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Inhibition of Intracellular Cathepsin Activities and Suppression of Immune Responses Mediated by Helper T Lymphocyte Type-2 by Peroral or Intraperitoneal Administration of Vitamin B_6

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We reported that pyridoxal phosphate (PAP), a coenzyme form of vitamin B_6, strongly inhibits activities of cathepsin B and weakly inhibits those of cathepsins S, K, and C *in vitro.* Either intraperitoneal injection or peroral administration of medication doses of vitamin B_6 in the diet caused dose-dependent inhibition of hepatic cathepsins B, L, S, and C, and the inhibition was exhibited much more significantly in the case of a high protein diet than in a low protein diet. Administration of vitamin B_6 induced the suppression of immune responses against ovalbumin (OVA) mediated by helper T lymphocyte type-2, based on the suppression of antigen processing by cathepsin B inhibition, as in the case of CA-074 administration, a cathepsin B specific inhibitor. Ovalbumin-dependent production of immunoglobulins IgE, IgG_1 and interleukin IL-4 was suppressed by administration of medication doses of pyridoxal (PA) or pyridoxine (PI), while the production of IgG_{2a} and interferon (INF)-γ mediated by helper T lymphocyte type 1 was not changed. Administration of medication doses of vitamin B_6 caused the inhibition of intracellular cathepsin B activity due to suppression of the functions of helper T lymphocyte type-2.

Key Words: **cathepsin B, immune suppression, vitamin B_6.**

Katunuma *et al.* developed the epoxysuccinyl-isoleucyl-proline derivatives CA-074 and CA-030 as specific inhibitors of cathepsin B [1, 2], and these synthetic inhibitors do not inhibit cathepsins L, S, K, or C. We also reported that these cathepsin B inhibitors suppressed immune responses such as antibody productions and the rechallenged antigen dependent blastogenesis of primed splenocytes against B-type hepatitis virus vaccine and rabies virus vaccine as antigens [3]. We reported that IgE production by inoculation of leishmania to the mouse foot pad was also specifically suppressed by treatment with CA-074 [4]. Ovalbumin dependent productions of IgE and IgG_1 in the sera and also cytokine IL-4 production in the culture medium of rechallenged splenocytes were suppressed by administration of CA-074 [4]. From this evidence, lysosomal cathepsin B may participate in the antigen presentation mediated by type-2 helper T cells. Administration of cathepsin B inhibitors may induce the changing of functional differentiation of helper T lymphocyte subclasses from helper T type-2 to that of type-1 by the inhibition of antigen processing. On the other hand, special aldehyde derivatives, such as leupeptin, antipain and methyl-ethyl ketone, having affinity with −SH group of cathepsins to form thiosemiathetal bonds, have been known as specific inhibitors of cysteine proteases [5, 6]. PAP, a coenzyme form of vitamin B_6, also has an active aldehyde group in the 4th position of the pyridine ring. We reported that PAP inhibits cathepsin B strongly and L, S, K, and C weakly *in vitro* by forming semithioathetal bonds [7]. However, PLP is not incorporated directly into cells due to difficulties of cell membrane penetration. We found that either intraperitoneal injection or peroral administration of pyridoxine (PI) or pyridoxal (PA) inhibited the hepatic cathepsin activities, and as a result, various immune responses against ovalbumin as an antigen were suppressed. This paper reports that the hepatic cathepsins

Abbreviations used: E-64, *N*-(L-3-*trans*-ethoxycarbonyloxirane-2-carboxyl)-L-leucine-4-aminobutylamide; CA-074, *N*-(L-3-*trans*-propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline; [^{3}H], tritium-labeled; FCS, fetal calf serum; ELISA, enzyme-linked immunosorbent assay; Th, helper T lymphocyte; Ig, immunoglobulin.

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TABLE 1

Synthetic Diet Composition of Standard Pyridoxine and Pyridoxine Excess in 20% Casein or 70% Casein

	Standard pyridoxine		Pyridoxine excess	
Vitamin-free casein (g)	70	20	70	20
Sucrose (g)	10	10	10	10
Cornstarch (g)	7	57	7	57
Oil (g)	8	8	8	8
Mineral (g)	4	4	4	4
Vitamin mixture (g) (omitted pyridoxine)	1	1	1	1
Pyridoxine (mg)	0.58	0.58	6	6
Choline chloride (ml)	0.2	0.2	0.2	0.2

Note. Data are presented as contents in 100 g diet.

and antigen presentation were inhibited by administration of not only synthetic inhibitor CA-074 but also natural vitamin B_6.

