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Actinobacillus actinomycetemcomitans Y4 Capsular-Polysaccharide-Like Polysaccharide Promotes Osteoclast-Like Cell Formation by Interleukin-1α Production in Mouse Marrow Cultures

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The mechanism of osteoclast-like cell formation induced by periodontopathic bacterium *Actinobacillus actinomycetemcomitans* Y4 (serotype b) capsular-polysaccharide-like polysaccharide (capsular-like polysaccharide) was examined in a mouse bone marrow culture system. When mouse bone marrow cells were cultured with *A. actinomycetemcomitans* Y4 capsular-like polysaccharide for 9 days, many multinucleated cells were formed. The multinucleated cells showed several characteristics of osteoclasts, including tartrate-resistant acid phosphatase (TRACP) and the ability to resorb the calcified dentine. In this study, we examined the effects of antisera to interleukins on the formation of osteoclast-like cells induced by *A. actinomycetemcomitans* Y4 capsular-like polysaccharide. Monospecific anti-mouse recombinant interleukin-1α (rIL-1α) serum completely inhibited the formation of osteoclast-like cells in the presence of *A. actinomycetemcomitans* Y4 capsular-like polysaccharide. However, anti-mouse rIL-1β and anti-mouse rIL-6 sera showed no effect on osteoclast-like cell formation. IL-1 receptor antagonist significantly inhibited the osteoclast-like cell formation mediated by *A. actinomycetemcomitans* Y4 capsular-like polysaccharide in mouse marrow cultures. The bioactive IL-1 was detected in the culture media of mouse bone marrow cells stimulated with *A. actinomycetemcomitans* Y4 capsular-like polysaccharide. These results indicate that IL-1α is involved in the mechanism of the formation of osteoclast-like cells induced by *A. actinomycetemcomitans* Y4 capsular-like polysaccharide. We sought to determine whether osteoclast-like cell formation induced by *A. actinomycetemcomitans* Y4 capsular-like polysaccharide could be modulated by the protein kinase inhibitors H8 and HA1004. The formation of osteoclast-like cells was suppressed by H8 and HA1004. These findings suggest that the signals by protein kinases may regulate osteoclast-like cell formation induced by *A. actinomycetemcomitans* Y4 capsular-like polysaccharide. Furthermore, a correlation between IL-1α and prostaglandin E_2 in the osteoclast recruitment induced by *A. actinomycetemcomitans* Y4 capsular-like polysaccharide is discussed.

Actinobacillus actinomycetemcomitans is a gram-negative, capnophilic, fermentative coccobacillus which has been implicated in the etiology and pathogenesis of several forms of periodontal disease (32). Clinical, microbiological, and immunological studies provide evidence of a correlation between *A. actinomycetemcomitans* and several types of periodontitis (19, 30). *A. actinomycetemcomitans* produces a multiplicity of tissue-damaging products such as leukotoxin (21, 25), collagenase (16), lipopolysaccharide (LPS), alkaline and acid phosphatases, an epitheliotoxin, a fibroblast-inhibitory factor, and a bone resorption-inducing toxin (19). Among these products, LPS and capsular materials from *A. actinomycetemcomitans* are potent mediators of bone resorption (8, 10, 29). However, the mechanism of bone resorption by capsular materials of *A. actinomycetemcomitans* is poorly understood.

Oral *A. actinomycetemcomitans* strains are serologically classified into five distinct groups, a through e (5, 17, 33). It has been reported that serotype b strains are recovered more frequently and may exhibit a greater periodontopathic potential than other serotype strains (30). Saarela et al. (17) indicated a rare simultaneous occurrence of multiple *A. actinomycetemcomitans* serotypes and the stability of infection by the same serotypes. Amano et al. (3) extracted a serotype-specific capsular-polysaccharide-like polysaccharide (capsular-like polysaccharide) antigen from whole cells of *A. actinomycetemcomitans* Y4 (serotype b) by autoclaving, purified it by ion-exchange chromatography and gel filtration, and showed that it is a polymer consisting of a disaccharide repeating unit, →3)-α-D-fucopyranosyl-(1→2)-β-L-rhamnopyranosyl-(1→. However, the periodontopathic mechanism of the serotype b-specific capsular-like polysaccharide is still unknown.

