Effect of Liposomal and Free Bisphosphonates on the IL-1β, IL-6 and TNFα Secretion from RAW 264 Cells in Vitro

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Received September 21, 1994; accepted January 9, 1995

Purpose. In order to evaluate the possible antiinflammatory action of bisphosphonates, the effect of the drugs on the secretion of proinflammatory cytokines (IL-1β, IL-6 and TNFα) from macrophages was studied. Liposomes or high concentration of extracellular calcium was used to enhance the intracellular delivery of bisphosphonates. ***Methods.*** RAW 264 cells were used as macrophage model, and they were induced with lipopolysaccharide to produce the cytokines. The cytokine concentrations in the culture supernatants were measured with time-resolved fluoroimmunoassay. ***Results.*** As a free drug, clodronate and pamidronate, but not etidronate, inhibited LPS-stimulated secretion of the cytokines from macrophage-like RAW 264 cells. Low concentrations of pamidronate, however, induced the IL-6 secretion, and the cytokine inhibitory action at the higher concentrations of pamidronate was attributed to cytotoxicity of the compound. The cytokine induction or toxic effects were not observed with clodronate or etidronate. When the drugs were encapsulated in negatively charged unilamellar liposomes, the inhibitory potency of both clodronate and etidronate enhanced by a factor of 10-20, while that of pamidronate was not increased. The complex formation of bisphosphonates with extracellular calcium, although enhancing the uptake of the compounds by macrophages, did not considerably increase their cytokine inhibitory potency. ***Conclusions.*** Bisphosphonates have inhibitory action on cytokine secretion by macrophages. The non-cytotoxic cytokine inhibition by liposome encapsulated clodronate could be beneficial in local inflammatory diseases, where the inflammation is sustained by the excessive amounts of inflammatory cytokines produced by activated macrophages.

KEY WORDS: bisphosphonates; liposomal drug delivery; macrophages; proinflammatory cytokines.

INTRODUCTION

Bisphosphonates are pyrophosphate analogues which inhibit osteoclastic bone resorption in disorders characterized by excessive bone loss. Clinically they have been used to treat ectopic calcification, Paget's disease, osteoporosis, and tumour-induced osteolytic bone disease (for review, see ref. 1). Bisphosphonates bind avidly to hydroxyapatite and accumulate mainly in calcified tissue in vivo, and, in normal conditions, soft tissues are exposed to the compounds for only a short period (2). The mechanism of action of bisphosphonates in bone resorption is not clear, but it is thought to be intracellular (1), and the uptake of bisphosphonates by bone resorbing osteoclasts is mediated probably by bone mineral when the cells engulf drug coated bone (3).

Liposomes have proved to be effective carriers of bisphosphonates to macrophages and other highly endocytotic cells in vitro (4,5). As a free drug, high concentration of bisphosphonates is required to arrest the growth of RAW 264 macrophages, while the encapsulation of the drugs in negatively charged unilamellar liposomes enhances their potency by a factor of 20-1000. High extracellular calcium also mediates the uptake of bisphosphonates by phagocytic cells in vitro (4,5). In vivo, liposome-encapsulated clodronate (dichloromethylene bisphosphonate) eliminates the macrophages from spleen, liver, lungs, and lymph nodes of mice when administered via the appropriate routes (6).

The macrophage suppressive effect of liposomal clodronate has evoked an interest on the possible use of bisphosphonates in inflammatory diseases, like rheumatoid arthritis (7), where macrophages and macrophage derived proinflammatory cytokines (interleukins 1 and 6; IL-1, IL-6, tumor necrosis factor α; TNFα) are involved in pathological processes (8). This approach is strongly supported by our preliminary findings that liposomal clodronate inhibits IL-6 and TNFα secretion by macrophages (9), and by the finding that clodronate possesses antiarthritic activity in animal models of arthritis (10).

IL-1β, IL-6 and TNFα are multifunctional cytokines with widely overlapping activities (11). These inflammatory cytokines have a central role in the pathology of chronic inflammatory diseases (12,13), and, further, potent antiinflammatory agents, like glucocorticoids, inhibit effectively the cytokine production and cytokine mediated inflammation (14). Thus, the inhibition of the cytokine production and secretion is a valuable marker for potential antiinflammatory drugs. In this study we have evaluated the effects of three bisphosphonates; clodronate, etidronate (1-hydroxyethylidene-1,1-bisphosphonate) and pamidronate (3-amino-1-hydroxypropylidene bisphosphonate), on IL-1β, IL-6 and TNFα secretion by activated macrophage-like RAW 264 cells in vitro, using liposomes and extracellular calcium as mediators of intracellular delivery of the compounds.