MATERIALS AND METHODS

Assay of cathepsins. Cathepsin B activity was measured with Z-Arg-Arg-MCA and Arg-MCA for cathepsin H by Barrett's method [9], and cathepsin L activity was measured with Z-Phe-Arg-MCA in the presence of the cathepsin B specific inhibitor CA-074, by Inubushi's method [10]. For the inhibition assay of various immune responses, 10–50 μg/ml of pyridoxal derivatives in the culture medium of primed splenocytes was used and 0.5–1.0 mg/30 g body weight of pyridoxal derivatives was injected intraperitoneally.

Assay of ovalbumin-dependent productions of antibodies and cytokines. Ovalbumin-dependent production of IgG_1 and IgE was assayed with enzyme-linked immunosorbent assay (ELISA) kits (Sulfo-NHS, Pierce Co., Rockford, IL) using goat anti mouse IgG_1 (Fc)-conjugated with peroxidase, and EDC (Sigma Chemicals, St. Louis, MO) using goat anti mouse IgE (Fc)-conjugate with peroxidase, respectively. IgG_{2a} was assayed using an assay kit (Cappel Products Organon Teknica, Durham, NC) with rabbit affinity purified anti mouse IgG_{2a} (Fc)-conjugated with peroxidase. The ovalbumin dependent IL-4 production was assayed by the ELISA system BIOTRAK (Amersham Life Sciences), using anti mouse IL-4 conjugate with peroxidase. The INF-γ assay system is based on a solid phase ELISA system BIOTRAK (Amersham Life Sciences), which utilizes an antibody for INF-γ bound to the wells of a plate together with an antibody to IFN-γ conjugated with horseradish peroxidase. The IL-4 and INF-γ productions by primed splenocytes in the culture medium were assayed. The passive cutaneous anaphylaxis (PCA) reaction induced primarily by IgE was assayed according to the method of Mota *et al.* [11]. Diluted mouse anti-ovalbumin serum (0.1 ml/site) was injected intradermally to the dorsal skin of male Wistar rats. After 48 h, 1 ml of saline containing 1 mg of ovalbumin and 5 mg of Evans blue dye was injected intravenously. Thirty minutes after the antigen injection, blue spots >5 mm in diameter on the removed skin were judged as PCA reaction positive.

Preparation of excess vitamin B_6 diet with high protein. The composition of the synthetic diet of excess vitamin B_6 with high protein for BALB/c mice is shown in Table 1. The standard diet contained 0.58 mg/100 g pyridoxine in a 20% casein diet and the excess vitamin B_6 diet contained 6.0 mg/100 g of pyridoxine in a 20% casein diet. A 70% casein diet was used as the high protein diet. These dietary controlled mice were used for the experiments on the inhibition of hepatic cathepsin activities and the suppression of immune responses by vitamin B_6.