Sims et al. (18) reported that most juvenile periodontitis patients respond to infection by *A. actinomycetemcomitans* by producing serum antibodies. Localized juvenile periodontitis is characterized by alveolar bone loss mainly affecting the permanent first molars and incisors (31). The aim of this study was to examine the bone-resorbing activity of *A. actinomycetemcomitans* Y4 capsular-like polysaccharide in mouse bone marrow cultures. In addition, the role of interleukin-1 (IL-1) in the formation of osteoclast-like cells mediated by *A. actinomycetemcomitans* Y4 capsular-like polysaccharide was investigated. These studies will give us basic information on the severe alveolar bone loss in periodontal diseases.

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MATERIALS AND METHODS

Microorganisms. *A. actinomycetemcomitans* Y4 (serotype b) was used in this study. This strain was grown in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) supplemented with 1% (wt/vol) yeast extract at 37°C for 3 days in an atmosphere of 5% CO_2 in air (15). Organisms were harvested by centrifugation, washed three times with pyrogen-free water, and lyophilized.

Preparation of capsular-like polysaccharide. Capsular-like polysaccharide was extracted from lyophilized cells of *A. actinomycetemcomitans* Y4 by autoclaving (3). The extract was purified by chromatography on DEAE–Sephadex A-25 (Pharmacia Fine Chemicals, Piscataway, N.J.) and Sephacryl S-300 (Pharmacia) columns and lyophilized. No protein, D-glycero-D-mannoheptose, L-glycero-D-mannoheptose, 2-keto-3-deoxyoctulosonic acid, ribose, deoxyribose, hexosamine, or phosphorus was detected in the purified capsular-like polysaccharide. The material from *A. actinomycetemcomitans* Y4 was designated Y4 capsular-like polysaccharide.

Mice. Female C3H/HeJ mice (6 weeks old) were obtained from Shizuoka Laboratories Animal Center (Shizuoka, Japan).

Mouse bone marrow cultures. Bone marrow cells were isolated from C3H/HeJ mice as described previously (22). Marrow cells were suspended to a density of 10^6 cells per ml in alpha minimal essential medium (GIBCO Laboratories, Grand Island, N.Y.) containing 10% fetal calf serum and antibiotics. The cells (10^6 per well) were cultured with Y4 capsular-like polysaccharide in 24-well flat-bottom tissue culture plates (1 ml per well) at 37°C in an atmosphere of 5% CO_2 in air. Cultures were fed every 3 days by replacing 0.8 ml of old medium with fresh medium containing stimulants. After being cultured for 9 days, the adherent cells were fixed with 10% formalin in phosphate-buffered saline (PBS; pH 7.2) for 10 min and treated with ethanol-acetone (50:50, vol/vol) for 1 min. Fixed cells stained for tartrate-resistant acid phosphatase (TRACP) by incubation in 0.1 M sodium acetate buffer (pH 5.0) containing naphthol AS-MX phosphate (Sigma Chemical Co., St. Louis, Mo.) and red violet LB salt (Tokyo Kasei Industry Co., Tokyo, Japan) in the presence of 50 mM sodium tartrate. The results were expressed as means ± standard deviations (SD) of cultures. For inhibition studies, anti-mouse recombinant IL-1α (rIL-1α; Genzyme, Cambridge, Mass.), anti-mouse rIL-1β (R&D Systems, Minneapolis, Minn.), anti-recombinant tumor necrosis factor alpha (anti-rTNF-α; R&D Systems), anti-mouse rIL-6 (Boehringer GmbH, Mannheim, Germany), mouse IL-1 receptor antagonist (IL-1ra) (15), salmon calcitonin (Sigma), and polymyxin B sulfate (Sigma) were added to the test and control bone marrow cultures. The amount of prostaglandin E_2 (PGE_2) in the medium was determined by using a bicyclic PGE_2 ^3H radioimmunoassay kit (TRK 800; Amersham Corp, Little Chalfont, Buckinghamshire, England). Mouse bone marrow cells were cultured with Y4 capsular-like polysaccharide in the presence of various concentrations of *N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H8; Seikagaku Kogyo Co., Tokyo, Japan) and *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide dihydrochloride (HA 1004; Seikagaku Kogyo Co.). Percent inhibition was calculated as 100 × [(number of TRACP-positive multinucleated cells with Y4 capsular-like polysaccharide − number of TRACP-positive multinucleated cells with Y4 capsular-like polysaccharide and protein kinase C {PKC} inhibitor)/number of TRACP-positive multinucleated cells with Y4 capsular-like polysaccharide]. In some experiments, mouse bone marrow cells were cultured with Y4 capsular-like polysaccharide in the presence of dentine slices for 9 days. The slices were then fixed, treated with 0.1% trypsin (type I; Sigma) to remove attached cells, and washed. The dentine slices were examined by scanning electron microscopy to demonstrate the resorption pits.