MATERIALS AND METHODS

Encapsulation of Bisphosphonates in Liposomes

The stock solutions of bisphosphonates were prepared by dissolving the drugs in deionized water at the concentration of 200 mM of clodronate and etidronate (Leiras Pharmaceutical Co., Tampere, Finland), and 100 mM of pamidronate (Ciba-Geigy, Basel, Switzerland). The pH of the solutions was adjusted to 7.2 by sodium bicarbonate powder or 1N hydrochloric acid and they were filter sterilized (0.2 μm syringe filter, Gelman, Ann Arbor, MI).

The bisphosphonates were encapsulated in liposomes made from distearoylphosphatidylglycerol (DSPG) (Orion Farmos Co., Turku, Finland) and cholesterol (Sigma, St. Louis, MO) (67:33) using reverse-phase evaporation (15), as described elsewhere (4,5), and are subsequently referred to

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as DSPG-liposomes. The lipid content of liposomes was measured using phosphorus assay (16), and the bisphosphonate concentration in the liposomes was determined spectrophotometrically using the property of bisphosphonates to form a chromophoric complex with copper(II) ions (5).

Cell Culture and Cytokine Induction

The murine macrophage-like RAW 264 cells were originally obtained from T.D. Heath, University of Wisconsin, Madison, WI. The cells were grown in Dulbecco's Modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) and 100 IU/ml penicillin and streptomycin in 7% CO_2 atmosphere at 37°C. In the cytokine experiments the cells were diluted to 10^6 cells/ml and dispensed to 96-well plates, 200 μl/well (2×10^5 cells/well). The cells were allowed to adhere for 2 hours and the non-adherent cells were washed away. Fresh medium with or without extra calcium ($CaCl_2$, final concentration 4.5 mM) was added to the cells, and the cells were treated with liposome encapsulated (0.3-100 μM) or free bisphosphonates (3-1000 μM for etidronate and clodronate, and 1-300 μM for pamidronate). These concentrations of the drugs were chosen for this study on the basis of the growth inhibition studies with RAW 264 cells (5). The effect of nonloaded liposomes at lipid concentrations used for drug delivery was also tested. After an overnight incubation, the cells were washed free of drugs and serum free DMEM supplemented with 10 μg/ml of lipopolysaccharide (LPS) (E.Coli, serotype 0127:B8, Sigma) was added to the wells. The plates were incubated for additional 24 hours in 37°C, and the cell supernatants were collected, centrifuged and stored at −80°C. The cell viability was evaluated with MTT (3,(4,5)-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide) (Sigma) assay (17) after the treatment with the drugs and after the LPS-induction.

Assay for IL-1β, IL-6 and TNFα

The cytokines were analysed using a non competitive immunoassay based on dissociation-enhanced lanthanide fluoroimmunoassay, which will be described in detail elsewhere (Pennanen et al., manuscript submitted). In short, the microtiter strips (Nunc, Roskilde, Denmark) were coated with the capture antibody, 1 μg/ml of IL-6 (Pharmingen, San Diego, CA), or 2 μg/ml of IL-1β (Immugenex, Los Angeles, CA) or 2 μg/ml of TNFα (Pharmingen, San Diego, CA), in 0.1 M sodium carbonate buffer pH 9.6 by incubating 100 μl/well for 2 h at room temperature in an orbital shaker at 200 rpm. After three washings the non-specific binding was blocked with 200 μl of 1% BSA in 50 mM TRIS-HCl-buffer containing 150 mM NaCl (pH 7.5) at room temperature for 1 h, and the washings were repeated. The samples and the standards in the assay buffer were added to the strips, 200 μl/well, and the strips were incubated at 4°C overnight. After washings, the biotinylated second antibody (250 ng/ml of IL-6, 500 ng/ml of TNFα or 5 μg/ml of IL-1β) was added to the wells in 200 μl of the assay buffer. After 1 h incubation at room temperature, the strips were washed once, and 200 μl of Eu-labelled streptavidin (100 ng/ml, Wallac/Pharmacia, Turku, Finland) in the assay buffer was dispensed to the wells. The strips were incubated for 30 minutes at room temperature with 30 rpm shaking, and washed six times. Europium was rendered fluorescent by releasing it from the streptavidin with enhancement solution (Wallac/Pharmacia). After equilibration the fluorescence was measured with LKB Wallac 1230 Arcus fluorometer (Wallac/Pharmacia). The standard curves were generated using spline smoothed algorithm of the RIA Calc software of the instrument.