RESULTS AND DISCUSSION

Inhibition of Intracellular Cathepsin B by Pyridoxal Derivatives in Vivo

As reported in a previous paper, PAP strongly inhibits activity of cathepsin B and weakly inhibits that of cathepsins L, K, S and C *in vitro* by the formation of thiosemiathetal bonds between the aldehyde of PAP and −SH of cathepsins, just as in the case of leupeptin and antipain [7]. For instance, 10^{-4} M of PAP inhibits 50% of purified cathepsin B activity and 10–30% of purified cathepsins L, K, S, and C [7]. Pyridoxine phosphate and pyridoxamine phosphate having no aldehyde radical, did not show any inhibitory activity. Although pyridoxal (PA) also contains the aldehyde, PA showed only weak inhibition of cathepsin B *in vitro* [7], that is, 10^{-3} M of pyridoxal inhibited only 30% of cathepsin B. Phosphate of PAP enhanced the binding affinity with cathepsins, and the reason is explained in our previous paper [7]. Either intraperitoneal injection or peroral administration of medication doses of vitamin B_6, PA, or PI caused significant inhibition of hepatic cathepsin B, but PAP did not, because PLP is unable to penetrate the cell membrane. It is known that the requirement amounts of vitamin B_6 increase in the case of a high protein diet, because the coenzyme form of vitamin B_6 participates in many enzymes of amino acid metabolism. Therefore, the inhibition of the activities of hepatic cathepsins B and L by a ten fold excess amount of vitamin B_6 (6.0 mg/100 g) in the diet was expressed much more significantly in the case of a high protein diet (70%) than that in a standard protein diet (20%), as shown in Fig. 1. The body weights were not significantly different between the standard PI diet group and the high PI diet group. The activities of cathepsins B, L, C, and S + K were suppressed dose dependently by the administration of PI 0.58 mg, 6.0 mg or 12 mg in the 70% casein diet for 3 weeks, as shown in Fig. 2. Intraperitoneal injection of PA (0.5 mg/30 g) in mice caused effective inhibition of hepatic cathepsin B (data not shown). More than 90% of intracellular total vitamin B_6 exists as a PAP form; therefore the PI administered must be converted to PAP in the cells. Since the intracellular physiological concentration of PAP is 20–50 μM, this concentration is much lower than that required to inhibit intracellular cathepsin activity. Therefore, it is unlikely that the intracellular PAP at a physiological concentration participates in the regulation of intracellular cathepsin activity *in vivo*. However, the antigen processing of ovalbumin by cathepsin B in antigen presenting cells is able to expected to be suppressed by the administra-

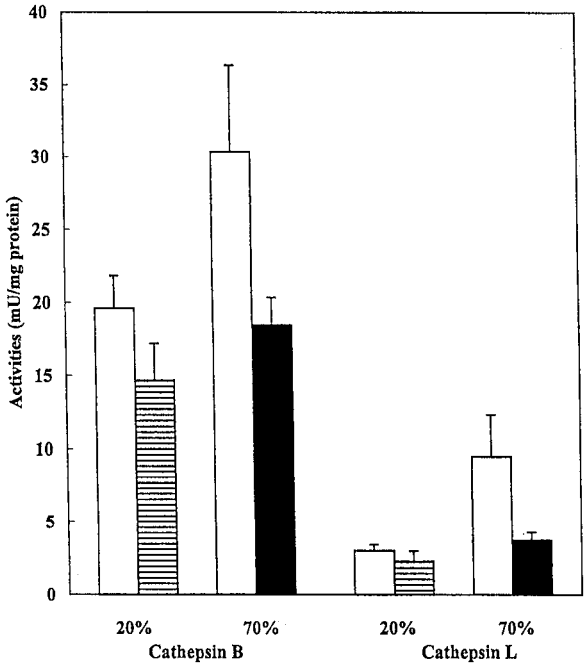

FIG. 1. Suppression of activities of hepatic cathepsins B and L by excess doses of pyridoxine in 20 or 70% casein diet. Pyridoxine content in diet was 6.0 mg/100 g diet. After 3 weeks feeding under excess pyridoxine diet, the sonicated mice livers were used for cathepsin activities. The activities of cathepsins B and L were assayed by Inubushi's method. □: standard 0.58 mg pyridoxine diet. ▤ and ■: 6.0 mg pyridoxine diet. The vertical axis is illustrated cathepsin activities of mU/mg protein. One group was consisted of 8 mice each.

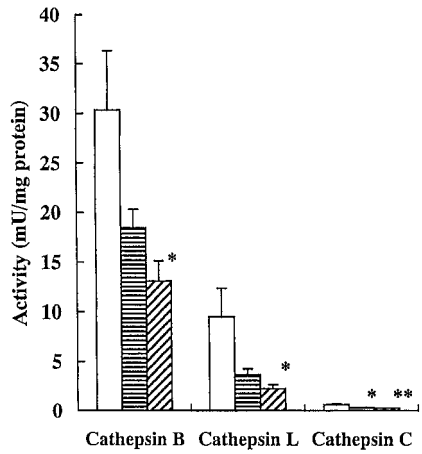

FIG. 2. Dose-dependent suppressions of various kinds of cathepsin activities by excess amount of pyridoxine in high protein diet. Pyridoxine 0.58 mg (standard diet), pyridoxine 6.0 mg and 12.0 mg (high pyridoxine diet) in 70% casein diet were administrated *ad libitum* for 3 weeks. The sonicated livers were used for cathepsin assay. The activities of cathepsins B, L, and C were assayed by Inubushi's differential cathepsin assay method. □, 0.58 mg pyridoxine diet; ▤, 6.0 mg pyridoxine diet; ▨, 12.0 mg pyridoxine diet. One group was consisted of 8 mice each. Statistically significant between standard and pyridoxine excess groups. $*P < 0.05$, $**P < 0.01$. Values are means ± SED for mice (□ ▤ ▨: $n = 6$).

tion of medication doses of vitamin B_6 based on the inhibition of intracellular cathepsin B.