RPMI 1788 assay. A human IL-1-dependent cell line, RPMI 1788 (5×10^2 cells), was cultured in 0.2 ml of RPMI 1640 medium (GIBCO) containing 5% fetal calf serum, antibiotics, 2 mM L-glutamine, 5×10^{-5} M β-mercaptoethanol, 1 mM sodium pyruvate, and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer with human rIL-1α (R&D Systems) or culture supernatants of mouse bone marrow cells. The supernatants from C3H/HeJ mouse bone marrow cells stimulated with Y4 capsular-like polysaccharide were serially diluted. Each dilution was then incubated with RPMI 1788 cells. Cultures were incubated for 116 h (28). Stock MTT (3-[4,5-dimethylthazol-2-yl]-2,5-diphenyl tetrazolium bromide; 2.5 mg/ml; Sigma) solution (20 μl per well) was added to the wells, and plates were incubated at 37°C for the final 4 h. Acid-isopropanol (100 μl of 0.04 N HCl in isopropanol) was added and mixed thoroughly to dissolve the dark blue crystals. After a few minutes at room temperature to ensure that all crystals were dissolved, the plates were read on an MPRA4 microplate reader (Tokyo Soda, Tokyo, Japan), using a test wavelength of 570 nm and a reference wavelength of 620 nm (12).

ELISA. We used the Inter Test-1αX mouse IL-1α and Inter Test-1βX mouse IL-1β enzyme-linked immunosorbent assay (ELISA) kits (Genzyme) to determine the amounts of IL-1α and IL-1β in culture supernatants of mouse bone marrow cells.

RESULTS

Osteoclast-like cell formation in mouse marrow cultures. No TRACP-positive multinucleated cells were detected in the untreated cultures during the entire culture periods (Fig. 1A). Addition of Y4 capsular-like polysaccharide (2.5 μg/ml) induced the formation of TRACP-positive multinucleated cells in C3H/HeJ mouse marrow cultures for 9 days (Fig. 1B, arrows). Y4 capsular-like polysaccharide induced the formation of TRACP-positive mononuclear and multinucleated cells in C3H/HeJ mouse marrow cultures in a dose-dependent manner (Table 1). Figure 2 shows the time course of change in the TRACP-positive multinucleated cell formation in C3H/HeJ mouse marrow cultures. The TRACP-positive multinucleated cells were induced by Y4 capsular-like polysaccharide (2.5 μg/ml) on day 6 and 9. TRACP-positive multinucleated cells which have three or more nuclei were defined as osteoclast-like cells and counted in the following experiments. We examined whether TRACP-positive osteoclast-like multinucleated cells derived from mouse mononuclear cells resorbed dentine. When mouse marrow cells were cultured with Y4 capsular-like polysaccharide on dentine slices for 9 days, resorption lacunae were formed on the surfaces (Fig. 1D). No resorption pits were detected on the dentine slices in the absence of Y4 capsular-like polysaccharide during the entire periods.

