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Contrast between effects of aminobisphosphonates and non-aminobisphosphonates on collagen-induced arthritis in mice

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1 Bisphosphonates (BPs) are inhibitors of bone resorption, and many derivatives have been developed for the treatment of enhanced bone resorption. Aminobisphosphonates (aminoBPs) are particularly potent in this respect. We have shown previously that aminoBPs, such as 4-amino-1-hydroxybutylidene-1,1-bisphosphonic acid (AHBuBP), induce histidine decarboxylase, the enzyme forming histamine, and increase macrophages, granulocytes and osteoclast numbers. Non-aminoBPs do not show this activity.

2 In the present study, an additional aminoBP, cycloheptyl-aminomethylene bisphosphonate (CHAMBP), was shown to have similar properties to AHBuBP suggesting that these actions are common among aminoBPs.

3 In experiments carried out to determine if aminoBPs affect immune responses, we found that CHAMBP and AHBuBP each exacerbated the arthritis induced in mice by the co-injection of type II collagen and an adjuvant, a model for rheumatoid arthritis. In contrast, dichloromethylene bisphosphonate (C12MBP), a typical non-aminoBP, did suppress the arthritis.

4 On the basis of these results, and those obtained previously, we propose that the exacerbating effects of CHAMBP and AHBuBP may be related to their ability to stimulate the synthesis of histamine and to increase macrophages and granulocytes. Conversely, we propose that the suppressive effect of C12MBP on arthritis is related to its cytotoxic action on macrophages or granulocytes.

Keywords: Bisphosphonates; arthritis; inflammation; histamine; histidine decarboxylase; rheumatoid arthritis

Introduction

Many derivatives of bisphosphonates (BPs) have been developed as promising agents for the therapeutic correction of enhanced bone resorption, such as occurs in Paget's disease, tumoural osteolysis, tumoural hypercalcaemia, osteoporosis, and rheumatoid arthritis (Green *et al.*, 1994; Bonjour *et al.*, 1994; Geddes *et al.*, 1994). Among these derivatives, aminobisphosphonates (aminoBPs) are particularly potent. We have reported that aminoBPs with a free amino group in their molecule, when injected into mice, enhanced the activity of histidine decarboxylase (HDC), the enzyme forming histamine, in the bone marrow, spleen, lung and liver (Endo *et al.*, 1993). However, non-aminoBPs showed no such activity. Compared to the transient enhancement of HDC activity after the injection of lipopolysaccharides (LPS) or cytokines (Endo, 1989; Endo *et al.*, 1992b), the enhancement of HDC activity by the aminoBPs was remarkably long-lasting, persisting for several days (Endo *et al.*, 1993).

In addition to its role in inflammation, histamine has been shown to stimulate haematopoietic precursor cells *in vitro* (Byron, 1977; Shounan & You-Heng, 1988; Nakaya & Tasaka, 1988; Schneider *et al.*, 1990; Dy *et al.*, 1993). Moreover, haematopoietic cytokines, such as interleukin-3 (IL-3), granulocyte-macrophage-colony stimulating factor (GM-CSF) and granulocyte-colony stimulating factor (G-CSF), induced HDC *in vivo* only in haematopoietic organs (Lebel *et al.*, 1990; Endo *et al.*, 1992b). These, and our recent results (Endo *et al.*, 1995), suggest that histamine is involved in haematopoiesis, probably with a role in the formation of macrophages and granulocytes.

It is known that the precursor cells of granulocytes, macrphages and osteoclasts are identical. In correspondence with the haematopoietic action of histamine, the aminoBPs which enhanced HDC activity increased granulocytes, macrophages and osteoclasts (Endo *et al.*, 1993). However, the activity and morphological properties of osteoclasts were impaired (Endo *et al.*, 1993). None of the non-aminoBPs tested showed such effects These results support the idea that histamine may be involved in the formation of granulocytes, macrophages and osteoclasts and, therefore, suggested to us that aminoBPs may affect immune responses.

Granuloyctes, macrophages and osteoclasts are believed to play important roles in the pathogenesis of rheumatoid arthritis. Because rheumatoid arthritis produces destruction of the bone around joints, and this destruction is generally believed to be due to enhanced bone resorption by osteoclasts, agents inhibiting bone resorption should be useful in the treatment of this disease. The inhibitory actions of aminoBPs on bone resorption are much stronger than those of non-aminoBPs (Mühlbauer *et al.*, 1991; Green *et al.*, 1994). Therefore, it seemed important to examine whether aminoBPs and non-aminoBPs might have different effects on a model of rheumatoid arthritis. Polyarthritis induced in mice or rats by the injection of type II collagen together with an adjuvant is widely used as a model for rheumatoid arthritis. In the present study, therefore, the effects were examined of two aminoBPs and a typical non-aminoBP on this model.

Methods

Mice and reagents

Male DBA/1 (6 weeks old) were obtained from the Mouse Centre of our University.

Bovine type II collagen prepared by Collagen Research Centre (Kiyose, Japan) was purchased from Cosmo-Bio Inc. (Tokyo, Japan). A peptidoglycan subunit from the cell wall of *Staphylococcus epidermidis* (designated as SEPS, Kawata *et al.*, 1984) was provided by Dainippon Pharmaceutical Co. (Osaka, Japan).