Suppression of Immune Responses by Intraperitoneal Injection of Vitamin B_6 Derivatives

Effects of intraperitoneal injection of various vitamin B_6 derivatives, PI, PA, or PAP on the production of immunoglobulins such as IgE and IgG_1, and also passive cutaneous anaphylaxis reaction (PAP) were assayed using the sera of mice primed with ovalbumin. One hour before and after antigen priming, 0.5–1.0 mg/30 g (body weight) of PA, PI, or PAP was administered intraperitoneally, and the injection was continued twice/day for 9 days. All values are illustrated as the % remaining activity in the cases with vitamin B_6 derivatives, comparing with that of the control as 100%. The rechallenged ovalbumin dependent production of IL-4 by the primed splenocytes (10^6 cells/ml) in the culture medium and the inhibition of IL-4 production by addition of the vitamin B_6 derivatives were assayed. The PCA reaction was assayed using the same sera for the antibody assay, and the suppression of ovalbumin induced PCA reaction by the administration of vitamin B_6 derivatives is shown in Fig. 3. The PCA titer of the control mice was 12 ± 3 ($\log_2$PCA titer = 3.5 ± 0.5), and that of the mice treated with PA was suppressed to about 50% of the control, and that with PI treatment was suppressed to 75%, while the treatment with PAP showed no effect. The PCA reaction by this method is induced primarily by IgE, and the PCA reaction is a representative model of allergic

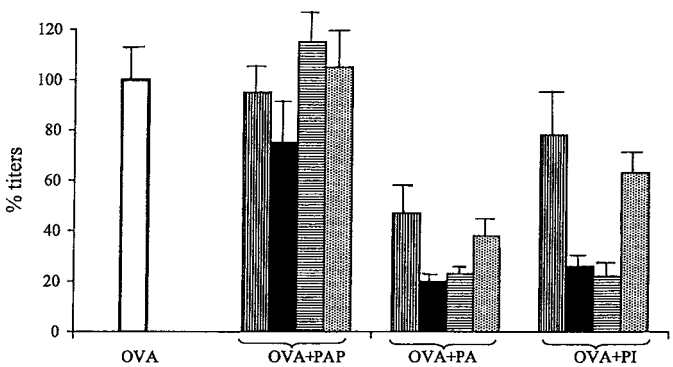

FIG. 3. Comparison of immune suppressions on various vitamin B_6 derivatives. 10 μg/mouse of ovalbumin with aluminum hydroxide gel was injected intraperitoneally into BALB/c male mice, and then the primed mice at 9th day after the priming were used for assay of various immune responses. The primed splenocytes produced 38 pg/ml of IL-4 by rechallenge of ovalbumin in the culture medium after 64 hours of culture. The assay method of PCA reaction employed the method mentioned in the text. All values were illustrated as the % remaining activity in the cases with vitamin B_6 derivatives, comparing with the control as 100%. Each group was consisted of 8 mice. ▥, PCA reaction; ■, IgE production; ▤, IgG_1 production; and ▨: IL-4 production.

dermatitis mediated by IgE. The productions of IgE, IgG_1, and IL-4 were markedly suppressed by the treatment with PA or PI, but not with PAP. The [^{3}H]-thymidine incorporations into primed splenocytes (blastogenesis) stimulated by rechallenge of OVA were inhibited by addition of PA or PI in the medium, but the addition of PAP showed no inhibition.

Dose-dependent suppression of various immune responses by intraperitoneal treatment of PA in vivo. The same primed splenocytes (10^6 cells/ml) were used for the assay of rechallenged ovalbumin dependent [^{3}H]-thymidine incorporation and the IL-4 production, under the same cultured conditions. The splenocytes produced 35 pg/ml of IL-4 in the culture medium at 64 hours after rechallenge of OVA. The [^{3}H]-thymidine incorporations and the IL-4 productions were suppressed by PA treatment in a dose dependent manner, as shown in Fig. 4. These suppressions were parallel to those of IgE and IgG_1 productions. The treatment of 1 mg of PA/per 30 g of body weight showed almost complete suppression of these immune responses.