Monospecific anti-murine rIL-1α serum greatly inhibited osteoclast-like multinucleated cell formation and resorption lacuna formation on the dentine slices in the presence of Y4 capsular-like polysaccharide (2.5 μg/ml) (Fig. 1C and 3A). This antibody did not induce the formation of TRACP-positive mononuclear and multinucleated cells. Anti-mouse rIL-1β serum (1 μg/ml) completely inhibited osteoclast-like multinucleated cell formation induced by mouse rIL-1β (1 ng/ml). However, this antibody showed no effect on osteoclast-like multinucleated cell formation induced by Y4 capsular-like polysaccharide (2.5 μg/ml) (Fig. 3B). Anti-murine rIL-6 serum also showed no effect on osteoclast-like multinucleated cell formation induced by Y4 capsular-like polysaccharide (2.5 μg/ml). Anti-murine rTNF-α (10 μg/ml) serum partially inhibited Y4 capsular-like polysaccharide-induced osteoclast-like multinucleated cell formation (36% inhibition). Salmon calcitonin (10 ng/ml) inhibited TRACP-positive multinucleated cell formation induced by Y4 capsular-like polysaccharide (2.5 μg/ml). Polymyxin B sulfate showed no effect on the formation of osteoclast-like multinucleated cells in the presence of Y4 capsular-like polysaccharide at a concentration of 5 μg/ml, which inhibited TRACP-positive multinucleated cell formation induced by Y4 LPS (1 μg/ml). Figure 4 shows the effect of mouse IL-1ra on osteoclast-like multinucleated cell formation induced by Y4 capsular-like polysaccharide. Mouse IL-1ra dose dependently inhibited Y4 capsular-like polysaccharide-induced osteoclast-like multinucleated cell formation.

IL-1 and prostaglandin production by mouse marrow cultures. To elucidate the ability of IL-1 to induce the formation of osteoclast-like multinucleated cells, the amount of bioactive IL-1 in culture supernatants of C3H/HeJ mouse bone marrow cells stimulated with Y4 capsular-like polysaccharide (2.5 μg/ml) was determined by using RPMI 1788 cells. IL-1 was quantitated from a standard curve set up with known amounts of rIL-1α. We prepared serial dilutions of culture supernatants from C3H/HeJ mouse bone marrow cells stimulated with Y4 capsular-like polysaccharide (2.5 μg/ml) to compare the levels of IL-1. The amounts of bioactive IL-1 in culture supernatants of bone marrow cells stimulated with Y4 capsular-like polysaccharide on days 3, 6, and 9 were 84.2 ± 4.1, 96.0 ± 3.5, and 97.3 ± 6.1 ng/ml, respectively. The bioactive IL-1 in culture supernatants of nonstimulated C3H/HeJ mouse bone marrow cells was below the limit of detection in the RPMI 1788 assay. Furthermore, Y4 capsular-like polysaccharide did not induce the proliferation of RPMI 1788 cells. Monospecific anti-mouse rIL-1α serum completely inhibited the ability of RPMI 1788

