ. Studies in humans showed that plasma PLP levels inversely correlate with C-reactive protein, TNF-α receptor 2, and IL-6 levels, all of them recognized as tumour markers (Lee et al., 2009). It has been reported that vit B6 was able to suppress the inflammatory serine protease inhibitor clade A member 3 (SPI-3) mRNA expression in TNF-α-stimulated HT-29 cells (Yanaka et al., 2011).

In a previous study we have shown that human colon cancer cells from HT-29 and RKO lines altered the immune balance between pro- and anti-inflammatory cytokine generation by human PBMC, indicating that malignant cells may further promote inflammatory processes and subsequent carcinogenesis (Bessler and Djaldetti, 2010). It was shown that deregulation of the inflammatory cytokine equilibrium depends on the number of

malignant cells – lower numbers of these cells caused initial high secretion of anti-inflammatory cytokines (IL-1ra and IL-10), and with their increasing number, a dose-dependent elevation of the pro-inflammatory cytokines (TNFα, Il-1β, IFNγ and IL-6) was observed. However, since vit B6 exerted a direct effect on cytokine secretion by PBMC, and the lack of an effect of the drug on malignant cell proliferation was shown by the XTT assay, the lower production of inflammatory cytokines found in the current study cannot be attributed to the reduced number of malignant cells in the cultures, but rather to an impaired immune cross-talk between PBMC and colon cancer cells. This equilibrium may be affected by a number of substances such as drugs, nutrients and spices, as reviewed by Djaldetti and Bessler (2014). The results of the present study demonstrate that although vit B6 did not directly affect the malignant cell viability, it exerted a pronounced dose-dependent inhibitory effect on all of the cytokines examined, except for IL-1ra. It should be emphasized that this effect was not related to the nature of the cell stimulator used in the study.

Conclusions

The present results show that vit B6 is actively engaged in the modulation of cytokine production by PBMC, and even more expressed when induced by both HT-29 and RKO human colon cancer cell lines. Generation of TNF-α, IL-1β, IL-6, IFN-γ and IL-10 was reduced, whereas that of IL-1ra was not affected. These findings indicate that vit B6 is capable of interfering with the cross-talk between immune and cancer cells. It is therefore conceivable that the results of the present work point to an additional way by which vit B6 may prevent tumour development and spreading.

Authors' contributions

Both authors have equally contributed to conception and design of the study. Prof. Bessler performed the laboratory investigations. Both authors were involved in analysis and interpretation of the results, drafting of the manuscript, and have agreed to send the final version of the work for publication. The authors declare that they have no financial and competing interests.

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