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Inhibition of inflammatory actions of aminobisphosphonates by dichloromethylene bisphosphonate, a non-aminobisphosphonate

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1 When injected intraperitoneally into mice in doses larger than those used clinically, all the amino derivatives of bisphosphonates (aminoBPs) tested induce a variety of inflammatory reactions such as induction of histidine decarboxylase (HDC, the histamine-forming enzyme), hypertrophy of the spleen, atrophy of the thymus, hypoglycaemia, ascites and accumulation of exudate in the thorax, and an increase in the number of macrophages and/or granulocytes in the peritoneal cavity of blood. On the other hand, dichloromethylene bisphosphonate (Cl_2MBP) a typical non-aminoBP, has no such inflammatory actions. In the present study, we found that this agent can suppress the inflammatory actions of aminoBPs.

2 Cl_2MBP, when injected into mice before or after injection of 4-amino-1-hydroxybutylidene-1,1-bisphosphonic acid (AHBuBP; a typical aminoBP), inhibited the induction of HDC activity by AHBuBP in a dose- and time-dependent manner. The increase in HDC activity induced by AHBuBP was largely suppressed by the injection of an equimolar dose of Cl_2MBP. Cl_2MBP also inhibited other AHBuBP-induced inflammatory reactions, as well as the inflammatory actions of two other aminoBPs. However, Cl_2MBP did not inhibit the increase in HDC activity induced by lipopolysaccharide (LPS).

3 We have previously reported that AHBuBP augments the elevation of HDC activity and the production of interleukin-1β (IL-1β) that are induced by LPS. These actions of AHBuBP were also inhibited by Cl_2MBP.

4 Based on these results and reported actions of bisphosphonates, the mechanisms underlying the contrasting effects of aminoBPs and Cl_2MBP, a non-aminoBP are discussed. The results suggest that combined administration of Cl_2MBP and an aminoBP in patients might be a useful way of suppressing the inflammatory side effects of aminoBPs.

Keywords: Bisphosphonates; aminobisphosphonates; histidine decarboxylase; histamine; lipopolysaccharide (LPS); interleukin-1 (IL-1)

Abbreviations: aminoBP, aminobisphosphonate; AHBuBP, 4-amino-1-hydroxybutylidene-1,1-bisphosphonic acid; BP, bisphosphonate; CHAMBP, cycloheptylaminomethylene bisphosphonate; Cl_2MBP, dichloromethylene bisphosphonate; HDC, histidine decarboxylase; IL, interleukin; LPS, lipopolysaccharide; HEBP, 1-hydroxyethylidene-1,1-bisphosphonate; MP-AHPrBP, 3-(N-methyl-N-pentyl)amino-1-hydroxypropane-1,1-diphosphonic acid; TNF, tumour necrosis factor

Introduction

Many derivatives of bisphosphonates (BPs), inhibitors of bone resorption, have been developed as promising agents for the treatment of conditions in which there is enhanced bone resorption, such as Paget's disease, tumoral osteolysis, tumoral hypercalcaemia, osteoporosis and rheumatoid arthritis (Green *et al*., 1994; Bonjour *et al*., 1994; Geddes *et al*., 1994). Among these derivatives, the aminobisphosphonates (aminoBPs) are particularly powerful (Geddes *et al*., 1994). However, undesirable acute phase responses, including fever, occur in humans after administration of aminoBPs (Adami *et al*., 1987). Recently, Sauty *et al*. (1996) suggested that an increase in the plasma levels of IL-6 and TNF may be responsible for the acute phase reactions induced by aminoPBs.

We found some time ago that injection of aminoBPs into mice, in doses larger than those used clinically, enhances the activity of histidine decarboxylase (HDC), the enzyme that forms histamine, in the bone marrow, spleen, lung and liver, resulting in an increase in histamine levels in these tissues (Endo *et al*., 1993). In addition, we have also found that aminoBPs induce hypertrophy of the spleen, atrophy of the thymus, ascites and accumulation of exudate in the thorax, and an increase in the number of granulocytes, macrophages and even osteoclasts (Endo *et al*., 1993), although the activity of osteoclasts is impaired. The inflammatory actions of aminoBPs described above are assumed to affect immune responses. Indeed, we have demonstrated that aminoBPs exacerbate the arthritis induced in mice by co-injection of a type II collagen and adjuvant, and we proposed the idea that this effect might be related to the ability of aminoBPs to increase HDC activity and/or to increase macrophages and granulocytes (Nakamura *et al*., 1996). Recently, we have shown that administration of an aminoBP augments both the production of interleukin-1 (IL-1) and the induction of HDC by a lipopolysaccharide (LPS) (Sugawara *et al*., 1998).

On the other hand, dichloromethylene bisphosphonate (Cl_2MBP), a typical non-aminoBP with a weak bone-resorbing action, has none of the effects described above and has an anti-inflammatory action on collagen-induced arthritis (Nakamura *et al*., 1996 and references cited therein). In the present study,

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we found that Cl_2MBP can inhibit or suppress the inflammatory actions of all three of the aminoBPs we tested.

