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Contrasting effects of an aminobisphosphonate, a potent inhibitor of bone resorption, on lipopolysaccharide-induced production of interleukin-1 and tumour necrosis factor α in mice

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1 Aminobisphosphonates (aminoBPs), potent inhibitors of bone resorption, have been reported to induce inflammatory reactions such as fever and an increase in acute phase proteins in human patients, and to induce the histamine-forming enzyme, histidine decarboxylase, in mice. In the present study, we examined the effect of aminoBP, 4-amino-1-hydroxybutylidene-1,1-bisphosphonic acid (AHBuBP), on the production of the pro-inflammatory cytokines, IL-1 and TNFα, in mice.

2 Intraperitoneal injection of AHBuBP did not itself produce detectable levels of IL-1 (α and β) and TNFα in the serum. However, the elevation of serum IL-1 induced by lipopolysaccharide (LPS) was greatly augmented in mice injected with AHBuBP 3 days before the LPS injection, whereas the LPS-induced elevation of serum TNFα was almost completely abolished.

3 Spleen and bone marrow cells taken from mice injected with AHBuBP produced IL-1β *in vitro* spontaneously, and the production was augmented following the addition of LPS. Cells that accumulated in the peritoneal cavity in response to AHBuBP produced a particularly large amount of IL-1β. However, AHBuBP treatment of mice did not lead to an impairment of the *in vitro* production of TNFα by these three types of cells.

4 Liposomes encapsulating dichloromethylene bisphosphonate (a non-amino BP) selectively deplete phagocytic macrophages. When an intraperitoneal injection of these liposomes was given 2 days after an injection of AHBuBP, there was a marked decrease in the LPS-induced elevation of serum IL-1 (α and β) (LPS being injected 3 days after the injection of AHBuBP).

5 These results indicate that AHBuBP has contrasting effects on the *in vivo* LPS-induced production of IL-1 and TNFα in mice, enhancing the production of IL-1 by phagocytic macrophages and suppressing the production of TNFα, although underling mechanisms remain to be clarified.

Keywords: Bisphosphonate; IL-1; TNFα; macrophages; lipopolysaccharide; histamine; histidine decarboxylase

Introduction

Bisphosphonates (BPs) strongly inhibit bone resorption, and a variety of derivatives of BPs have been developed in the search for treatments for enhanced bone resorption, such as occurs in Paget's disease, tumoral osteolysis, tumoral hypercalcaemia, osteoporosis and rheumatoid arthritis (Bonjour *et al*., 1994; Geddes *et al*., 1994). Among these derivatives, the aminobisphosphonates (aminoBPs) exhibit a particularly strong activity level. However, undesirable inflammatory reactions, including fever, occur in human patients after administration of aminoBPs (Adami *et al*., 1987). Indeed, fever has been documented in many papers and involvement of some cytokines has been suggested (see References in Sauty *et al*., 1996).

We have reported that a single intraperitoneal injection of aminoBPs into mice, admittedly in doses larger than those used clinically, induces an elevation of the activity of the histamine-forming enzyme, histidine decarboxylase (HDC), and an increase in the number of granulocytes and macrophages in the blood, spleen, bone marrow and peritoneal cavity (Endo *et al*., 1993; Nakamura *et al*., 1996).

Interleukin-1 (IL-1) and tumour necrosis factor α (TNFα) are endogenous pyrogens (Dinarello, 1991) and are both capable of inducing HDC activity in mice (Endo *et al*., 1986; 1992; Endo, 1989; Endo & Nakamura, 1992). In the present study, we examined the effect of 4-amino-1-hydroxybutylidene-1, 1-bisphosphonic acid (AHBuBP), a typical aminoBP, on the *in vivo* production of IL-1α, IL-1β and TNFα in mice.

Methods

Mice and reagents

Male BALB/c mice (6–7 weeks old) were obtained from the Mouse Centre of our University. All experiments complied with the Guidelines for Care and Use of Laboratory Animals in Tohoku University. 4-Amino-1-hydroxybutylidene-1,1-bisphosphonic acid (AHBuBP) and dichloromethylene bisphosphonate (Cl_2MBP) were synthesized by ourselves and dissolved in sterile saline. The pH of the solutions of AHBuBP was adjusted to 7.0 with NaOH, the solutions were passed through a filter (0.4 μm), and they were injected intraperitoneally (0.1 ml 10 g^{-1}). Lipopolysecchariide (LPS) from *Escherichia coli* O55:B5, prepared by Boivin's method, was purchased from Difco Lab. (Detroit, MI, U.S.A.). The lethal dose of AHBuBP and the dose needed to induce maximum HDC activity are approximately 160 μmol kg^{-1} (52 mg kg^{-1}) and 40 μmol kg^{-1}, respectively (Endo *et al*., 1993). In the present study, therefore, we examined the effect of this agent at 40 μmol kg^{-1}.