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The bisphosphonates used in this study were cycloheptylaminomethylene bisphosphonate (CHAMBP or YM-175) from Yamanouchi Pharmaceutical Co. (Tokyo, Japan), dichloromethylene bisphophonate (C12MBP) from Kissei Pharmaceutical Co. (Matsumoto, Japan), 4-amino-1-hydroxybutylidene-1,1-bisphophonic acid (AHBuBP) and 3-amino-1-hydroxypropylidene-1,1-bisphosphonate (AHPrBP) from Teijin Pharmaceutical Co. (Tokyo, Japan). Bisphosphonates were dissolved in sterile saline (or water, in the case of high concentrations) and their pH adjusted to 7 with NaOH or HCl. These solutions were injected intraperitoneally (i.p., 0.1 ml per 10 g body weight).

Assay of HDC activity

Mice were killed by decapitation at the indicated times, and tibias and femurs rapidly removed and kept at −80°C until assayed. HDC activity, i.e. the activity of the enzyme contained in tissues that produces histamine from histidine, was assayed as described previously (Endo, 1983). HDC activity in the bone marrow was expressed in terms of nmol histamine produced during 1 h incubation by the enzyme contained in the bone marrow of 1 g tibia and/or femur (nmol h^{-1} g^{-1}) (Endo *et al.*, 1992a).

Induction of arthritis

Arthritis was induced in DBA/1 mice by co-injection of type II collagen and an adjuvant. In some experiments, SEPS was used as a bacterial component of the adjuvant. Its molecular properties were described by Kawata *et al.* (1984). In such experiments, 1 ml of 0.2% type II collagen in 0.01 M acetic acid, 1 ml of Freund's incomplete adjuvant and 4 mg of SEPS were mixed homogeneously by use of a sonicator, and 50 μl of the mixture was injected intradermally at the root of the tail of DBA/1 mice of 6 to 8 weeks of age (1st sensitization). Three weeks later, the same mixture was again injected in the same way (2nd sensitization). In other experiments, we used Freund's complete adjuvant (FCA) as follows: 1 ml of the type II collagen solution described above was mixed with 1 ml of FCA, and the mixture was injected as described above.

The severity of the joint inflammation in each mouse was quantified by scoring each paw on a scale of 0 to 4 (Wood *et al.*, 1969). The incidence of arthritis and total of the score for all four paws were used as indices of the arthritis produced. For convenience, the mean score per mouse in each experimental group has been used to illustrate the effects of the drugs. However, statistical analysis of the scores was performed by use of a non-parametric method as described below.

Administration of bisphosphonates

CHAMBP (0.32 and 1.6 μmol kg^{-1}), AHBuBP (1.6 and 8.0 μmol kg^{-1}) and C12MBP (160 μmol kg^{-1}) were each injected intraperitoneally once per week. The reasons for using this regimen were as follows (1) Doses: the doses of C12MBP used in many reports to inhibit bone resorption are 20 to 200 μmol kg^{-1} per day, but the minimum effective doses of AHBuBP and CHAMBP are about 1/100 of that of C12MBP, and CHAMBP is more potent than AHBuBP (Isomura *et al.*, 1990; Fujimoto *et al.*, 1990; Mühlbauer *et al.*, 1991). (2) Interval between injections: bisphosphonates bind strongly and almost irreversibly to the bone and tend to accumulate there (Lin *et al.*, 1991).

The lethal doses in mice within 1 week after a single intraperitoneal injection of AHBuBP or CHAMBP were about 80 and 160 μmol kg^{-1}, respectively. C12MBP did not induce any macroscopic symptoms even at 800 μmol kg^{-1}.

Histological observation of the bone

Tissues were stained with haematoxylin-eosin as described previously (Endo *et al.*, 1993). Macrophages and granulocytes in the peritoneal cavity were detected by nonspecific esterase staining (Yam *et al.*, 1971).

Table 1 Effects of weekly i.p. injections of C12MBP and CHAMBP on normal DBA/1 mice

	Dose (μmol kg^{-1})	*body* (g)	*Weight spleen* (mg)	*tibia*[a] (mg)	*HDC activity in the tibia* (nmol h^{-1} g^{-1})
Saline		28±2	64±7	56±3	1.2±0.2
C12MBP	160	28±2	68±11	66±4*	1.1±0.3
CHAMBP	1.6	28±3	68±10	68±5*	1.6±0.2*

HDC activity in the tibia of each DBA/1 mouse was assayed 4 days after the 5th weekly injection. Each value is the mean±s.d. from 5 mice. [a]from two legs of each mouse. *$P<0.05$ *vs* saline group (unpaired *t* test).

Table 2 Effect of bisphosphonates on the activity of histidine decarboxylase (HDC) in mice treated with a mixture of Freund's complete adjuvant and type II collagen (FCA-collagen)

Treatment		*HDC activity* (nmol h^{-1} g^{-1})	
1st injection	*2nd injection*	*Spleen*	*Bone*
Saline	Saline	1.0±0.4	2.0±0.3
Saline	C12MBP	0.8±0.3	1.8±0.3
Saline	ABHuBP	1.3±0.3	1.8±0.4
Saline	CHAMBP	1.1±0.5	2.0±0.3
FCA-collagen	Saline	1.2±0.3	2.6±0.5
FCA-collagen	C12MBP	1.0±0.2	2.3±0.5
FCA-collagen	AHBuBP	2.1±0.2*	3.4±0.5*
FCA-collagen	CHAMBP	2.1±0.3*	4.4±0.6*

FCA-collagen or saline was injected intradermally into DBA/1 mice as described in the Methods. Six days later, C12MBP (160 μmol kg^{-1}), CHAMBP (1.6 μmol kg^{-1}), AHBuBP (8 μmol kg^{-1}) or saline was injected intraperitoneally. HDC activities in the spleen and bone (tibia plus femur) of the mice were assayed 3 days after the 2nd injection. Each value is the mean±s.d. from 6 mice. *$P<0.01$ *vs* the corresponding control, in which bisphosphonate was replaced by saline (unpaired *t* test).