Methods

Mice and reagents

Male BALB/c mice (6–7-weeks-old) were obtained from the facility for experimental animals in our university. Dichloromethylene bisphosphonate (Cl_2MBP) and 4-amino-1-hydroxybutylidene-1,1-bisphosphonic acid (AHBuBP) were synthesized by ourselves. Cycloheptylaminomethylene bisphosphonate (CHAMBP) and 3-(N-methyl-N-pentyl)amino-1-hydroxypropane-1,1-diphosphonic acid (MP-AHPrBP) were provided by Yamanouchi Pharmaceutical Co. (Tokyo, Japan) and Boehringer Mannheim (Mannheim, Germany), respectively. Bisphosphonates (BPs) were dissolved in sterile saline and adjusted to pH 7 with NaOH or HCl. Lipopolysaccharide (LPS) from *Escherichia coli* O55:B5 prepared by Boivin's method was obtained from Difco Laboratories (Detroit, MI, U.S.A.). These solutions were injected intraperitoneally (i.p.) (0.1 ml per 10 g body weight) as indicated in the text. All experiments complied with the guidelines for care and use of laboratory animals in Tohoku University.

Assay of HDC activity

HDC activity was assayed by a previously described method (Endo *et al*., 1992a). HDC activity in the liver is expressed as nmol of histamine formed in 1 h by the enzyme contained in 1 g of each tissue (nmol h^{-1} g^{-1}). HDC activity in the bone marrow is expressed as the activity in 1 g of tibia plus femur, because these bone tissues were themselves assayed for HDC activity (Endo *et al*., 1992a).

Determination of cytokines in the serum

IL-1β and TNFα were assayed using ELISA kits (Endogen, Cambridge, MA, U.S.A.), the assay procedures being performed exactly as described by the manufacturer. The blood was collected directly into test tubes following decapitation of the mice. Serum was recovered by centrifugation at $2000 \times g$ at 4°C, then stored at −80°C until used. The amount of each cytokine is expressed as pg per ml serum.

Determination of exudate in thorax

After the thorax had been opened with scissors, the exudate present in the thorax was absorbed using small pre-weighed pieces of filter paper and the amount of exudate was measured as the increase in the weight of the filter paper.

Determination of cell number of peritoneal cavity

Peritoneal exudate cells were obtained as follows. Sterile saline (10 ml) was injected into the peritoneal cavity of ether-anaesthetized mice, and the cavity was massaged. Then, the suspension of cells in the saline (5 ml) was recovered using a syringe, and the number of cells in the suspension was counted after appropriate dilution.

Determination of serum glucose

Serum was prepared as described above, and serum glucose levels were measured by the glucose oxidase method using a glucometer (Accutrend; Boehringer-Mannheim, Mannheim, Germany).

Data analysis

Experimental values are given as mean ± standard deviation. The statistical significance of differences was analysed by Dunnett's multiple comparison test after ANOVA, P values less than 0.05 being considered to indicate significance.

Results

Effects of Cl_2MBP and AHBuBP on HDC activity in mouse tissues

The effects of Cl_2MBP and AHBuBP (separately or in combination) on HDC activity in mouse tissues are shown in Figure 1. Three days after a single intraperitoneal injection of AHBuBP (40 μmol kg^{-1}) there was a marked elevation of HDC activity in the lung, liver, spleen and bone marrow. On the other hand, Cl_2MBP had no such effects, even at 800 μmol kg^{-1}. The combined injection of these agents (injection of Cl_2MBP immediately after injection of AHBuBP) completely abolished the AHBuBP-induced elevation of HDC activity in all the tissues examined. Bone marrow is the tissue with the highest HDC activity in normal mice (Endo *et al*., 1992a). It should be noted that Cl_2MBP did not decrease the normal level of HDC activity in the bone marrow or in the other tissues examined.

Dose-dependent inhibitory effect of Cl_2MBP on the AHBuBP-induced elevation of HDC activity

Injection of Cl_2MBP immediately after the injection of AHBuBP produced a dose-dependent inhibition of the AHBuBP-induced elevation of HDC activity in the lung, liver, spleen and bone marrow (Figure 2). Cl_2MBP at 32 μmol kg^{-1} strongly inhibited the HDC elevation induced by AHBuBP (40 μmol kg^{-1}). The inhibitory effect of Cl_2MBP in the spleen was significant even at 6.4 μmol kg^{-1}.

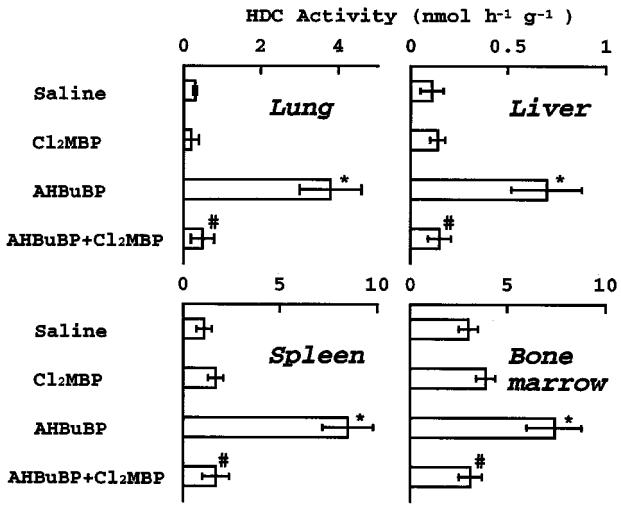

Figure 1 Effects of Cl_2MBP and AHBuBP on HDC activity in mouse tissues. Cl_2MBP (800 μmol kg^{-1}) was injected (i.p.) immediately after injection of AHBuBP (40 μmol kg^{-1}, i.p.), and the mice were sacrificed 3 days later. Each value is the mean ± s.d. from four mice. *$P<0.001$ vs saline. #$P<0.001$ vs AHBuBP.