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Assay of HDC activity

HDC activity was assayed by a previously described method (Endo *et al*., 1993). HDC activity in each tissue was expressed as nmol of histamine formed h^{-1} by the enzyme contained in 1 g of the tissue (nmol h^{-1} g^{-1}). HDC activity in the bone marrow was expressed as the activity in 1 g of the tibia plus femur, because both these bones were subjected to the assay for HDC activity (Endo *et al*., 1993).

Determination of cytokines by ELISA

Preparation of serum Blood was collected directly into test tubes following decapitation of mice. Serum was recovered by centrifugation at $2000 \times g$ at 4°C, then stored at −80°C until used.

Preparation of culture supernatant Spleen and bone marrow cells were prepared by a method currently in common use (Dennert *et al*., 1990). In brief, spleens were minced in phosphate buffered saline (PBS) and filtered through cotton gauzes to obtain single cell suspensions. Bone marrow cells were collected by flushing femur and tibia with PBS. Peritoneal exudate cells were obtained as follows. Sterile saline (10 ml) was injected into the peritoneal cavity of ether-anaesthetized mice, the cavity was massaged and the suspension of cells recovered. Cells were centrifuged and re-suspended in RPMI 1640 medium (5×10^6 cells ml^{-1}) supplemented with 10% foetal calf serum, 5×10^{-5} M 2-mercaptoethanol, 0.2 mM glutamine, 0.1 mM non-essential amino acids, 100 iu ml^{-1} penicillin and 100 μg ml^{-1} streptomycin, with or without LPS (10 μg ml^{-1}). The cell suspension was distributed into 96-well flat-bottomed plates (0.2 ml^{-1} per well, 1×10^6 cells per well). After incubation at 37°C for the indicated times in a humidified atmosphere containing 5% CO_2, the culture medium was centrifuged at $10{,}000 \times g$ at 4°C. The supernatant was collected and stored at −80°C until used.

Determination of cytokines IL-1α, 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 assay of each sample was carried out in duplicate. The amount of each cytokine was expressed as pg or ng ml^{-1} of serum or culture medium.

Depletion of macrophages

Van Rooijen and his co-workers developed a macrophage 'suicide' technique, using liposomes encapsulating Cl_2MBP (Van Rooijen & Sanders, 1994). This method has been shown to be specific for phagocytic cells of the mononuclear phagocyte system and, within a day or two of the intravenous injection of such liposomes into mice or rats, phagocytic macrophages have been depleted almost completely (Van Rooijen & Sanders, 1994, 1996; Salkowski *et al*., 1995). A suspension of liposomes encapsulating Cl_2MBP (Cl_2MBP-liposomes) was prepared by a method similar to that used by Van Rooijen & Sanders (1994), as described previously (Endo *et al*., 1995). Briefly, 75 mg of phosphatidylcholine and 11 mg of cholesterol were dissolved in chloroform in a round-bottomed flask. The thin film that formed on the walls of the flask after rotary evaporation at 37°C was dispersed by gentle shaking for 10 min in 10 ml of Cl_2MBP solution (200 mg ml^{-1}) in 10 mM sodium phosphate buffer (pH 7.4). This suspension was kept for 2 h at room temperature, then sonicated for 3 min (50 Hz) and kept for another 2 h. To the resulting suspension of liposomes, PBS was added to give a final volume 50 ml and mixed gently. Then, the suspension was centrifuged at $2000 \times g$ for 5 min. The precipitated liposomes were finally suspended in 4 ml of PBS. We have confirmed that within 24 h of a single intravenous injection of 0.2 ml of this suspension, a complete depletion of F4/80 positive cells (mature macrophages) occurs in the liver and splenic red pulp, though these cells are not significantly affected in the lung (Endo *et al*., 1995).

Data analysis

Experimental values are given as means ± s.d. The statistical significance of differences was analysed using Dunnett's multiple comparison test, and P values less than 0.05 were considered to be significant.