RESULTS

Effect of Free and Calcium Bound Bisphosphonates on Cytokine Secretion

Free clodronate and pamidronate inhibited IL-1β, IL-6 and TNFα secretion from macrophages (Figs. 1 and 2). At the highest concentration (300 μM), pamidronate blocked almost totally the secretion of the cytokines, TNFα being the most sensitive (Fig. 2). Pamidronate was about 10 times more potent than clodronate in inhibiting IL-1β and TNFα, whereas for IL-6 they were about equipotent (Table I). Low concentrations of pamidronate augmented the IL-6 secretion from macrophages over 50% compared to untreated LPS-stimulated cells used as a control (Fig. 2). Clodronate affected the IL-6 secretion most effectively suppressing it almost completely at 1000 μM concentration (Fig. 1). On the contrary, free etidronate did not affect significantly the cytokine secretion from the cells at the concentrations studied (Fig. 3).

Etidronate and pamidronate were approximately two times more potent inhibitors of IL-6 secretion in calcium supplemented medium (Ca-conc. 4.5 mM) than in normal medium (Ca-conc. 1.8 mM) (Table I), but the potentiation of the effect on other cytokines was not observed. Extra calcium could not enhance the effect of free clodronate on cytokine secretion (Table I).

The viability of RAW 264 cells was tested both after drug treatments and subsequent LPS stimulation. The viability of the cells was over 80% after all the drug treatments (data not shown). Figure 4 shows the viability of RAW 264 cells after the exposure to bisphosphonates and subsequent LPS-induction. The LPS-treatment did not produce any additional decrease in cell viability, when the cells were pretreated with clodronate or etidronate (Fig. 4A and B). However, pamidronate sensitized the cells to a toxic effect of LPS (Fig. 4C), suggesting that the decrease of cytokine secretion by pamidronate was due to cell death. The results presented here are from one experiment, but similar results were obtained from two parallel experiments.

Effect of Liposome-Encapsulation on the Potency of the Bisphosphonates

Liposome-encapsulated clodronate was over ten times more potent inhibitor of cytokine secretion from RAW 264 cells than the free drug (Fig. 1, Table I). The inhibitory potency of etidronate was also considerably increased for IL-1β and IL-6, but not for TNFα (Fig. 3, Table I), whereas the potency of pamidronate could not be enhanced by liposome-encapsulation (Fig. 2, Table I).

The viability of the macrophages after the treatment with liposomal clodronate and etidronate before (data not shown) and after LPS-induction was over 70% (Fig. 4A and