Time-dependent inhibitory effect of Cl_2MBP on the AHBuBP-induced elevation of HDC activity

The magnitude of the inhibition of the AHBuBP (40 μmol kg^{-1})-induced elevation of HDC activity was dependent on the time at which Cl_2MBP (160 μmol kg^{-1}) was injected (Figure 3). The inhibitory effect of Cl_2MBP was greatest when it was injected simultaneously with AHBuBP, and it declined as the interval between the injections was increased. In the liver and spleen, there was still a significant inhibition when Cl_2MBP was injected as much as 2 h after the injection of AHBuBP.

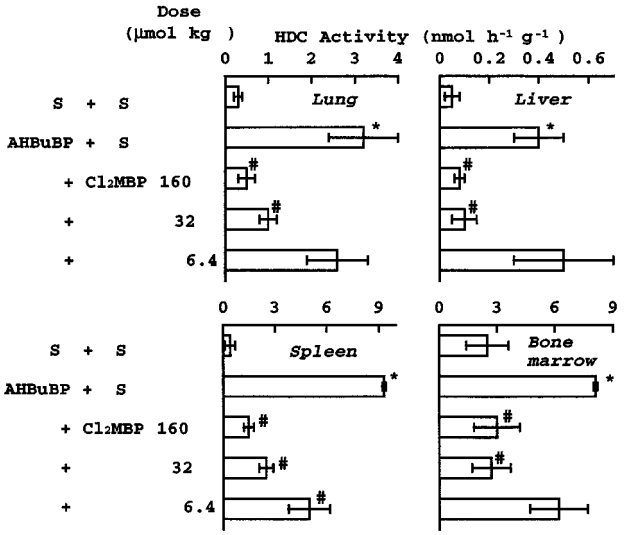

Figure 2 Dose-dependent inhibitory effect of Cl_2MBP on the AHBuBP-induced elevation of HDC activity. Saline (S) or Cl_2MBP (6.4–160 μmol kg^{-1}) was injected (i.p.) immediately after injection of saline or AHBuBP (40 μmol kg^{-1}, i.p.). The mice were sacrificed 3 days later. Each value is the mean ± s.d. from four mice. *$P<0.001$ vs S + S. #$P<0.001$ vs AHBuBP + S.

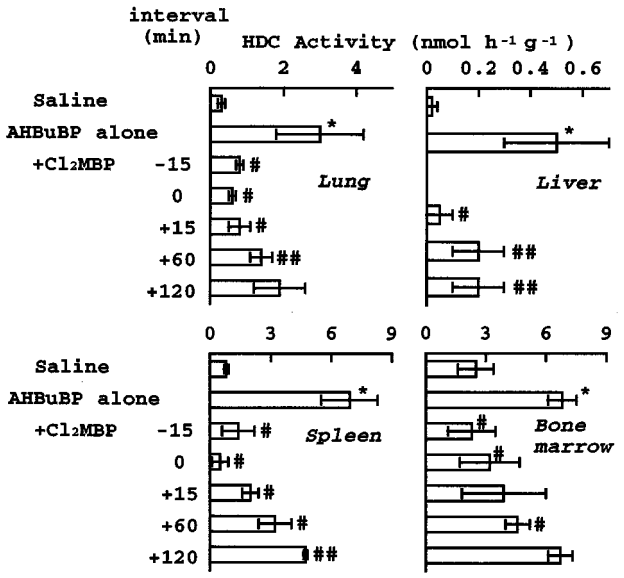

Figure 3 Time-dependent inhibitory effect of Cl_2MBP on the AHBuBP-induced elevation of HDC activity. Cl_2MBP (160 μmol kg^{-1}) was injected (i.p.) into mice 15 min before (−), simultaneously with, or 15–120 min after (+) injection of AHBuBP (40 μmol kg^{-1}, i.p.). The mice were sacrificed 3 days later. Each value is the mean ± s.d. from four mice. *$P<0.001$ vs normal control. #$P<0.01$, ##$P<0.05$ vs AHBuBP alone.

Effects of Cl_2MBP on other inflammatory reactions induced by AHBuBP

AHBuBP induces in mice a variety of inflammatory reactions, such as hypertrophy of the spleen, atrophy of the thymus, accumulation of exudate in the thorax and peritoneal cavity, and accumulation of macrophages and granulocytes in the peritoneal cavity. The AHBuBP-induced hypertrophy of the spleen was also inhibited (both dose-dependently and time-dependently) by Cl_2MBP (Figure 4). The atrophy of the thymus, the accumulation of exudate in the thorax, and the accumulation of cells in the peritoneal cavity were also suppressed by Cl_2MBP (Table 1). In the present study, it was found that AHBuBP induced hypoglycaemia, and this effect was also prevented by Cl_2MBP (Table 1).

Effects of Cl_2MBP on inflammatory reactions induced by other aminoBPs

Like AHBuBP, two other aminoBPs (CHAMBP and MP-AHPrBP) induced elevations of tissue HDC activity and other inflammatory reactions. Cl_2MBP (160 μmol kg^{-1}) also inhibited these reactions. (Figure 5 and Table 2).