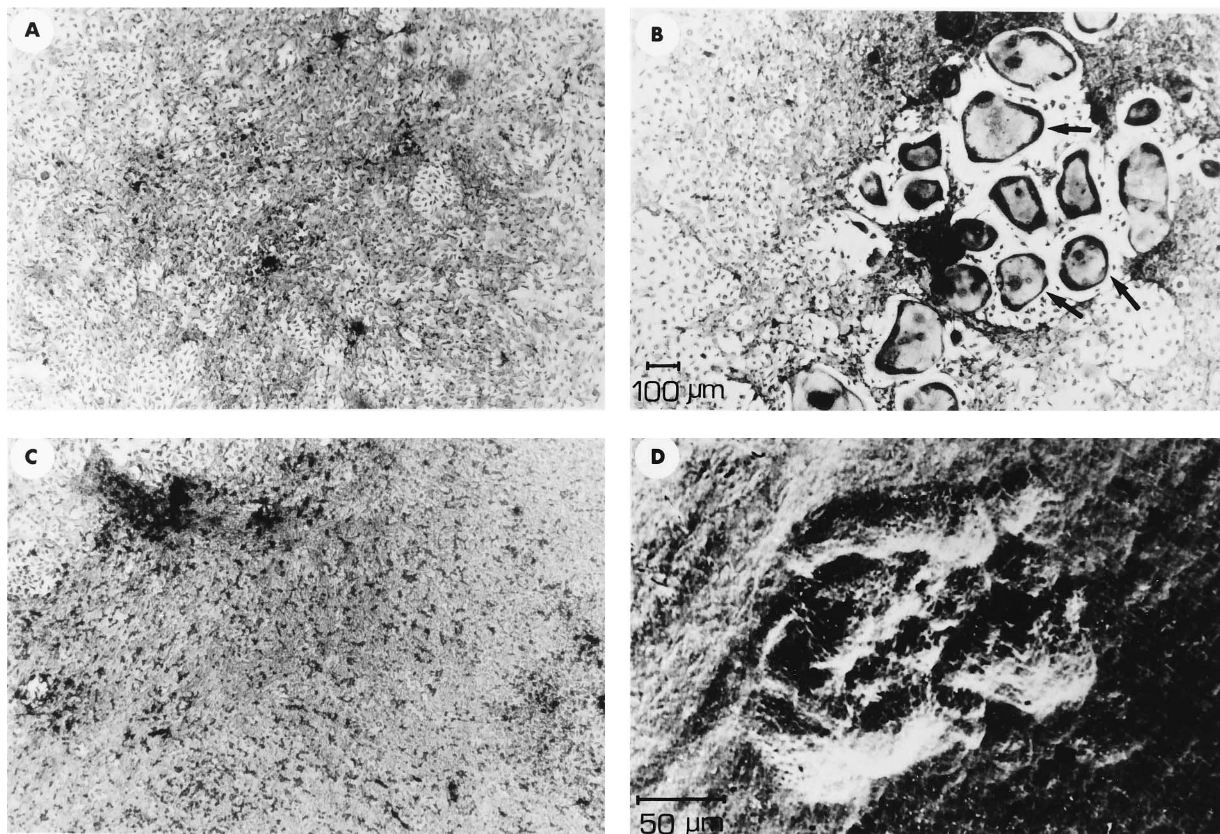

FIG. 1. TRACP-positive multinucleated cell formation in C3H/HeJ mouse bone marrow cultures. Mouse marrow cells (10^6 per well) were cultured without stimulant (A), with Y4 capsular-like polysaccharide (2.5 μg/ml) (B), or with Y4 capsular-like polysaccharide (2.5 μg/ml) and anti-mouse rIL-1α serum (1/1,000 dilution) (C). Mouse marrow cells (10^6 per well) were cultured with Y4 capsular-like polysaccharide (2.5 μg/ml) on a dentine slice for 9 days. The dentine slice was examined by scanning electron microscopy (D). Magnifications: A to C, ×125; D, ×200.

cells to proliferate in response to culture supernatants of bone marrow cells stimulated with Y4 capsular-like polysaccharide, whereas monospecific anti-mouse rIL-1β showed no effect on the proliferation of RPMI 1788 cells induced by culture supernatants of bone marrow cells stimulated with Y4 capsular-like polysaccharide (Fig. 5). The amounts of IL-1α and IL-1β in the culture supernatants were determined by ELISA. The amounts of IL-1α in culture supernatants of mouse bone marrow cells stimulated with Y4 capsular-like polysaccharide (2.5 μg/ml) on days 3, 6, and 9 were 107.3 ± 9.5, 115.3 ± 8.7, and 118.9 ± 10.3 ng/ml, respectively. In contrast, IL-1β levels in culture supernatants prepared under the same conditions were less than 0.1 ng/ml. The amounts of PGE_2 in culture supernatants of C3H/HeJ mouse marrow cells stimulated with Y4 capsular-like polysaccharide was also determined by radioimmunoassay. After incubation for 9 days, 193.5 ± 5.8 ng of PGE_2 per ml was detected in the culture supernatant of marrow cells stimulated with Y4 capsular-like polysaccharide. PGE_2 production by marrow cells stimulated with Y4 capsular-like polysaccharide

TABLE 1. TRACP-positive cell formation in C3H/HeJ mouse bone marrow cultures[a]

Y4 capsular-like polysaccharide (μg/ml)	No. of TRACP-positive cells/well	
	Mononuclear	Multinucleated
0	28.7 ± 3.7	0
0.1	67.7 ± 8.3	7.3 ± 1.2
1	92.2 ± 12.5	16.1 ± 2.2
2.5	138.3 ± 21.3	27.3 ± 3.9

[a] Mouse marrow cells (10^6 per well) were cultured with Y4 capsular-like polysaccharide, and the numbers of TRACP-positive mononuclear and multinucleated cells formed were calculated. Data are shown as the means ± SD of six cultures.