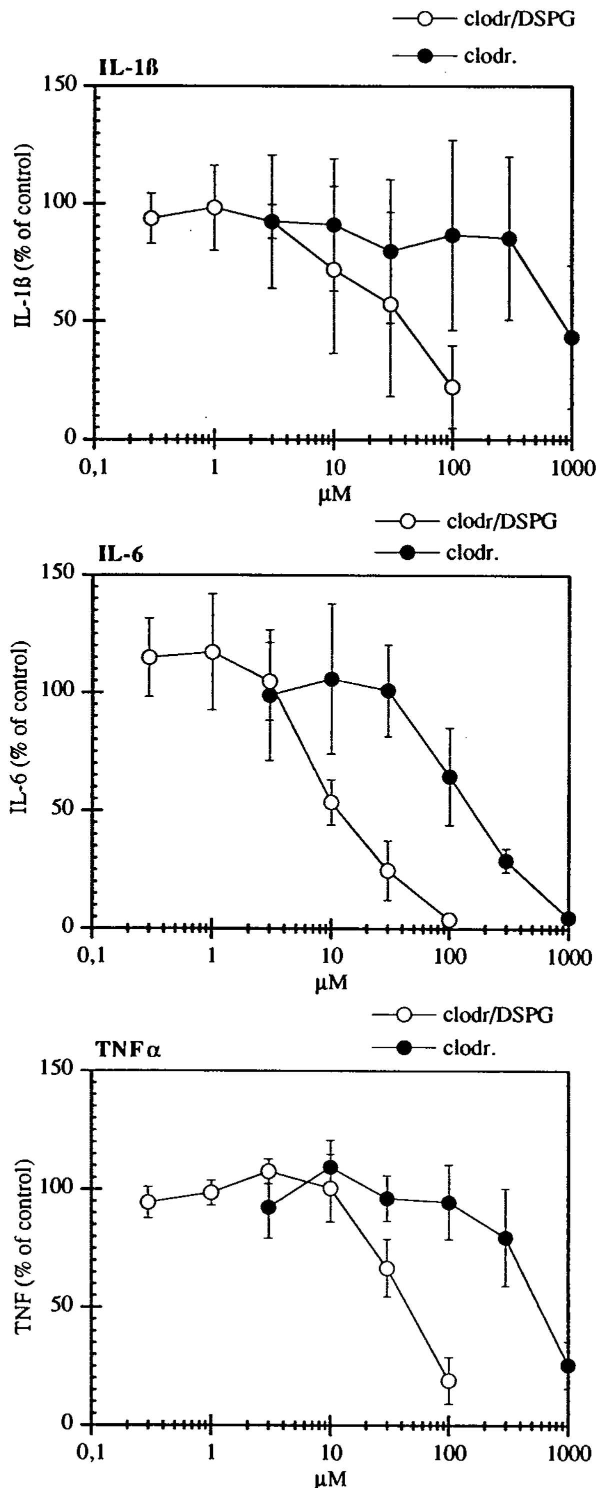

Fig. 1. The effect of free clodronate and clodronate encapsulated in DSPG-liposomes (clodr/DSPG) on the IL-1β, IL-6 and TNFα secretion from RAW 264 cells. The results are average from three separate experiments (mean±SD). The cells (2×10^5/well) were preincubated with the drugs for 20 h, and, thereafter, were incubated for 24 h with LPS (10 μg/ml) supplemented DMEM. The untreated LPS-stimulated cells (control) produced IL-1β 2.3±0.6 ng/ml, IL-6 30.4±14.7 ng/ml and TNFα 399.5±238.0 ng/ml (mean±SD, n=7).