Effect of Cl_2MBP on the induction of HDC by LPS

LPS is a potent inducer of HDC in various tissues, as well as in haematopoietic organs (Endo, 1982; 1983). Some cytokines have been implicated in the induction of HDC by LPS (see Discussion). In contrast to the HDC induction induced by AHBuBP, the induction by LPS is transient: HDC activity induced by an injection of LPS in mice peaks within 3–5 h of

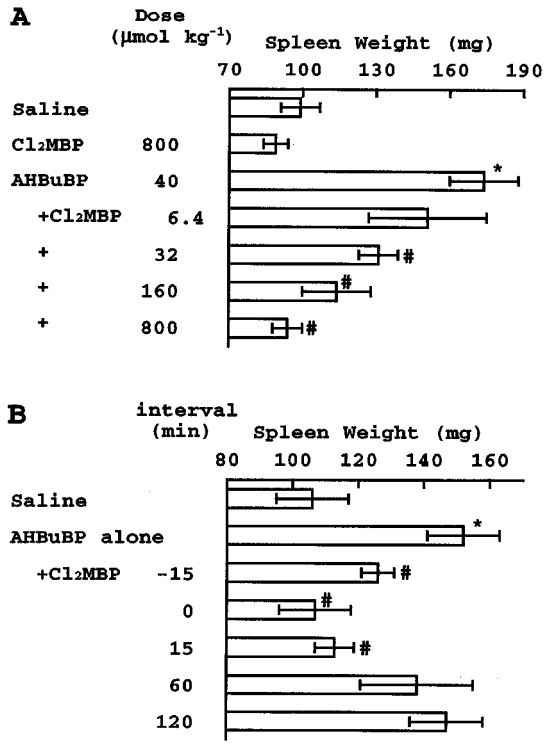

Figure 4 (A) Dose-dependent inhibitory effect of Cl_2MBP on the AHBuBP-induced hypertrophy of spleen. Spleen weights in the experiments shown in Figures 1 and 2 are shown. *$P<0.001$ vs Saline. #$P<0.001$ vs AHBuBP. (B) Time-dependent inhibitory effect of Cl_2MBP on the AHBuBP-induced hypertrophy of spleen. Spleen weights in the experiment shown in Figure 3 are shown. *$P<0.001$ vs Saline. #$P<0.01$ vs AHBuBP alone.

Table 1 Inhibition of Cl_2MBP of inflammatory reactions induced by other AHBuBP

	Body (g)	*Weight of Spleen* (mg)	*Thymus* (mg)	*Exudate in thorax* (mg)	*Cells in p. cavity* (10^6/*mouse*)	*Serum glucose* (mg dl^{-1})
Control	24.8±1.1	102±5	85±14	30±10	1.1±0.2	168±13
Cl_2MBP	24.5±0.6	100±2	77±4	25±8	0.6±0.1	180±20
AHBuBP	21.9±0.7	146±9*	49±7*	77±25*	25.9±1.3*	118±17*
+Cl_2MBP	25.1±1.1	109±6#	73±10#	33±15#	4.5±0.6#	180±20#

Cl_2MBP (160 μmol kg^{-1}) was injected into mice immediately after an injection of AHBuBP (40 μmol kg^{-1}, i.p.) and the mice were sacrificed 3 days later. Control mice were given saline. Each value is the mean±s.d. from four mice. *$P<0.01$ vs control. #$P<0.01$ vs AHBuBP.

Table 2 Inhibition by Cl_2MBP of inflammatory reactions induced by other AminoBPs

	Body (g)	*Weight of Spleen* (mg)	*Thymus* (mg)	*Exudate in thorax* (mg)	*Cells in p. cavity* (10^6/*mouse*)	*Serum glucose* (mg dl^{-1})
Control	24.5±0.5	95±12	74±5	28±9	2.0±0.7	186±16
CHAMBP	23.0±1.2	132±10*	57±2*	455±150*	14.3±0.4*	107±19*
+Cl_2MBP	24.2±1.1	101±6#	72±9#	95±29#	5.9±1.9#	156±12#
MP-AHPrBP	24.2±1.1	137±12*	67±9	195±88*	20.1±4.1*	141±13*
+Cl_2MBP	24.2±0.4	106±7†	70±7	55±16†	7.8±2.1†	175±11†

Cl_2MBP (160 μmol kg^{-1}) was injected into mice immediately after an injection of CHAMBP or MP-AHPrBP (each 40 μmol kg^{-1}, i.p.) and the mice were sacrificed 3 days later. Control mice were given saline. Each value is the mean±s.d. from four mice. *$P<0.01$ vs central. $P<0.01$ vs CHAMBP. †$P<0.01$ vs. MP-AHPrBP.