Results

Potentiation of LPS-induced HDC activity in AHBuBP-injected mice

As described in our previous study (Endo *et al*., 1993), AHBuBP produces a long-lasting elevation of HDC activity, the maximum elevation occurring about 3 days after the i.p. injection of AHBuBP. However, we could not detect IL-1 (both α and β) or TNFα in the serum of control mice or of mice given an i.p. injection of AHBuBP, even after 3 days (data not shown).

Next, we decided to examine the effect of AHBuBP on the LPS-induced production of IL-1 and TNFα, because LPS is a potent inducer of these cytokines and HDC activity. By measuring HDC activity as an index, we tried to establish the time at which the production of these cytokines might be augmented. It should be noted that in contrast to the effect of AHBuBP, the induction of HDC by LPS is transient, the maximum elevation occurring about 4 h after the injection of LPS (Endo, 1989). The value shown with a closed circle at time 0 in Figure 1 is the elevated HDC activity at 4 h after the injection of LPS alone. As also shown in Figure 1, HDC activity (measured at 4 h after the injection of LPS) was greatly augmented in AHBuBP-injected mice, the effect being at its

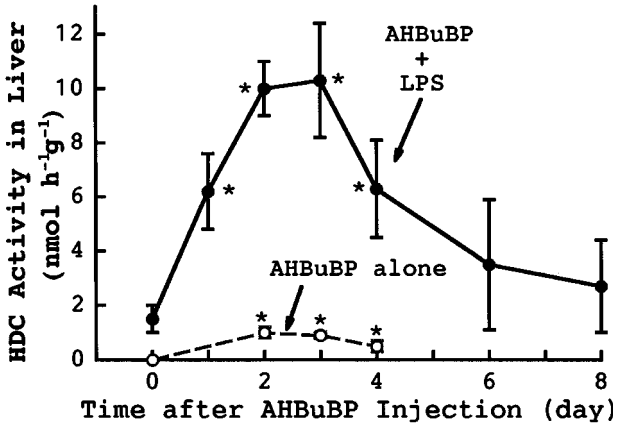

Figure 1 Effect of interval after AHBuBP injection on the LPS-induced elevation of HDC activity in the liver. AHBuBP (40 μmol (13 mg) kg^{-1}) was injected i.p. At various times after this injection (abscissa), LPS (100 μg kg^{-1}) was injected i.p. into one group of mice and they were sacrificed 4 h later. Each value is the mean ± s.d. from four mice. *$P < 0.01$ vs time 0.

greatest when LPS was injected 2 or 3 days after the injection of AHBuBP. This corresponds to the time at which the elevation of HDC activity induced by AHBuBP is itself at a maximum (Figure 1 and Endo *et al*., 1993). In the next set of experiments, therefore, we examined the effect of AHBuBP on the LPS-induced production of IL-1 and TNFα at 3 days after the injection of AHBuBP.

Potentiation of LPS-induced IL-1 production and impairment of the production of TNFα in AHBuBP-injected mice

As mentioned above, IL-1 and TNFα were below detectable levels in the serum of both control unstimulated mice and AHBuBP-injected mice. However, their levels in control mice were elevated within a few h of an LPS injection, as shown in Figure 2 (broken lines), the levels being elevated to about 30 pg of IL-1β at 4 h and to about 700 pg of TNFα at 2 h. In AHBuBP-injected mice (solid lines), the LPS-induced elevation of IL-1β was greatly augmented, the maximum elevation (to 120 pg) occurring at 2 h after the injection of LPS. In contrast, the LPS-induced elevation of TNFα was largely abolished (decreased to about 100 pg) in AHBuBP-injected mice.

Production of IL-1 and TNFα in vitro *by cells from AHBuBP-injected mice*

To try to identify the cells responsible for the production of IL-1 in AHBuBP-injected mice, the production of IL-1 by the cells of the spleen, bone marrow and peritoneal cavity was examined *in vitro* (Figure 3). The spleen cells and bone marrow cells from mice injected with AHBuBP spontaneously produced a slightly higher level of IL-1β than cells from control saline-injected mice (left-hand panels, Figure 3). Further, as shown in the right-hand panels in Figure 3, in response to LPS these cells all produced a larger amount of IL-1β, the cells from AHBuBP-injected mice producing a markedly higher level of IL-1β than cells from the control saline-injected mice when both were stimulated by LPS.