Suppression of Immune Responses by High Pyridoxine Diet

As shown in Fig. 5, the immune responses mediated by helper T lymphocyte type-2, such as productions of IgE, IgG_1 and IL-4, were suppressed by a high PI diet, while that by helper T lymphocyte type-1, IgG_{2a} and INF-γ productions, were not changed. These results obtained by peroral administration of an excess vitamin B_6 diet were the same as those obtained by the intraperitoneal injection of vitamin B_6. These effective doses of vitamin B_6 are rather high, but they are within medication doses.

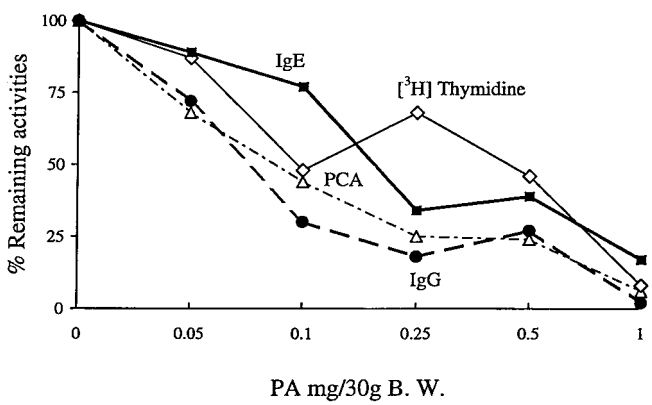

FIG. 4. Dose-dependent suppressions of various immune responses by PA injection. Assay methods of immune responses were the same as those of Figs. 2 and 3. The thick solid line (■) and the thick broken line (●) indicate the IgE formed and IgG_1 formed, respectively. The thin solid line (△) and the thin broken line (◇) indicate the PCA reaction and the [^{3}H]-thymidine incorporation, respectively.

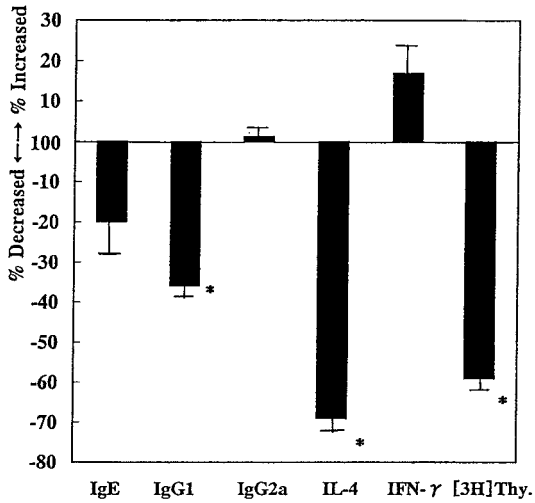

FIG. 5. Changes of ovalbumin-dependent immune responses by peroral administration of excess amount of pyridoxine in high protein diet. Percent changes (standard as the 100%) of productions of immunoglobulins and cytokins by pyridoxine 6.0 mg diet in 70% casein diet were compared with pyridoxine 0.58 mg diet in 70% casein diet. One group was consisted of 8 mice. Statistically significant between standard and pyridoxine excess diet. *$P < 0.05$.

The suppression profiles of these immune responses by vitamin B_6 administration were principally the same as that by CA-074 administration; however, some minor differences were observed between them. The immune suppression of productions of IgE and IgG_1 by vitamin B_6 administration were slightly weaker than that by CA-074 administration and the immune enhancement of productions of IgG_{2a} and IFN-γ were much weaker than that by CA-074 [4] as Fig. 5 shows. The differences may be due to the following two reasons: (i) the inhibition of cathepsin B by vitamin B_6 is weaker than that by CA-074: (ii) the inhibition by PAP is not strictly specific than that by CA-074, that is, vitamin B_6 also inhibits cathepsins L, S, K and C weakly. And it is reported that cathepsin S participates in the removal (degradation) of the invariant chain of MHC class II [13, 14]. In fact, all immune responses against OVA were suppressed by addition of CLIK-060, a specific inhibitor of cathepsin S [12, 15].

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