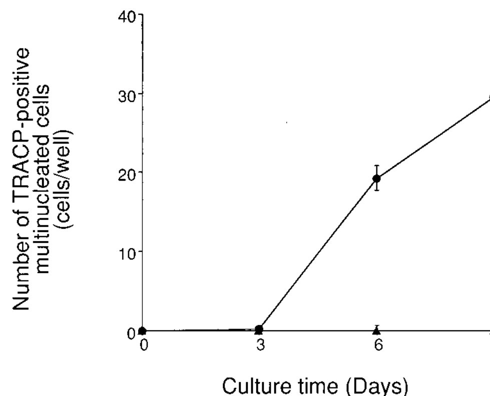

FIG. 2. Time course for the formation of TRACP-positive multinucleated cells in C3H/HeJ mouse bone marrow cultures. Mouse marrow cells (10^6 per well) were cultured with (●) or without (▲) Y4 capsular-like polysaccharide (2.5 μg/ml). At indicated times, cells were fixed and stained for TRACP. Data are expressed as the means ± SD of six cultures.

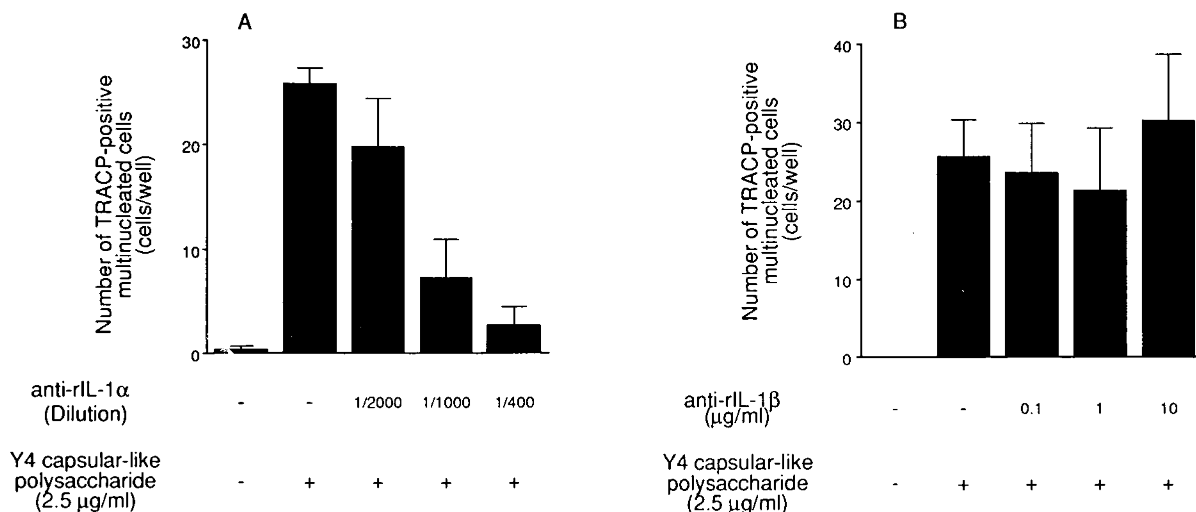

FIG. 3. Effects of antisera on the formation of TRACP-positive multinucleated cells induced by Y4 capsular-like polysaccharide (2.5 μg/ml) in C3H/HeJ mouse bone marrow cultures. Mouse bone marrow cells (10^6 per well) were cultured with Y4 capsular-like polysaccharide in the presence of various concentrations of anti-mouse rIL-1α serum (A) or anti-mouse rIL-1β serum (B). Data are expressed as the means ± SD of six cultures.

was inhibited markedly by the addition of anti-mouse rIL-1α during the culture periods (21.7 ± 3.0 ng/ml).