As mentioned previously (Endo *et al*., 1993), i.p. injection of AHBuBP into mice results in an accumulation of granulocytes and macrophages in the peritoneal cavity; a total of $2-3\times10^7$ cells per mouse are recovered. In the present study, it was found that the cells that accumulated in the peritoneal cavity produced an extremely high level of IL-1β both spontaneously and in response to LPS (Figure 3). In this study, we did not carry out this experiment using peritoneal cavity cells from control mice, because very few cells (less than 1×10^6 cells per mouse) were recovered.

As mentioned above, the LPS-induced elevation of TNFα in the serum was suppressed almost completely in AHBuBP-injected mice (Figure 2). However, there was no evidence of such a suppression by AHBuBP of the *in vitro* production of TNFα by the cells from the three sources; that is to say, there was no marked difference between saline-injected and AHBuBP-injected mice (data not shown).

Effect of macrophage depletion on the LPS-induced production of IL-1 and TNFα in control and AHBuBP-injected mice

Figure 4 shows the effect of Cl_2MBP-liposomes (which deplete phagocytic macrophages) on the production of IL-1α, IL-1β and TNFα in control mice. The LPS-induced production of IL-1α and TNFα was reduced by an i.p. injection of the liposomes 24 h before the LPS injection, but the LPS-induced production of IL-1β was not reduced.

In AHBuBP-injected mice, the intraperitoneal injection of Cl_2MBP-liposomes caused a decrease in the LPS-induced production of both IL-1α and IL-1β (Figure 5). In these experiments, it was also confirmed that the LPS-induced

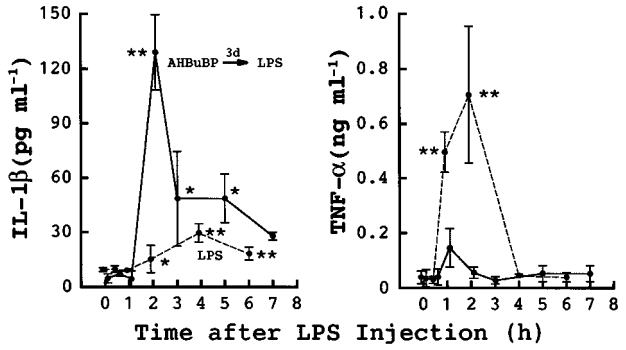

Figure 2 Effects of LPS on the levels of IL-1β and TNFα in the serum of normal and AHBuBP-injected mice. LPS (100 μg kg^{-1}) was injected i.p. into mice 3 days after injection of AHBuBP (40 μmol (13 mg) kg^{-1}) and blood was collected at the indicated times (solid line). Control mice received LPS without a prior injection of AHBuBP (broken line). Each value is the mean ± s.d. from three mice. $^*P<0.05$; $^{**}P<0.01$ vs time 0.