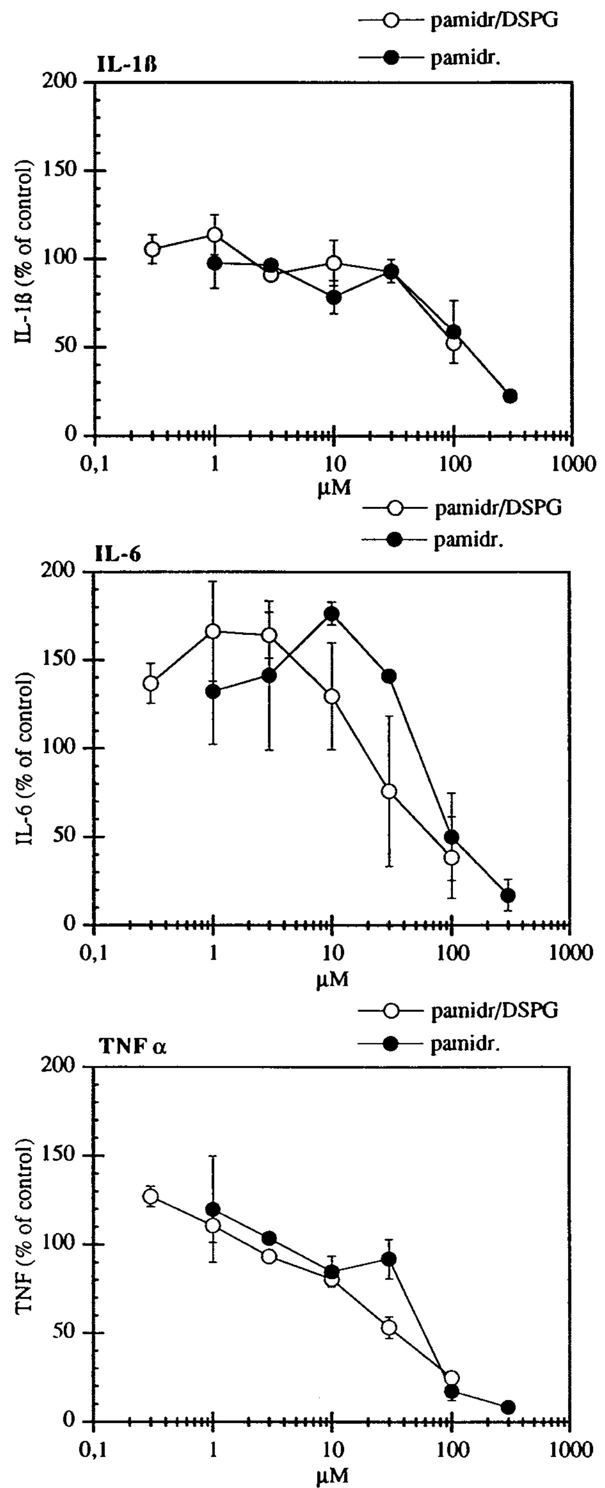

Fig. 2. The effect of free pamidronate and pamidronate encapsulated in DSPG-liposomes on IL-1β, IL-6 and TNFα secretion from RAW 264 cells. The results are average from two separate experiments (mean±SD). Details in Fig. 1.

B), demonstrating that the effect on the cytokine secretion was not due to the cytotoxic effects of the drugs. Liposomal pamidronate itself was only slightly toxic for the cells (data not shown), but sensitized the cells to the toxic effects of LPS (Fig. 4C).

Table I. The Potency of Bisphosphonates in Suppressing LPS-Induced Cytokine Secretion from RAW 264 Cells in Vitro[a]

	IC_{50} (μM)[b] (mean ± SD)		
	Encapsulated	Free	Free + Ca^{2+} (4.5 mM)
IL-1β			
Clodronate	44 ± 28	>1000	>1000
Etidronate	33 ± 3	>1000	>1000
Pamidronate	>100	126 ± 35	>300
IL-6			
Clodronate	12 ± 4	153 ± 55	120 ± 46
Etidronate	51 ± 4	>1000	540 ± 260
Pamidronate	>100	119 ± 37	64 ± 6
TNFα			
Clodronate	46 ± 11	568 ± 151	582 ± 288
Etidronate	>100	>1000	>1000
Pamidronate	34 ± 5	59 ± 2	47 ± 12

[a] The cells (2×10^5/well) were preincubated with free drugs in normal and calcium (4.5 mM) supplemented DMEM and with drugs in DSPG-liposomes (DSPG:cholesterol 67:33) for 20 h, whereupon, they were incubated with DMEM containing LPS 10 μg/ml for 24 h. Data represents mean ± SD from three (clodronate) or two (etidronate, pamidronate) separate experiments.

[b] The concentration required to produce 50% inhibition of cytokine secretion.

The effect of nonloaded DSPG-liposomes on the cytokine secretion at the lipid concentrations used for the drug delivery is shown in Figure 5. Low lipid concentrations increased the IL-1β and IL-6 secretion to about 50% over the amount produced by control cells, but the level of TNFα increased only slightly. Negatively charged phospholipids have previously been shown to augment the release of TNF from LPS triggered macrophages at low concentrations (18), and this effect was also seen here. Taking into account the molar drug/lipid-ratios of the liposomes (clodronate for 1.33, etidronate for 1.5 and pamidronate for 0.75), the lipid used for the clodronate and etidronate delivery (65-75 μM at most) was not involved in observed cytokine inhibition by these compounds. However, the lipid concentration (133 μM) of pamidronate/DSPG-liposomes at the highest drug concentration (100μM) could partially inhibit the IL-1β secretion by the macrophages. The viability of the cells was over 70% at every lipid concentration studied (data not shown).