Statistical evaluation

The change in the arthritic score within each experimental group during an experimental period was analyzed using a one-way analysis of variance (ANOVA). The difference between two experimental groups in terms of their arthritic scores at a given time was analyzed using a Ridid test, a non-parametric test.

Any changes in arthritic incidence throughout an experimental period among or between experimental groups were analyzed by a two-factor ANOVA test. The difference, in terms of incidence, between two experimental groups at a given time was analyzed by Fisher's exact probability test.

The values sampled from a population that followed a Gaussain distribution were analyzed by Student's unpaired t test, a parametric test.

Ridid analysis was carried out using a hand calculator and other analyses using a Macintosh computer and InStat and Super ANOVA software.

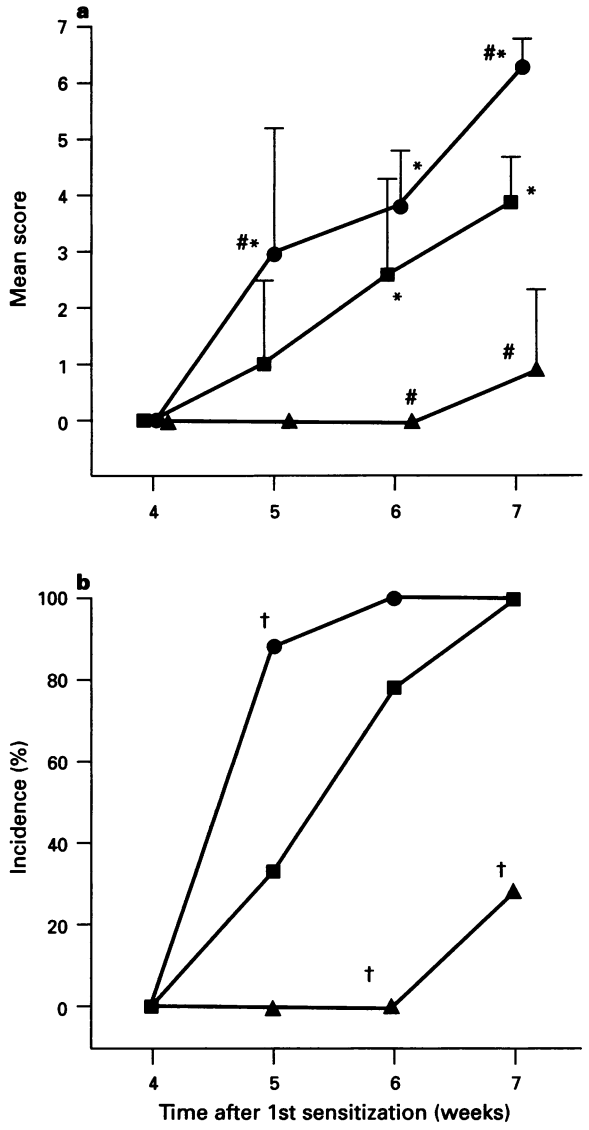

Figure 1 Effects of CHAMBP and C12MBP on the arthritis induced by co-injection of type II collagen, Freund's incomplete adjuvant and SEPS. CHAMBP (●, 1.6 μmol kg^{-1}), C12MBP (▲, 160 μmol kg^{-1}) or saline (■) was injected i.p. once each week for 8 weeks. The injection sequence was started 7 days before the 1st sensitization. Data are from 7 to 9 mice. (a) The arthritic scores for saline and CHAMBP groups increased significantly with time ($P<0.0001$ by one-way ANOVA test and $^{*}P<0.01$ vs. week 4 by Dunnett' multiple comparison test). In each week, the scores for the CHAMBP and C12MBP groups were compared to that of the saline group using a Ridid test ($^{\#}P<0.05$ vs. saline group). (b) The incidence for the C12MBP group as a whole was significantly lower than for the saline or CHAMBP group ($P<0.05$, two-factor ANOVA). The incidences in each group were compared by Fisher's exact probability test ($^{\dagger}P<0.05$ vs. saline group). Note: although there was no significant difference betwen the CHAMBP group and the saline group in a two-factor ANOVA test, the incidence for the CHAMBP group for week 5 was significantly higher than that for the saline group when Fisher's exact probability test was used.

Results

Effects of weekly injections of CHAMBP and C12MBP in normal mice

Before starting experiments on the arthritis model, the effects of weekly i.p. injection of these agents on normal DBA/1 mice were tested (Table 1). Under our conditions, these agents caused no decrease in body weight, no depression of behavioural activity, no increase in the weight of spleen, and no detectable increase in macrophages and granulocytes in the peritoneal cavity. However, there was a significant increase in the weight of the tibia, which was due to hypertrophy in the metaphyseal portion, i.e. the bone developed a club-like shape, indicating a strong inhibition of bone resorption. Under these conditions, only CHAMBP significantly increased HDC activity in the tibia.