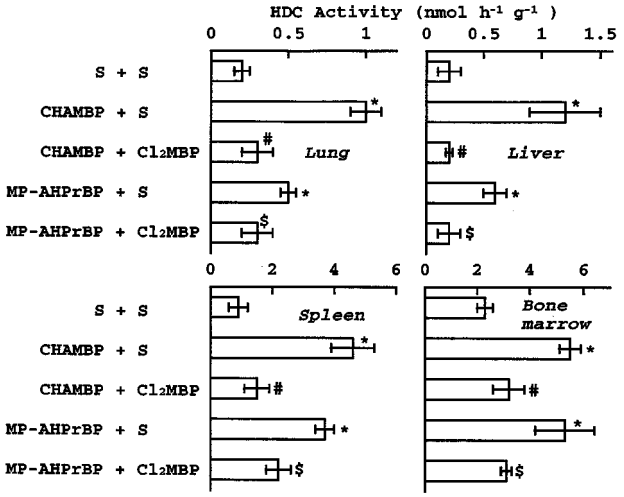

Figure 5 Effects of Cl_2MBP on the elevation of HDC activity induced by other aminoBPs. Saline (S) or Cl_2MBP (160 μmol kg^{-1}) was injected (i.p.) into mice immediately after injection of saline or of CHAMBP or MP-AHPrBP (each 40 μmol kg^{-1}, i.p.). The mice were sacrificed 3 days later. *$P<0.001$ vs saline+saline. #$P<0.01$ vs CHAMBP+S. \$$P<0.01$ vs MP-AHPrBP+S.

the injection and returns to the normal level within 20 h (Endo, 1982). Cl_2MBP (500 μmol kg^{-1}), when injected (i.p.) 10, 30 min or 3 days before LPS injection (0.1 mg kg^{-1}, i.p.), did not inhibit the LPS-induced production of IL-1β and elevation of HDC activity in the liver, spleen, lung and bone (Table 3).

Effects of Cl_2MBP on the augmentation by AHBuBP of the elevation of HDC activity and production of IL-1 induced by LPS

Recently, we have shown that AHBuBP pretreatment augments the LPS-induced elevation of HDC activity and production of IL-1 (both α and β), but suppresses the LPS-induced production of TNF (Sugawara *et al*., 1998). As shown in Figure 6, there were elevations of HDC activity in the lung, liver and spleen at 2 h after an i.v. injection of LPS. In mice treated with AHBuBP 3 days before the injection of LPS, there was a very considerable augmentation of the effect of LPS on HDC activity (note that the scale for HDC activity in Figure 6 is different from those in Figures 1, 2, 3 and 5). This augmentation was largely inhibited by Cl_2MBP. In the present experiment, the contrasting effects of AHBuBP on the production of IL-1 and TNF were confirmed (Figure 7). The AHBuBP-induced augmentation of IL-1 production was completely prevented by Cl_2MBP. Interestingly, Cl_2MBP showed a tendency to reverse the inhibition of TNFα production induced by AHBuBP, although this effect of Cl_2MBP was not statistically significant.

Discussion

Intraperitoneal injection of any of the aminoBPs we have tested so far induces inflammatory reactions. The doses used in the present study are higher than those used clinically. Therefore, our experimental system is indeed an animal model to induce inflammation by aminoBPs in mice. However, it is the fact that even at clinical doses, aminoBPs have inflammatory side effects. By contrast, the non-aminoBPs tested did not produce such inflammatory reactions. In the present study, we found that the development of the inflammatory reactions induced by these aminoBPs was largely prevented by Cl_2MBP, a typical non-aminoBP. Our findings highlight several interesting and important areas, all of which, however, will require more work before they can be clarified. We discuss these points in the following paragraphs.

Inhibition by Cl_2MBP of inflammatory actions of aminoBPs

AminoBPs and Cl_2MBP are structurally related analogues of pyrophosphate with a non-hydrolyzable P-C-P structure (Figure 8). It has been shown that Cl_2MBP injected i.v. into

Table 3 Effect of Cl_2MBP on the induction of IL-1β and HDC by LPS

	Serum IL-1 (pg mg^{-1})		*HDC activity* (nmol h^{-1} g^{-1})			
	IL-1α	*IL-1β*	*Liver*	*Lung*	*Spleen*	*Bone*
Control	5±5	6±4	0.1±0.1	0.6±0.3	0.6±0.4	3.8±0.8
LPS alone	36±17	25±7	3.3±0.3	10.6±2.3	18.2±2.3	14.2±1.7
Cl_2MBP+LPS						
10 min#	57±20	22±7	2.7±0.8	8.1±1.7	15.4±1.4	12.3±0.5
30 min	32±12	20±6	3.4±0.6	11.9±3.5	20.9±2.4	16.5±0.7
3 days	36±14	28±4	3.4±0.5	12.3±3.8	19.3±1.0	12.1±1.2

Cl_2MBP (500 μmol kg^{-1}) was i.p. injected into mice 10, 30 min or 3 days before i.p. injection of LPS (0.1 mg kg^{-1}). For the assay of IL-1α and IL-1β, the mice were sacrificed 3 h after the injection of LPS or saline (control). For the assay of HDC activity, the mice were sacrificed 4 h after the injection of LPS or saline. #Pretreatment time before LPS injection. Each value is the mean±s.d. from four mice.