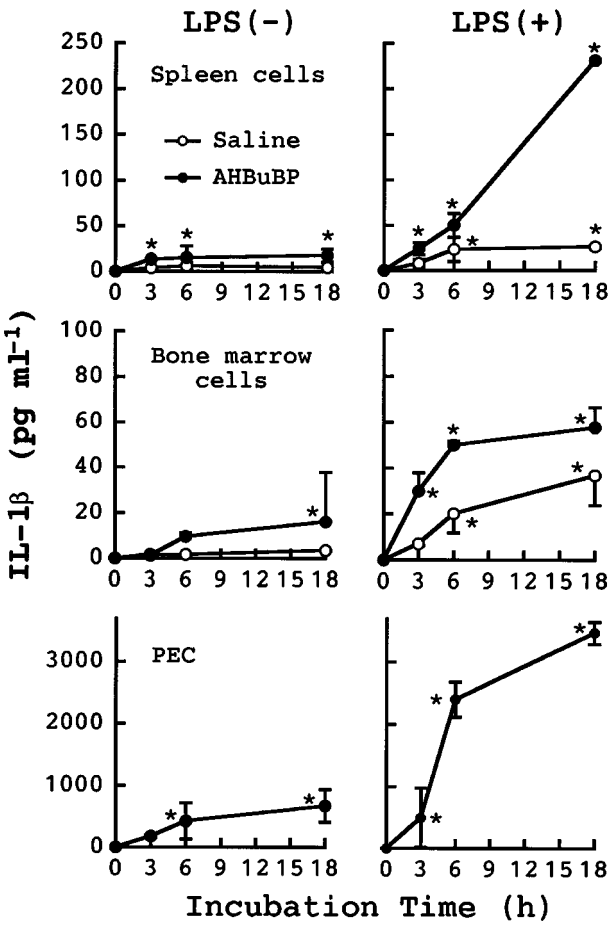

Figure 3 *In vitro* production of IL-1β by spleen cells, bone marrow cells and peritoneal exudate cells (PEC). Cells were taken from saline-injected (control) or AHBuBP-injected mice and incubated with (right-hand panels) or without (left-hand panels) LPS. The experiment was carried out as described in Methods. Each value is the mean ± s.d. from three mice. Note different vertical scales. $^*P<0.01$ vs time 0.

production of IL-1 was greatly augmented in AHBuBP-injected mice: from about 25 pg–130 pg for IL-1α and from about 15 pg–85 pg for IL-1β (data in Figures 4 and 5).

In these experiments, the almost complete prevention by AHBuBP of the LPS-induced production of TNFα (see Figure 2) was also confirmed. In fact, the LPS-induced levels of TNFα were about 950 pg (Figure 4) in control mice and only about 40 pg (Figure 5) in AHBuBP-injected mice. In AHBuBP-injected mice, Cl_2MBP-liposomes caused a recovery in the LPS-induced TNFα production: from about 40 pg–230 pg (Figure 5). In fact, this value was approximately the same as that seen in control mice treated with Cl_2MBP-liposomes and then with LPS (compare Figure 5 with Figure 4).

Discussion

In the present study, we found that the LPS-induced elevation of serum IL-1 (both α and β) was markedly augmented in AHBuBP-injected mice. By contrast, the LPS-induced elevation of serum TNFα was almost completely prevented in AHBuBP-injected mice. *In vitro* experiments using cells from the spleen, bone marrow and peritoneal cavity showed that there was a slight spontaneous production of IL-1β by these cells in AHBuBP-injected mice and that their production of IL-1β was greatly augmented by LPS. In particular, the cells that had accumulated in the peritoneal cavity of AHBuBP-injected mice produced a