Effects of C12MBP, CHAMBP and AHBuBP on the activity of histidine decarboxylase (HDC) in mice treated with an arthritis-inducing mixture

A single i.p. injection of CHAMBP (1.6 μmol kg^{-1}), AHBuBP (8 μmol kg^{-1}) or C12MBP (160 μmol kg^{-1}) produced no significant elevation in HDC activity in either the spleen or bone marrow of sham-immunized mice. However, CHAMBP and AHBuBP, but not C12MBP, significantly elevated HDC activity in these tissues in mice treated with the arthritis-inducing mixture of Freund's complete adjuvant and type II collagen (Table 2).

Effects of CHAMBP, AHBuBP and C12MBP on arthritis

Figure 1 shows the effects of CHAMBP and C12MBP on the arthritis induced by type II collagen combined with SEPS and Freund's incomplete adjuvant. Compared with the saline-group, the C12MBP-group showed a significantly lower arthritic score at 6 and 7 weeks after the first sensitization, whereas CHAMBP significantly increased the score at 5 and 7 weeks. The incidence of arthritis in the C12MBP-group at 6 and 7 weeks was significantly lower than in the saline-group. In contrast, the incidence in the CHAMBP-group at 5 weeks was significantly higher than in the saline-group.

Our result with C12MBP is essentially the same as those described in the literature. However, the effect of CHAMBP is different from those of many other bisphosphonates. In an attempt to confirm this unusual effect of CHAMBP, we next examined the effect of CHAMBP and ABHuBP on the arthritis induced by collagen type II and Freund's complete adjuvant, because this adjuvant is commonly used by other investigators. In these experiments, CHAMBP and AHBuBP were administered 7 days after the 1st sensitization.

As shown in Figure 2, the arthritic score at 5 weeks in the C12MBP group was significantly lower than that in the saline group, whereas the scores in the CHAMBP group at 6, 7 and 8 weeks were all significantly higher than those in the saline group. However, any difference in incidence among these groups did not reach significance. CHAMBP at a lower dose (0.32 μmol kg^{-1}) did not induce such an exacerbating effect

(data not shown). AHBuBP also worsened the arthritis at 8 μmol kg^{-1} (Figure 3), but not at 1.6 μmol kg^{-1} (data not shown).

Effects of CHAMBP administered 3 weeks after the 1st sensitization

In the above experiments, the administration of CHAMBP was started one week before or after the 1st sensitization. In the next experiment (Table 3), the injection of CHAMBP was started 3 weeks after the 1st sensitization. In this experiment, at six weeks after the 1st sensitization the incidence in the saline group was 0/5, but the incidence in the CHAMBP-group was 5/5.

Table 3 also shows HDC activity in the tibia. The HDC activity of the saline-group was significantly higher than that of age-matched normal DBA/1 mice. In turn, the HDC activity of the CHAMBP-group was markedly higher than that of the saline group.

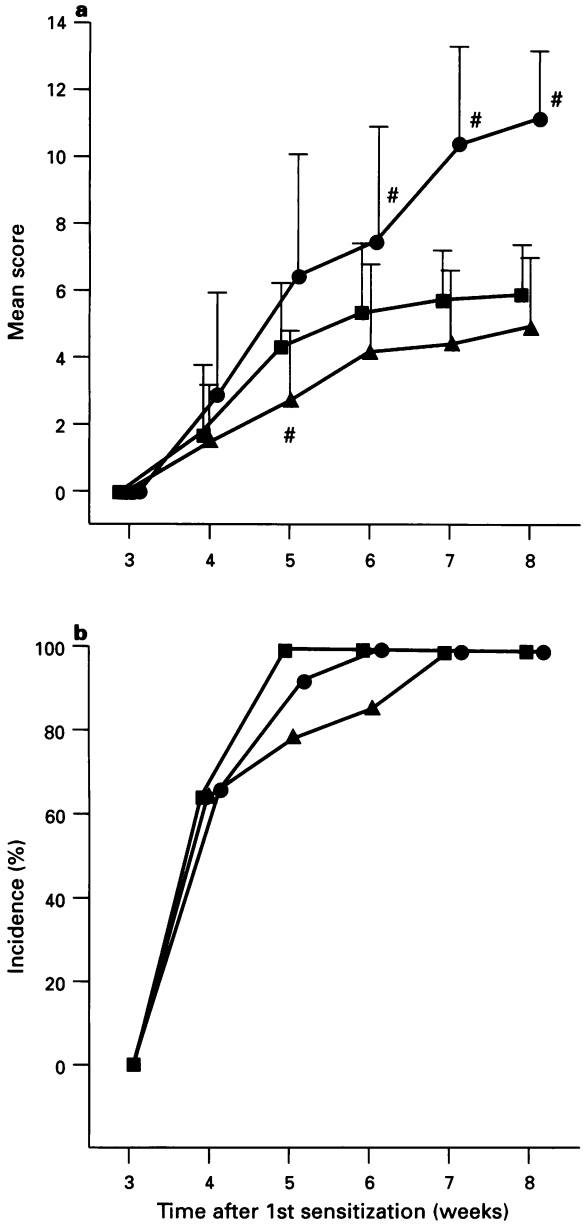

Figure 2 Effects of C12MBP and CHAMBP on the arthritis induced by co-injection of type II collagen and Freund's complete adjuvant. C12MBP (▲, 160 μmol kg^{-1}), CHAMBP (●, 1.6 μmol kg^{-1}) or saline (■) was injected once each week for 8 weeks, the injection sequence being started 7 days after the 1st sensitization. Data are from 14 mice of each group. (a) The arthritic score for each group increased significantly with time ($P<0.0001$ by one-way ANOVA test). In each week, the score for the CHAMBP and C12MBP groups was compared to that of the saline group using a Ridid test ($^{\#}P<0.05$). (b) In this experiment, there was no statistically significant difference between the 3 groups in terms of the incidence of arthritis.