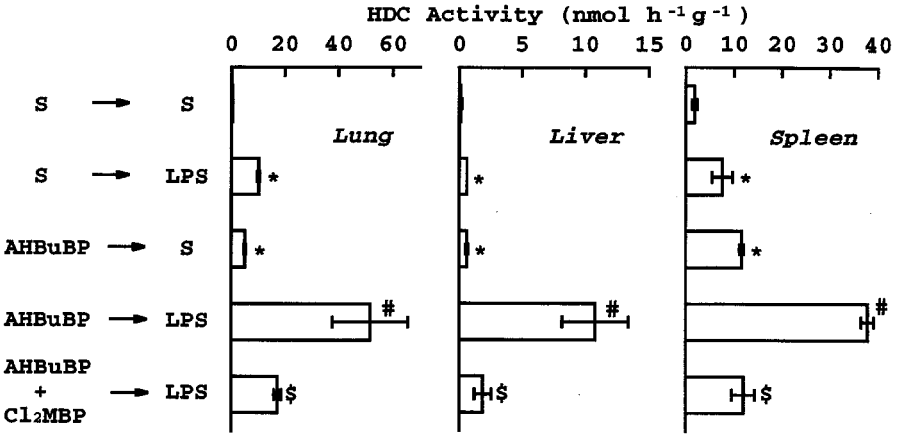

Figure 6 Effect of Cl_2MBP on the augmentation by AHBuBP of the LPS-induced elevation of HDC activity. Saline (S), AHBuBP (40 μmol mg kg^{-1}) or AHBuBP+Cl_2MBP (160 μmol kg^{-1}, immediately after AHBuBP) was injected intraperitoneally. Three days later, LPS (100 μg kg^{-1}) or S was injected intravenously into the mice and tissues were removed 2 h later. Each value is the mean±s.d. from four mice. *$P<0.01$ vs S+S. #$P<0.01$ vs S+LPS and AHBuBP+S. \$$P<0.01$ vs AHBuBP+LPS.

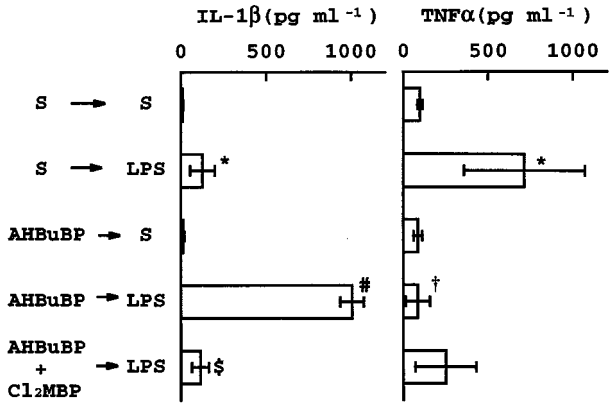

Figure 7 Effect of Cl_2MBP on the influence of AHBuBP on the LPS-induced production of IL-1β and TNFα. The blood of mice in the experiment shown in Figure 6 was assayed for the cytokines. *$P<0.01$ vs S+S. #$P<0.01$ vs S+LPS and AHBuBP+S. \$$P<0.01$ vs AHBuBP+LPS. †$P<0.01$ vs S+LPS.

mice (100 μmol kg^{-1}) is cleared rapidly from the plasma, spleen and liver and disappears almost completely within 2 h of its injection (Mönkkönen *et al*., 1989). In contrast, a high concentration of aminoBPs (AHPrBP and AHBuBP) have been shown to be retained for a much longer time in the liver, spleen and lung of mice and rats (Mönkkönen *et al*., 1989; Lin *et al*., 1992). Therefore, the suppressive effect of Cl_2MBP may depend on its concentration immediately after its injection. In addition, the effect of Cl_2MBP was greatest when it was injected simultaneously with AHBuBP and was markedly lowered when it was injected 2 h after injection of AHBuBP (Figures 3 and 4). Therefore, our findings are likely to suggest the following two possibilities. First, aminoBPs and Cl_2MBP may bind to common receptors involved in the induction of inflammatory reactions, and they may act on these receptors as an agonist or as an antagonist, respectively. Second, both aminoBPs and Cl_2MBP may positively or negatively affect a step in a biochemical pathway(s) in which a certain kind of phosphate may be involved. For example, Schmidt *et al*. (1996) reported that AHBuBP inhibits protein-tyrosine phosphatase, and they suggested that the activity of this enzyme may be important in the function and formation of osteoclasts. However, this pathway seems unlikely to be the common step on which aminoBPs act to induce inflammation, because 1-hydroxyethylidene-1,1-bisphosphonate (HEBP, a non-aminoBP) inhibits the activity of this enzyme with an IC_{50} value similar to that of AHBuBP (Schmidt *et al*., 1996), and yet HEBP does not induce inflammatory reactions (Endo *et al*., 1993). However, the testing of these hypotheses will require further experiments.

On the other hand, Mönkkönen and his co-workers found that non-aminoBPs, clodronate (Cl_2MBP) and tiludronate, inhibit LPS-induced production of IL-1β, IL-6 and TNFα (Pennanen *et al*., 1995; Mönkkönen *et al*., 1998) by a macrophage cell line (RAW 264) *in vitro*. They explained that