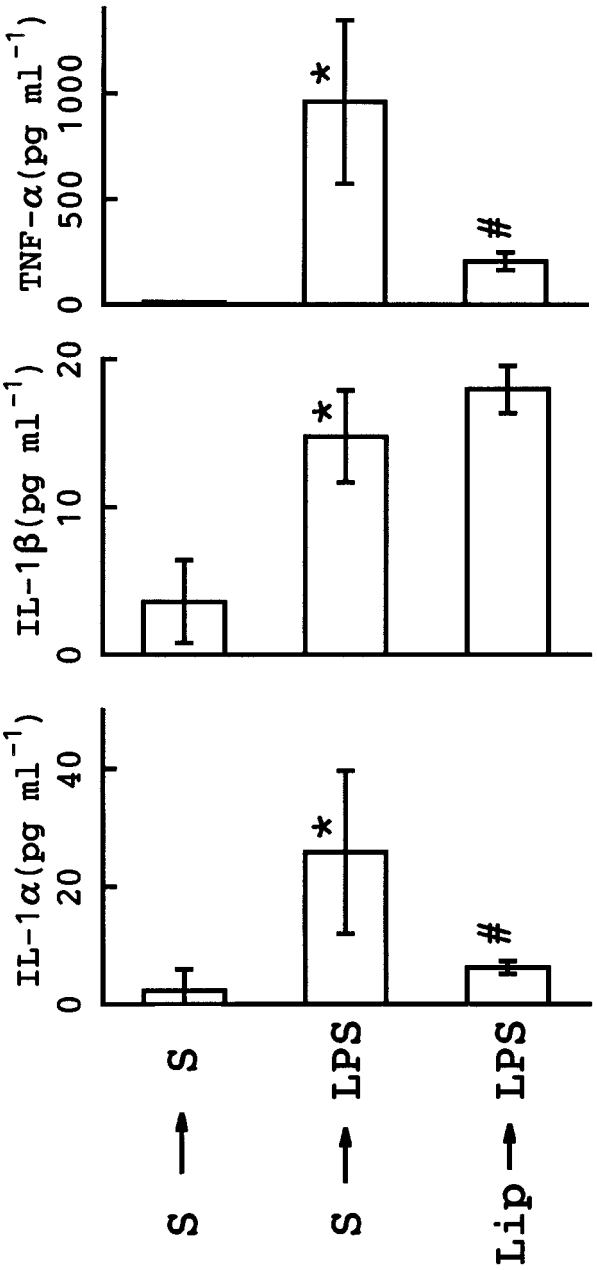

Figure 4 Effect of a depletor of phagocytic macrophages on the LPS-induced production of cytokines in normal mice. Cl_2MBP-liposomes (Lip) or saline (S) was injected i.p. (0.2 ml per mouse). The next day, LPS (100 μg kg^{-1}) or S was injected i.p. into the mice, blood was collected 2 h later, and serum cytokines were assayed. Each value is the mean ± s.d. from three mice. *$P<0.001$ vs S + S, #$P<0.01$ vs S + LPS.

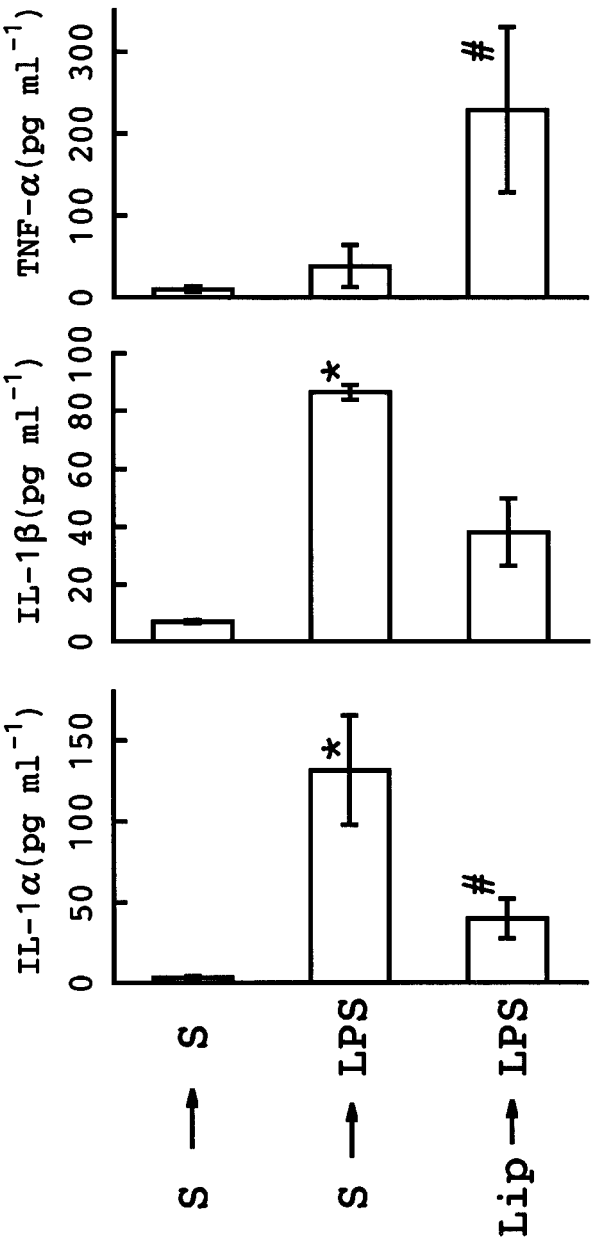

Figure 5 Effect of a depletor of phagocytic macrophages on the LPS-induced production of cytokines in AHBuBP-treated mice. On day 0, AHBuBP (40 μmol (13 mg) kg^{-1}) was injected i.p. into all groups. Two days later (day 2), Cl_2MBP-liposomes (Lip) or saline (S) was injected i.p. (0.2 ml^{-1} mouse). On day 3, LPS (100 μg kg^{-1}) or S was injected i.p.into the mice, blood was collected 2 h later, and serum cytokines were assayed. Each value is the mean ± s.d. from three mice. *$P<0.01$ vs S + S, #$P<0.01$ vs S + LPS.

remarkably large amount of IL-1β both spontaneously and in response to LPS.

As shown in Figure 4, in control mice (not treated with AHBuBP), the elevations of both IL-1α and TNFα induced by the i.p. injection of LPS were markedly decreased by i.p. injection of Cl_2MBP-liposomes (which specifically deplete phagocytic macrophages). This result suggests that the production of these cytokines induced by i.p. injection of LPS was mainly a function of phagocytic macrophages. On the other hand, the production of IL-1β seen in response to LPS in control mice seems to be from different cells, because its production was not reduced by the injection of Cl_2MBP-liposomes.