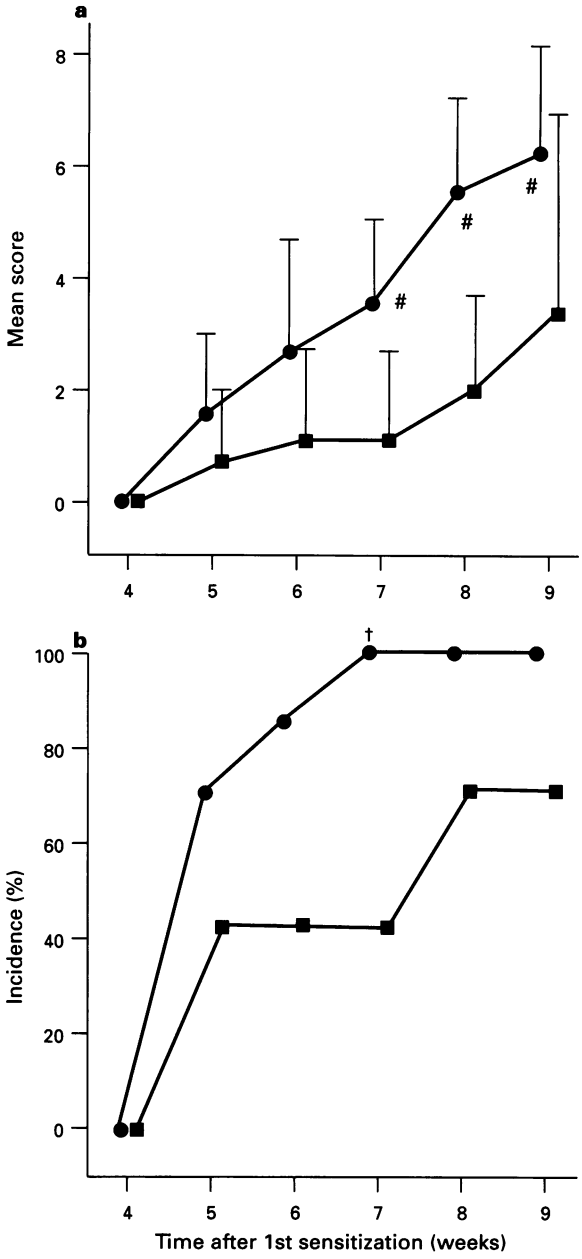

Figure 3 Effects of AHBuBP on the arthritis induced by co-injection of type II collagen and Freund's complete adjuvant. AHBuBP (●, 8 μmol kg^{-1}) or saline (■) was injected once each week. The injection sequence was started 7 days after the 1st sensitization. Data are from 8 mice of each group. (a) The arthritic scores for saline and AHBuBP groups increased significantly with time ($P<0.0001$ by one-way ANOVA test). In each week, the arthritic score for the AHBuBP group was compared to that of the saline group using a Ridid test ($^{\#}P<0.05$ vs. saline group). (b) The incidence for the AHBuBP groups as a whole was significantly higher than that for the saline group ($P<0.05$, two-factor ANOVA). In week 7, the incidence for the AHBuBP group was significantly higher than that for the saline group by Fisher's exact probability test ($^{\dagger}P<0.05$).

Histochemical analysis of joints and growth plates of tibias

Joints and tibias from the arthritic mice were compared histologically. In these experiments, joints from paws that had a severity score of three in the experiment shown in Figure 1 were selected from each group. There was marked bone destruction (arrow head) around the joints of the saline-group (Figure 4b). Similar bone destruction also occurred in the joints of the CHAMBP-group (Figure 4c). Although C12MBP exerted a suppressive effect on the arthritis, bone destruction was also evident in the joints of the C12MBP group (Figure 4d). Synovial proliferation with pannus formation, cartilage erosion, and infiltration of polymorphonuclear cells and mononuclear cells (macrophages and lymphocytes) into the joints were also evident in all of the arthritic groups. Thus, no apparent differences were detected among the joints of the saline, CHAMBP and C12MBP groups.

The effect of bisphosphonates on bone resorption in the growth plate of tibias from arthritic mice is illustrated in Figure 5. The number of bone trabeculae was markedly increased in the groups treated with either CHAMBP (Figure 5b) or C12MBP (Figure 5c). In addition, there was a club-shaped hypertrophy of the metaphysis in the tibias of these groups (seen in the experiment shown in Table 1 as an increase in weight). These results indicate that CHAMBP and C12MBP strongly inhibited the physiological bone resorption which is mediated by osteoclasts.

Table 3 Effects of CHAMBP administered 3 weeks after 1st sensitization on arthritis and histidine decarboxylase (HDC) activity in the tibia

	Incidence of arthritis	*Mean arthritis score*	*HDC activity in the tibia* (nmol h^{-1} g^{-1})
Normal mice			1.4±0.5
Sensitized mice			
Saline	0/5	0	2.3±0.2*
CHAMBP	5/5	4.6±1.3	4.9±0.4#

The method used to induce arthritis was the same as in the experiment shown in Figure 2. The injection sequence using CHAMBP (1.6 μmol kg^{-1}) or saline was started 3 weeks after the 1st sensitization and they were injected once every 7 days. Six weeks after the 1st sensitization, the incidence and scores were each determined and tibias were assayed for histidine decarboxylase (HDC) activity. Each value is the mean±s.d. from 5 mice. *$P<0.05$ *vs* normal mice, #$P<0.05$ *vs* saline group (unpaired *t* tests).