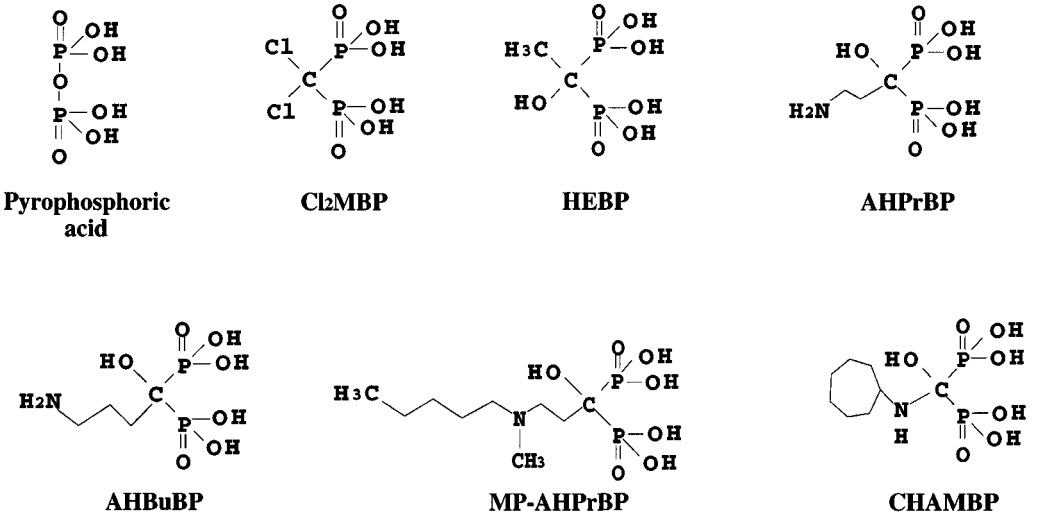

Figure 8 Structure of the bisphosphonates described in this paper.

this effect may be attributed to toxic metabolites formed after non-aminoBPs are ingested within cells (Frith *et al*., 1997; Auriola *et al*., 1997). We found that administration of Cl_2MBP-liposomes, which selectively deplete phagocytic macrophages *in vivo* (Van Rooijen & Sanders, 1994), largely abolishes the augmentation of LPS-induced production of IL-1 in AHBuBP-treated mice (Sugawara *et al*., 1998). Therefore, the formation of the cytotoxic metabolite of Cl_2MBP may underlie the depletion of macrophages. However, it is not clear whether the toxic metabolite is formed when Cl_2MBP itself is given to mice as a free drug. As described above, Cl_2MBP is cleared rapidly from the plasma, spleen and liver (Mönkkönen *et al*., 1989). Moreover, when free Cl_2MBP exerts its inhibitory effect, 10–20 times larger doses of Cl_2MBP are required than when it is encapsulated in liposomes (Pennanen *et al*., 1995). Therefore, to clarify whether the toxic metabolite of Cl_2MBP is involved in its suppressive effect on the inflammatory actions of aminoBPs, its formation *in vivo* following the injection of Cl_2MBP itself, requires investigation.

Cl_2MBP does not inhibit the inflammatory actions of LPS

Cl_2MBP itself, when injected 10, 30 min or 3 days before LPS-injection, was entirely ineffective in suppressing both the LPS-induced production of IL-1β and elevation of HDC activity. Although Cl_2MBP accumulates almost irreversibly in bone, it does not accumulate in soft tissues including liver and spleen of mice (Mönkkönen *et al*., 1989). Therefore, the reasons why Cl_2MBP does not inhibit the actions of LPS in mice not treated with an aminoBP are likely that (i) Cl_2MBP may be not ingested in macrophages in a sufficient amount to induce its toxic effect and/or (ii) the major cells in the mice responsible for both the LPS-induced production of IL-1β and elevation of HDC activity may be not phagocytic macrophages as suggested by previous studies (Salkowski *et al*., 1995; Endo *et al*., 1995).

Mechanisms underlying the inflammatory actions of aminoBPs

Among the cytokines capable of inducing HDC activity (including IL-1, TNF, granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF)), IL-1 is the most potent (Endo, 1989; Endo *et al*., 1992b). In the present study, it was found that aminoBPs induce hypoglycaemia, and it is known that injection of a low dose of IL-1 is also capable of inducing hypoglycaemia (Endo *et al*., 1985; Rey & Besedovsky, 1989; Endo, 1991). We found that AHBuBP greatly augments *in vivo* both the LPS-induced production of IL-1 (both α and β) and the LPS-induced elevation of HDC activity (Sugawara *et al*., 1998). In contrast, the LPS-induced production of TNFα was completely inhibited in AHBuBP-treated mice (Sugawara *et al*., 1998 and Figure 7 in the present study). Although IL-1 is barely detectable by current methods in the serum of mice injected with AHBuBP alone, we found that the cells of the peritoneal cavity, spleen and bone marrow in AHBuBP-injected mice spontaneously produced a higher level of IL-1 *in vitro* than the cells of normal mice (Sugawara *et al*., 1998). In addition, Northern blot analysis showed that there is a large increase in IL-1 mRNA, but not TNF- and GM-CSF-mRNAs, in the spleen of AHBuBP-injected mice (unpublished data). Moreover, our recent study using IL-1 (α/β)-deficient mice prepared by gene targeting indicated that in these mice, the elevation of HDC activity and most of other inflammatory reactions induced by aminoBPs are very small (unpublished data). These results suggest that a very slight but prolonged production of IL-1 by macrophages and/or other cells capable of producing IL-1 (such as endothelial cells) might be an important cause of the inflammatory action of aminoBPs, although the underlying mechanisms remain to be clarified.