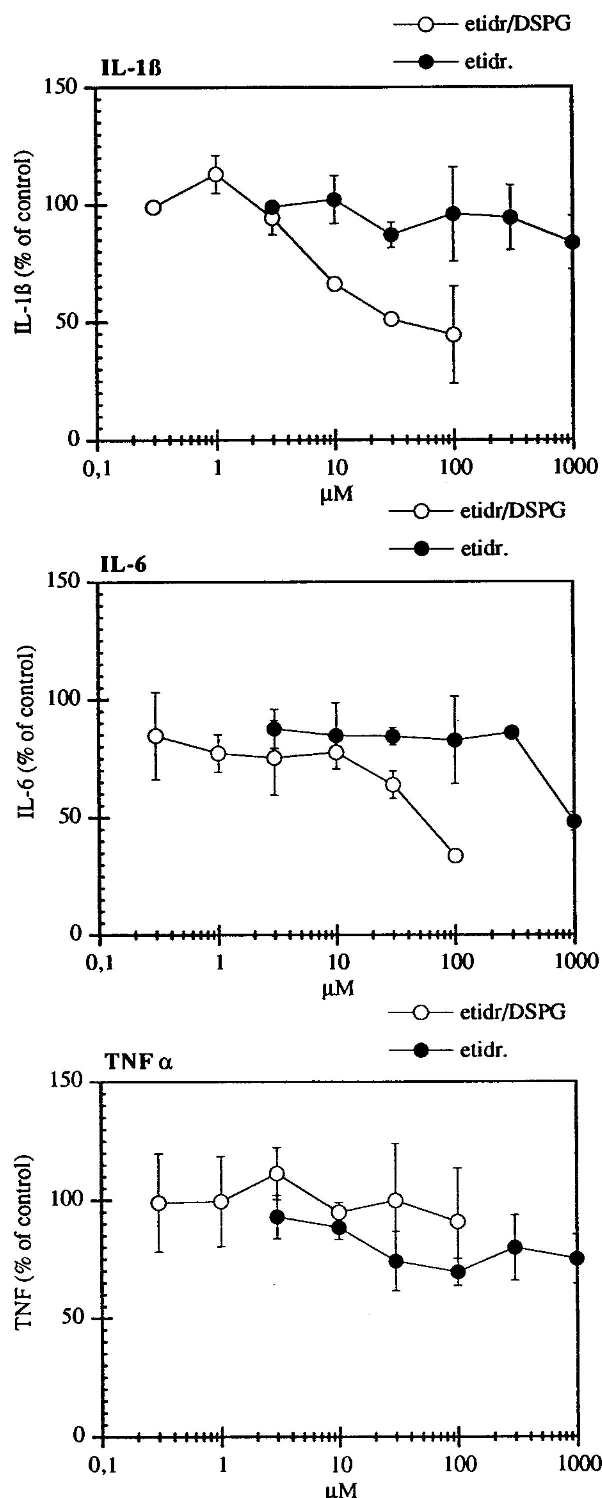

Fig. 3. The effect of free etidronate and etidronate encapsulated in DSPG-liposomes on IL-1β, IL-6 and TNFα secretion from RAW 264 cells. The results are average from two separate experiments (mean±SD). Details in Fig. 1.

DISCUSSION

This study clearly demonstrates the inhibitory effect of clodronate and pamidronate on IL-1β, IL-6 and TNFα release by activated macrophages, while etidronate is less potent in this respect. The inhibition of macrophage IL-1 production by very high concentration (about 4 mM) of etidronate has been reported before (19), but the studies with other bisphosphonates have failed to show any inhibition of IL-1 like activity produced by macrophages (20). Recently, clodronate, etidronate, and aledronate have been shown to inhibit IL-1 and TNF induced IL-6 production by human osteoblast-like cells (21). In addition to our preliminary report (9), this is the first report on the cytokine inhibitory activity of bisphosphonates on macrophages.

The present findings strongly support the idea of use of bisphosphonates as macrophage suppressors in chronic in-