Discussion

AHBuBP and AHPrBP each have a free amino group in their molecule, and we have previously shown that they induce prolonged elevation of HDC activity in various tissues including spleen and bone marrow, and an increase in macrophages and granulocytes (Endo *et al.*, 1993). Although the amino group of CHAMBP is cycloheptylated, the present study indicates that this agent also had essentially the same actions. Conversely, non-aminoBPs did not show such actions even at higher doses.

The reported effects of various bisphosphonates (BPs) on experimental arthritis and on human rheumatoid arthritis are summarized in Table 4. In the table, we note that BPs other than aminoBPs are effective in suppressing experimental arthritis. In our present study, C12MBP was also shown to have such a suppressive effect. However, both CHAMBP and AHBuBP, i.e. aminoBPs, were clearly shown to have an exacerbating effect on the development of the arthritis. The data on AHPrBP and N-dimethylAHPrBP reported by Markusse *et al.* (1990) indicate that these aminoBPs are also ineffective on, or may worsen, arthritis induced in rats. Therefore, with regard to experimental arthritis, and in contrast to non-aminoBPs, aminoBPs seem either not to affect it or to worsen it.

Francis *et al.* (1989) have proposed two phases in the development of experimental arthritis: (i) initial inflammation in periarticular soft tissues, and (ii) magnification of the inflammation by calcium released by osteolysis, leading ultimately to destruction of the joint. They suggest that BPs suppress the second phase by inhibiting bone resorption. However, our doses of CHAMBP or AHBuBP as well as the doses of AHPrBP and N-dimethylAHPrBP used by Markusse *et al.* (1990) (4 μmol kg^{-1} given daily by s.c. injection for 3 weeks) are sufficient to inhibit physiological bone resorption (Mühlbauer *et al.*, 1991). Indeed, our morphological observations confirmed their strong inhibition of bone resorption in the growth plate. Therefore, the contrast between the sup-

Table 4 Reported effects of bisphosphonates on arthritis

Bisphosphonate	*Arthritis*	*Effects*	*References*
Non-AminoBPs			
HEBP (etidronate)	rat AA	E	1,2
C12MBP (clodronate)	rat AA	E	2,6
	mouse AA	E	6,12
SR-41319 (tildronate)	rat AA	E	3
Pyridine-BP			
NE-58095 (risedronate)	rat AA	E	4
Pyrazoline-BPs	rat AA	E	5,6
	mouse CA	E	5,6
Amino-BPs			
AHPrBP (pamidronate)	rat CA	NE	7
	human RA	NE	8,9
	human RA	E	10,11
N-dimethylAHPrBP	rat CA	NE or W	7
CHAMBP (YM-175)	mouse CA	NE or W	12
AHBuBP (alendronate)	mouse CA	NE or W	12

AA, adjuvant-induced arthritis; CA, collagen-induced arthritis; RA, rheumatoid arthritis; E, effective; NE, not effective; W, worsening. References: (1) Francis *et al.*, 1972; (2) Flora, 1979; (3) Babier *et al.*, 1986; (4) Francis *et al.*, 1989; (5) Nugent *et al.*, 1993; (6) Dunn *et al.*, 1993a; (7) Markusse *et al.*, 1990; (8) Ralston *et al.*, 1989; (9) Tan *et al.*, 1989; (10) Bijvoet *et al.*, 1980; (11) Maccagno *et al.*, 1994; (12) present results.