Mönkkönen's group reported that aminoBPs are not converted to the toxic metabolite (Frith *et al*., 1997). However, they reported that pamidronate (AHPrBP) at higher concentrations inhibited the LPS-induced production of IL-1β *in vitro* by a macrophage cell line, RAW264, although this agent showed no significant effect at lower concentrations (Pennanen *et al*., 1995). In their experiments, they showed that the production of IL-6 was enhanced by the agent at its lower concentrations. By contrast, using the same experimental system, they recently reported that ibandronate (MP-AHPrBP) did not inhibit but did markedly enhance the LPS-induced secretion of IL-1β (Mönkkönen *et al*., 1998). Therefore, whether the effects of aminoBPs observed in these *in vitro* experiments can explain the *in vivo* inflammatory actions of aminoBPs is unclear. However, their finding that AHBuBP, but not non-aminoBPs, deposits in a large amount

in the liver and spleen of mice (Mönkkönen *et al*., 1989) seems to be an important cause to induce its inflammatory actions.

Possible involvement of histamine in the inflammatory and haematopoietic actions of aminoPBs

In addition to the actions on the microcirculation, histamine has been shown to stimulate the proliferation of haematopoietic precursor cells *in vitro* (Byron, 1977; 1980; Gross & Worthington-White, 1984; Shounan & You-Heng, 1988; Nakaya & Tasaka, 1988; Schneider *et al*., 1990; Dy *et al*., 1993). Indeed, the HDC activity in the bone marrow is the highest found in the tissues of normal mice (Endo *et al*., 1992a). Moreover, the haematopoietic cytokines, interleukin-3 (IL-3), GM-CSF and G-CSF, induce HDC activity *in vivo* only in the haematopoietic organs of the mouse (spleen and bone marrow) (Lebel *et al*., 1990; Endo *et al*., 1992b). It is known that haematopoiesis in the spleen is inactive in adult rats and, although LPS strongly induces HDC activity in the spleen of adult mice, it does not have this effect in the spleen of adult rats (Endo *et al*., 1995). Moreover, histamine has been shown to exert various effects on the immune system (Beer *et al*., 1983; Falus & Merétey, 1992). Collectively, these data suggest that histamine may be involved in the inflammatory and haematopoietic actions (increase in macrophages and granulocytes) of aminoBPs. However, further work is required to investigate this.

Relationship between inflammatory or anti-inflammatory actions of BPs and their inhibitory actions on bone-resorption

The potency with which Cl_2MBP inhibits bone resorption *in vivo* has been shown to be much less than those of aminoBPs (Mühlbauer *et al*., 1991; Geddes *et al*., 1994). Reitsma *et al*. (1982) have reported that the *in vitro* action on bone resorption of 3-amino-1-hydroxypropylidene-1,1-bisphosphonate (AHPrBP), an aminoBP which also induces inflammatory reactions (Endo *et al*., 1993), is different from that of Cl_2MBP. Various mechanisms have been proposed to explain the inhibitory action of BPs on bone resorption. These include a physico-chemical stabilization of the bone resulting from the binding of BPs to hydroxyapatite, a cytotoxic action on osteoclasts, an inhibition of osteoclast formation or recruitment, a metabolic inhibition that is specific to osteoclasts (Geddes *et al*., 1994; Rodan & Fleisch, 1996), an osteoblast-mediated inhibition (Sahni *et al*., 1993), apoptosis (Hughes *et al*., 1995) and inhibition of a protein-tyrosine phosphatase (Schmidt *et al*., 1996). In our present study, the inflammatory reactions induced by 40 μmol kg^{-1} of aminoBPs were all significantly inhibited by co-injection of an equimolar dose of Cl_2MBP (Figures 2 and 4). It could be of considerable interest to determine whether the mechanism by which aminoBPs inhibit bone resorption is independent of their inflammatory actions, and also whether the mechanism by which Cl_2MBP inhibits bone resorption is independent of its anti-inflammatory actions. It is also of interest to examine whether the suppressed production of TNF might be involved in the action of aminoBPs to inhibit bone resorption.

Possibility that other non-aminoBPs have anti-inflammatory actions

We have pointed out previously (Nakamura *et al*., 1996) that while aminoBPs are not effective at suppressing experimental arthritis in rats and mice (in fact, they exacerbate the arthritis), non-aminoPBs are effective. Dunn *et al*. (1993a and b) and Nugent *et al*. (1993) have reported that pyrazoline BPs (non-aminoBPs) resemble Cl_2MBP in having anti-inflammatory and anti-arthritic activities. On this basis, it seems likely that other non-aminoBPs, including pyrazoline BPs, will prove, like Cl_2MBP, to suppress the inflammatory actions of aminoBPs.

Proposal of a combined clinical use of an aminoBP and non-aminoBP

Finally, it has been shown that administration of aminoBPs produces inflammatory responses, including fever, in 10–50% of treated patients (Adami *et al*., 1987; Sauty *et al*., 1996). In addition, inflammatory side effects have also been reported in the field of ophthalmology (Siris, 1993; Macarol & Frauenfelder, 1994). Our findings suggest that the combined administration of an aminoBP (a potent inhibitor of bone resorption) with Cl_2MBP (a less potent inhibitor of bone resorption) may be a useful regimen in that it might produce much weaker inflammatory side effects than use of an aminoBP alone, while possibly producing a stronger inhibitory effect on bone resorption than that obtained by the separate use of either agent.

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