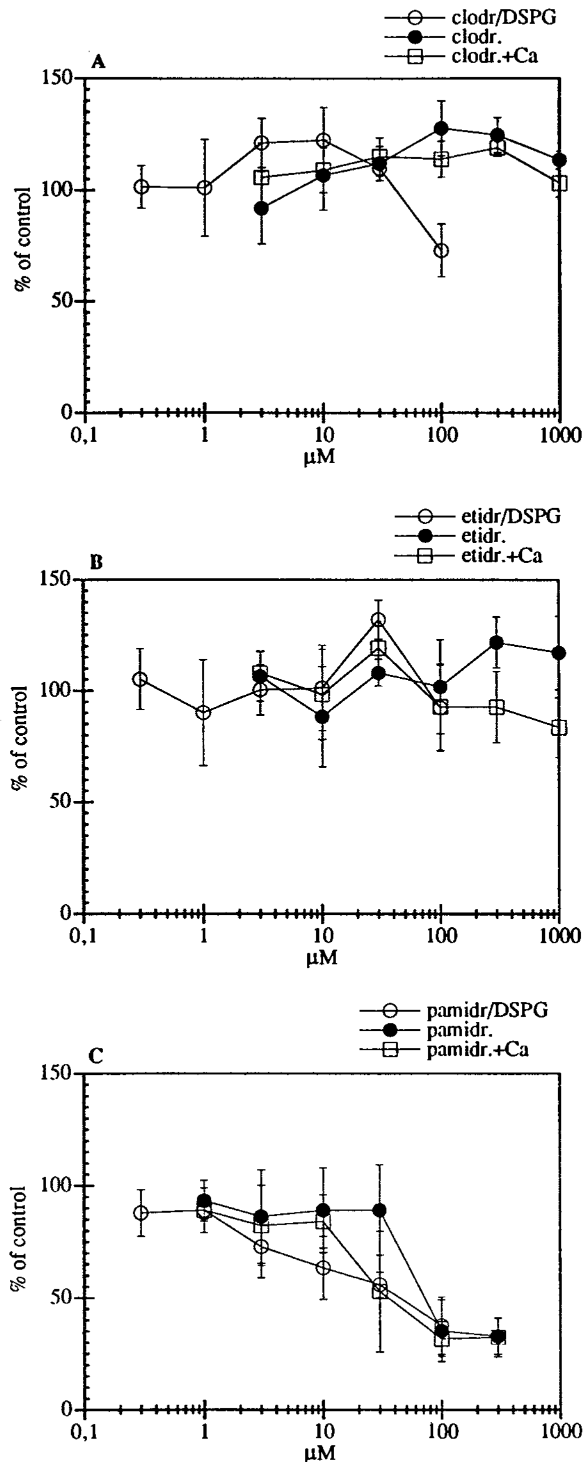

Fig. 4. The viability of RAW 264 cells after the pretreatment with free bisphosphonates in normal and calcium supplemented medium (+Ca), or with the drugs encapsulated in DSPG-liposomes, and subsequent LPS (10μg/ml) induction. The viability of the cells was studied with MTT-assay after 20 hr exposure of the cells to drugs and subsequent LPS-induction for additional 24 hr (mean±SD from one experiment carried out in quadruplicate). Similar results were obtained in two parallel experiments.

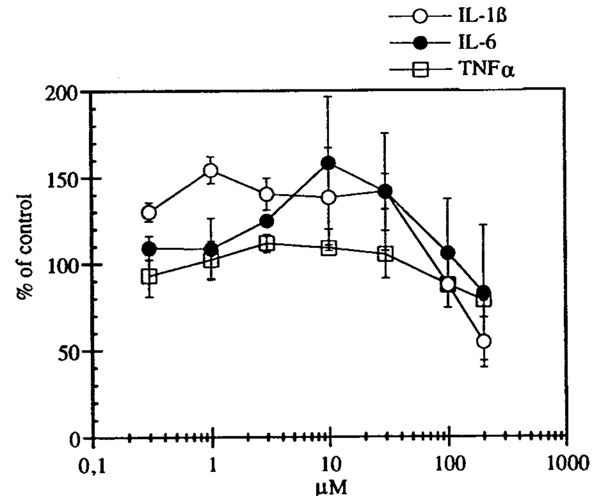

Fig. 5. The effect of nonloaded DSPG-liposomes at different lipid concentrations on IL-1β, IL-6 and TNFα secretion from RAW 264 cells. The results are average from two separate experiments (mean±SD). Details in Fig. 1.