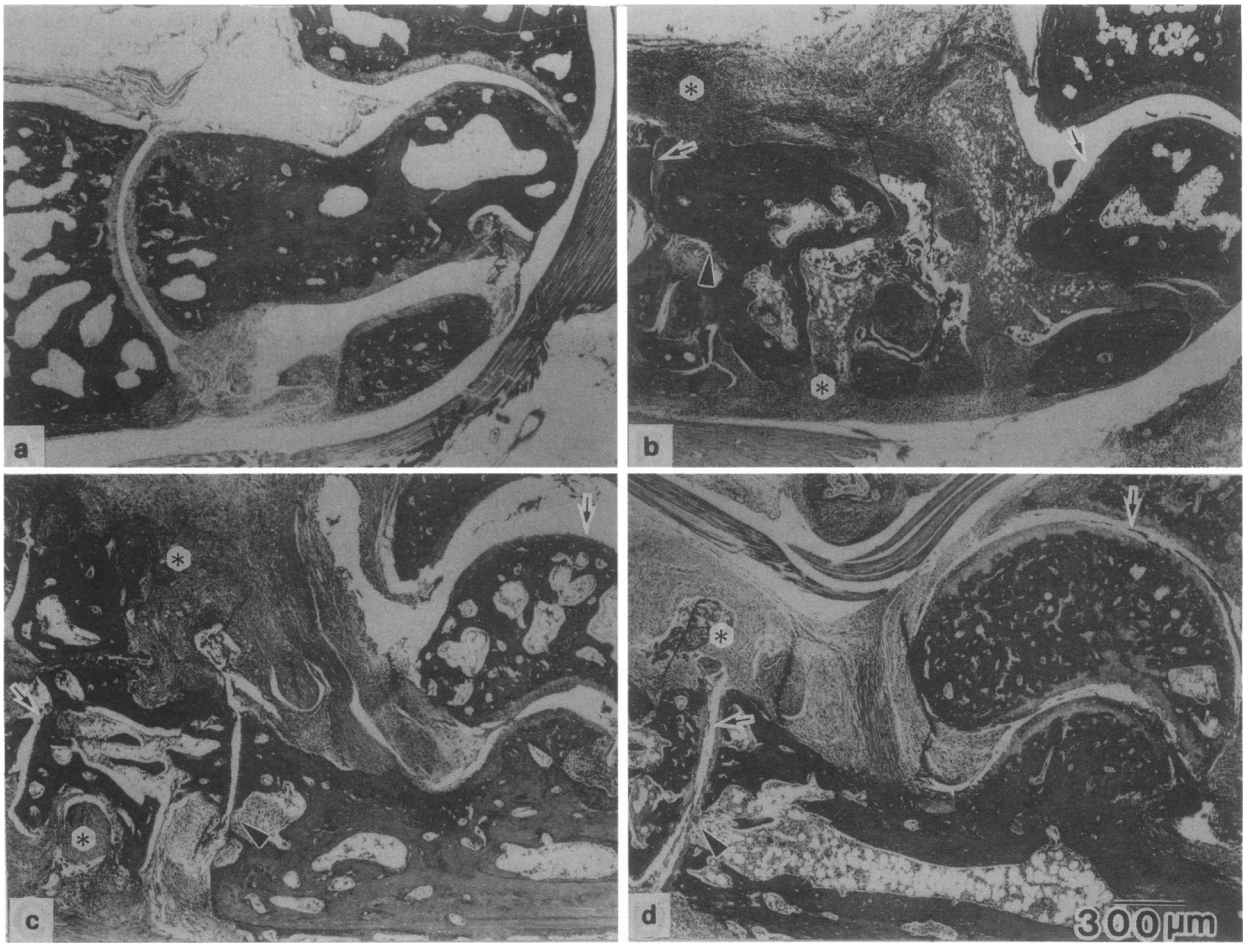

Figure 4 Haematoxylin-eosin staining of the area around the heel of normal and score-3-arthritic hind paw joints in the experiment of Figure 1. Joints of (a) normal mouse, (b) arthritic mouse from the saline group, (c) arthritic mouse from the CHAMBP group, (d) arthritic mouse from the C12MBP group. In arthritic joints, pannus formation (*), erosion of articular cartilage (arrow) and bone destruction (arrow head) are evident. Bar = 300 μm.

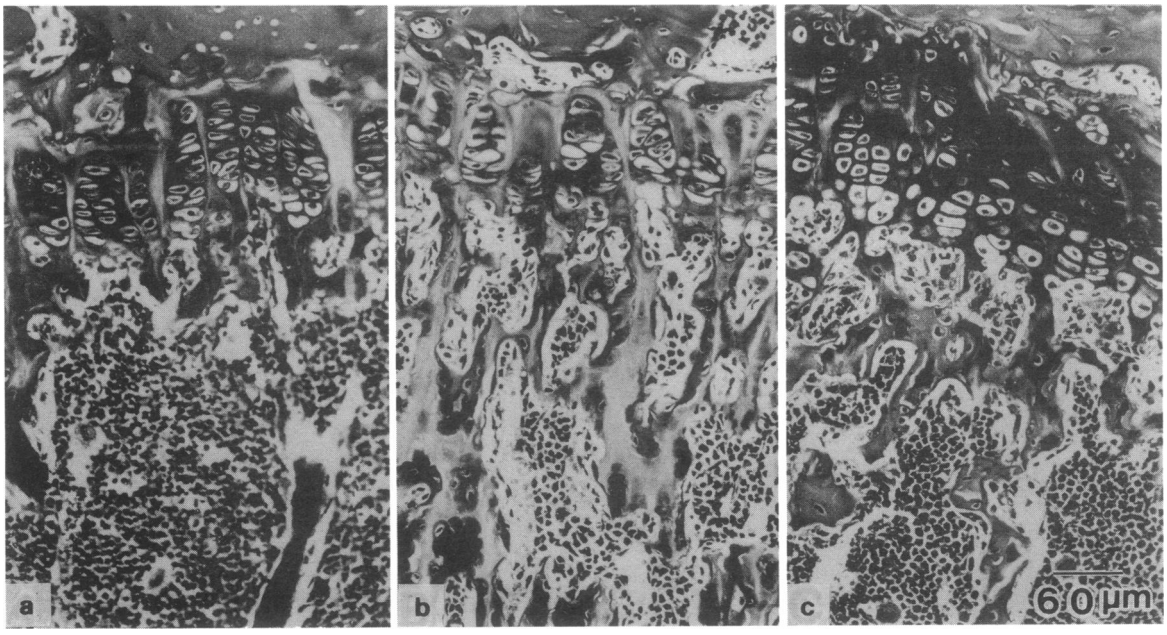

Figure 5 Haematoxylin-eosin staining of tibial growth plates from score-3-arthritic joints in the experiment of Figure 1. Tibias are from arthritic mice of (a) saline group, (b) CHAMBP group, and (c) C12MBP group. A marked increase in trabecular bone is evident in the growth plates of CHAMBP and C12MBP groups, indicating a strong inhibiton of osteoclast-mediated bone resorption. Bar = 60 μm.

pressive effect of non-aminoBPs and the ineffectiveness of aminoBPs on arthritis cannot be attributable to effects on bone resorption.