In mice treated with AHBuBP, a large number of cells accumulated in the peritoneal cavity, and these cells produced a high level of IL-1β *in vitro* when they were stimulated with LPS. The time course of the change in the serum level of IL-1β *in vivo* did not correspond to that observed in *in vitro* experiments, possibly because (a) the *in vivo* level of IL-1β is determined by both the rates of its formation and degradation, and (b) the *in vitro* data in the present study is likely to represent an accumulation of IL-1β. Therefore, the findings that (i) an extremely high production of IL-1β by the peritoneal cells, (ii) an accumulation of a large number of these cells ($2-3\times10^7$ cells per mouse) and (iii) a marked effect of i.p injection of Cl_2MBP-liposomes in decreasing IL-1 (α and β) in mice treated with AHBuBP, support the idea that the increased population of phagocytic macrophages in the peritoneal cavity in AHBuBP-treated mice may be responsible for the production of both IL-1α and IL-1β.

Intravenous injection of LPS into AHBuBP-treated mice resulted in an elevation of serum IL-1 (both α and β) that was 2–3 times greater than that induced by its i.p. injection into such mice (data not shown). In the former case, spleen cells and bone marrow cells, in addition to the cells in peritoneal cavity, may also have been involved in the marked production of IL-1, because, in the *in vitro* experiment, these cells showed that they produced a larger amount of IL-1 in AHBuBP-injected mice than in control mice.

The suppression of TNFα production by AHBuBP *in vivo* was not reproduced in the *in vitro* experiments on cells taken from mice treated with AHBuBP. Therefore, it seems likely that the suppressive effect seen *in vivo* is indirect, or possibly an unknown factor is involved in this action of AHBuBP, although we have no additional data explaining this phenomenon. In addition, at present, we cannot explain why Cl_2MBP-liposomes caused a recovery in LPS-induced TNFα production in AHBuBP-injected mice (Figure 5).

In mice, aminoBPs induce inflammatory reactions and a prolonged elevation of HDC activity. IL-1 and TNFα are widely recognized as pro-inflammatory cytokines, and we have shown that these cytokines are capable of inducing HDC in various tissues in mice (Endo, 1989; Endo *et al*., 1992). In addition, we have recently reported that repeated injections of a smaller dose of aminoBPs, including AHBuBP, exacerbate the arthritis induced in DBA/1 mice by a type II collagen (Nakamura *et al*., 1996). Interestingly, Hom *et al*. (1988, 1990) have reported that prolonged treatment with IL-1β accelerates the development of the arthritis induced by collagen in DBA/1 mice or in MRL/lpr mice. These results strongly suggest that a prolonged production of IL-1 might be an important underlying cause of the aminoBP-induced inflammation and exacerbation of arthritis as well as of the increased HDC activity. In the present study, however, we could not detect IL-1 and TNFα in the serum of mice injected with AHBuBP alone. On this basis, the inflammatory actions of AHBuBP cannot be attributed to these cytokines. However, the observation that cells taken from AHBuBP-injected mice produced IL-1 spontaneously might suggest that a very slight but prolonged elevation of IL-1 *in vivo* is responsible for the inflammatory actions of AHBuBP. To clarify these issues, we are now trying to detect the mRNAs of these cytokines and we are conducting experiments using IL-1 deficient mice. Our preliminary results suggest that IL-1 is the key cytokine responsible for the inflammatory actions of aminoBPs (including AHBuBP).

Recently, Sauty *et al*. (1996) showed that there is a very slight increase in the plasma levels of IL-6 and TNFα in patients treated with aminoBPs, suggesting that these cytokines may be causally involved in the fever and increase in acute phase proteins induced in human patients by aminoBPs. Although we cannot explain the difference between their results and ours, the two studies indicate that aminoBPs do have an effect on the production of pro-inflammatory cytokines in human patients and mice.

In conclusion, our results indicate that AHBuBP has contrasting effects on the *in vivo* production of IL-1 and TNFα in mice, enhancing the production of IL-1 by phagocytic macrophages and suppressing the production of TNFα, although the mechanisms remain to be clarified.

Note added in proof

The finding described in this paper had been reported by Sugawara *et al*. at the 4th Conference of the International Endotoxin Society, October 22–25, 1996, Nagoya, Japan. Abstracts in *J. Endotoxin Res*. 3-S1:55, 1996.

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