flammatory diseases. Especially liposome-encapsulated clodronate seems to be promising in this approach, since it strongly inhibited all the three cytokines studied. The higher potency of liposomal clodronate and etidronate is explained by much more effective delivery of the drugs to cells. Free clodronate and etidronate do not readily enter the cells, while liposomal drugs are taken up through the endocytosis of liposomes (4,5), resulting in the stronger suppression of cytokine secretion. Interestingly, the effect of pamidronate was not enhanced by liposome-encapsulation, suggesting either different delivery of the compound as a free drug or different mechanism of action for pamidronate. Pamidronate is less water soluble than clodronate and etidronate, and it can contribute to a better diffusion of the compound into cells, consequently reducing the difference between the potencies of free and encapsulated drug (5). Further, the cytokine inhibition by pamidronate was attributed to the decreased viability of macrophages after the drug and LPS treatment, suggesting that pamidronate has secundary effect on cytokine secretion due to toxicity on macrophages, while liposomal clodronate appears to affect the cytokine secretion through some specific mechanism not due to cytotoxicity.

Although this study was not addressed to examine the antiresorptive activity of bisphosphonates, the results may have some relevance also to that issue. It has been proposed that bisphosphonates may act in vivo, in addition to their effect on osteoclast activity, by affecting monocyte-macrophage poiesis from bone marrow precursors and by decreasing the number and activity of bone resident macrophages, a source of cytokines stimulating bone resorption (22). The results show that at least clodronate and pamidronate can affect the function of activated macrophages in vitro. These compounds when released from resorbed bone by osteoclasts (23) can diffuse to the fluid around the bone tissue and, thereupon, affect bone resident macrophages and inhibit their cytokine production.

An interesting observation in this study was also the consistent increase in IL-6 production by macrophages by low concentrations of pamidronate, but not by clodronate

and etidronate. Pamidronate, unlike clodronate and etidronate, induces a transient fever and acute phase response in up to 50 % of patients, which has been suggested to involve the stimulation of macrophages or monocytes to release IL-1 and IL-6 (24, 25). The serum levels of IL-6 correlate well with the levels of acute phase proteins suggesting that IL-6 is one of the major inducers of these proteins. Hence, although the stimulation of IL-1β was not observed in this study, the increase in IL-6 secretion is in good accordance with the interpretation.

The mechanism of action of bisphosphonates in the cytokine inhibition remains to be determined. Bisphosphonates have high affinity for metal ions (e.g. calcium, iron), and the depletion of intracellular iron seems to be involved in the growth inhibitory action of clodronate in RAW 264 cells (4). Iron may have a role in the regulation of immune response and the disruption of iron homeostasis has suggested to be involved in chloroquine-induced inhibition of TNF production by macrophages (26). On the other hand, the intracellular calcium complexation by bisphosphonates may partially account for the inhibition of the cytokine release; bisphosphonates complexed with extracellular calcium (calcium supplemented medium) were not more potent IL-1β and TNFα inhibitors than the free drugs, although they are taken up by the cells more effectively, and are 10-20 more potent macrophage growth inhibitors than free drugs (5). On the contrary, noncomplexed liposomal clodronate and etidronate were more potent inhibitors of the cytokine secretion than the free or calcium complexed drugs, suggesting that the compounds effectively delivered to the cells in the form capable to chelate intracellular calcium affect the cytokine release most strongly. Whether the iron and/or calcium chelation in the cells, or some other(s), yet unknown mechanism(s), are involved in the cytokine inhibitory action of bisphosphonates, warrants further examination.

The elimination of macrophages in vivo with liposome encapsulated clodronate is believed to be mediated by the cytotoxicity of the drug (27). Recently, this application has been used to deplete the phagocytic lining cells from the mouse knee joint by intra-articular clodronate-liposome injection in an experimental arthritis, and this treatment has prevented the onset of inflammation in the joint (7). Since macrophage-like and fibroblast-like cells are highly activated in the synovium of an arthritic joint expressing high amounts of inflammatory cytokines (8, 28), liposomal clodronate could have beneficial effects on the inflammation by inhibiting the cytokine production already at sublethal doses without the depletion of macrophage population.

NOTATION

DSPG: distearoylphosphatidylgrycerol; IL: interleukin; LPS: lipopolysaccharide; MTT: (3,(4,5)-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide; TNF: tumor necrosis factor.

ACKNOWLEDGMENTS

The authors wish to thank M.Sc. Terhi Nissinen for technical assistance. The study was financially supported by Technical Development Centre of Finland (TEKES), the Finnish Ministry of Education, the Academy of Finland, and Rheumatism Research Foundation, Finland

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