In DBA/1 mice treated with the mixture of Freund's complete adjuvant and type II collagen (i.e. sensitized in respect of the arthritis), a single i.p. injection of CHAMBP (1.6 μmol kg^{-1}) or AHBuBP (8 μmol kg^{-1}) elevated HDC activity in both bone and spleen. In these experiments, C12MBP was inactive in elevating HDC activity. In our study, weekly injections of CHAMBP and AHBuBP, at doses of 1.6 and 8 μmol kg^{-1}, respectively, worsened the arthritis. These results suggest that the ability of aminoBPs to induce HDC activity may be related to their worsening effect on arthritis.

As described above, aminoBPs have the ability to increase macrophages and granulocytes. They can even increase osteoclasts, although the activity and morphological properties of these osteoclasts are impaired (Endo *et al.*, 1993). BM21.0955, an N-alkyl derivative of ABHuBP and the most potent BP in terms of its ability to inhibit bone resorption, also increases osteoclasts (Mühlbauer *et al.*, 1991). Because the precursor cells of osteoclasts, macrophages and granulocytes are known to be identical, these results suggest that aminoBPs may stimulate the precursor cells.

As described in the Introduction, histamine may be involved in haematopoiesis, including the formation of macrophages and granulocytes. Substances with adjuvant activity such as lipopolysaccarides and SEPS can also increase HDC activity in the bone marrow and spleen (Endo *et al.*, 1992b; Ando *et al.*, 1984). Freund's complete adjuvant also increases HDC activity in these organs (data not shown). Therefore, it is likely that the enhanced HDC activity (leading to enhanced histamine formation) induced by aminoBPs or by their combination with substances capable of inducing HDC, activity, may be responsible, at least in part, for the stimulation of the precursor cells described above. This would promote the formation of macrophages and granulocytes, contributing to an enhanced inflammatory reaction.

C12MBP has been shown to be highly cytotoxic to macrophages and osteoclasts in the presence of bone particles *in vitro* (Chambers, 1980; Flanagan & Chambers, 1989). Moreover, intravenous injection of C12MBP encapsulated in liposomes can eliminate macrophages (Van Rooijen & Nieuwmegen, 1984; Van Rooijen *et al.*, 1990; Endo *et al.*, 1995). Reitsma *et al.* (1982) have demonstrated that the cytotoxicity of C12MBP is greater than that of AHPrBP, and the ability of liposome-encapsulated C12MBP to decrease macrophages is far greater than that of AHPrBP (Van Rooijen & Kors, 1989). However, the inhibitory action of C12MBP on bone resorption is 1/10 or less that of AHPrBP (Mühlbauer *et al.*, 1991). Therefore, in addition to its inhibition of bone resorption, it seems possible that the cytotoxic action of C12MBP may be involved in its suppressive effect on arthritis. For example, C12MBP (a strongly acidic substance) might conceivably bind to basic components such as histones (or their fragments) derived from degraded cells around arthritic tissues or to other basic materials, and such complexes might then be ingested by phagoctyic macrophages or granulocytes with a cytotoxic effect on these cells. Dunn *et al.* (1993a, b) proposed a similar idea; namely, that the antiinflammatory action of C12MBP may be related to a prevention of the development and subsequent recruitment of monocytes (i.e. macrophages).

As to the effects of BPs on human rheumatoid arthritis, only that of AHPrBP has been reported (Table 4). Ralston *et al.* (1989) and Tan *et al.* (1989) have found that it is not effective in restricting the progression of periarticular bone erosion, although the bone resorption was suppressed. These results seem to be in agreement with those of our animal experiments. On the other hand, Bijvoet *et al.* (1980) and Maccagno *et al.* (1994) reported AHPrBP was effective in suppressing rheumatoid arthritis. By way of an explanation for this discrepancy, Maccagno *et al.* (1994) pointed out differences between the groups in terms of dose and period of administration. However, we think it is more relevant that the former two groups used intravenous infusion of a small dose, whereas the latter two groups used oral administration of a large dose. Indeed, Adami *et al.* (1987) have shown that intravenous injection of aminoBPs, but not of C12MBP, induces inflammatory responses in human patients. We, therefore, suspect that the de-aminated metabolites of AHPrBP (i.e. non-aminoBPs), possibly produced in the liver after a large dose given by the oral route, may be involved in the reported suppression of the arthritis.

Unexpectedly, in the present study there was a severe destruction of the bone around the joints of the arthritic mice treated with CHAMPB, AHBuBP or even C12MBP. It is of considerable interest why such bone destruction occurs, even where there is a strong inhibition of physiological bone resorption in the growth plate. Ralston *et al.* (1989) discussed the possibility that cells other than osteoclasts might be involved in the erosion of the bone in rheumatoid arthritis. We suggest that macrophages and/or granulocytes, in addition to their inflammatory roles in soft tissues, may also have a role in the destruction of bone.

Finally, in the clinical application of aminoBPs, the closest attention must be paid to their inflammatory actions, since these could exacerbate autoimmune diseases, such as rheumatoid arthritis. Moreover, it should also be noted that bisphosphonates accumulate in the bone almost irreversibly (Lin *et al.*, 1991). Therefore, clinical doses must be lowered as low as possible. Fortunately, some aminoBPs can suppress bone resorption at much lower doses than those used in the present study (Mühlbauer *et al.*, 1991), and recently it was shown that oral administration of low doses of AHBuBP is effective in suppressing bone resorption in patients with osteoporosis (Liberman *et al.*, 1995).

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