Role of PKC in the mechanism of osteoclast-like cell formation. We examined the effects of the two protein kinase inhibitors in C3H/HeJ mouse bone marrow cultures. H8 and HA1004 (specific inhibitors of cyclic AMP [cAMP]-dependent protein kinases) virtually inhibited the formation of osteoclast-like multinucleated cell formation induced by Y4 capsular-like polysaccharide (2.5 μg/ml) in a dose-related manner (Fig. 6). However, H8 and HA1004 did not affect the generation of PGE_2 and IL-1α in response to Y4 capsular-like polysaccharide in mouse marrow cultures.

DISCUSSION

We demonstrated previously that capsular-like polysaccharide purified from *A. actinomycetemcomitans* Y4 either induces osteoclastic bone resorption in mouse organ cultures or promotes osteoclast formation in mouse marrow cultures (27). Indomethacin, an inhibitor of prostaglandin synthesis, strongly inhibited the osteoclast-like multinucleated cell formation induced by Y4 capsular-like polysaccharide, suggesting that it stimulates osteoclast recruitment by a mechanism involving PGE_2. It is well known that LPS from *A. actinomycetemcomitans* can stimulate macrophages to secrete PGE_2 (6). It may be possible that the osteoclast-forming ability of *A. actinomycetemcomitans* capsular-like polysaccharide is the result of contamination with LPS. However, Y4 capsular-like polysaccharide did not exhibit *Limulus* amebocyte lysate clotting activity (23). It stimulated the osteoclast-like cell formation in LPS-nonresponsive C3H/HeJ mouse marrow cultures (Fig. 1B).

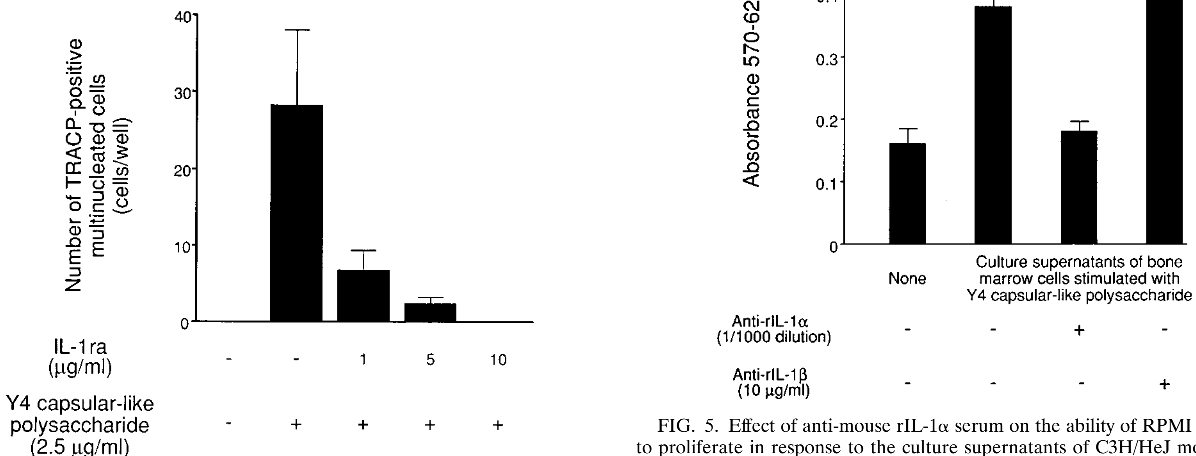

FIG. 4. Effect of mouse IL-1ra on the formation of TRACP-positive multinucleated cells induced by Y4 capsular-like polysaccharide (2.5 μg/ml) in C3H/HeJ mouse bone marrow cultures. Mouse bone marrow cells (10^6 per well) were cultured with Y4 capsular-like polysaccharide in the presence of various concentrations of IL-1ra. Data are expressed as the means ± SD of six cultures.

FIG. 5. Effect of anti-mouse rIL-1α serum on the ability of RPMI 1788 cells to proliferate in response to the culture supernatants of C3H/HeJ mouse bone marrow cells. Mouse bone marrow cells (10^6 per well) were cultured with Y4 capsular-like polysaccharide (2.5 μg/ml). The diluted samples (1/200 dilution) of the supernatants were incubated with RPMI 1788 cells in the presence or absence of antiserum to rIL-1α or rIL-1β, and its effect on the RPMI 1788 cell proliferation was determined by using the MTT reagent as described in Materials and Methods.

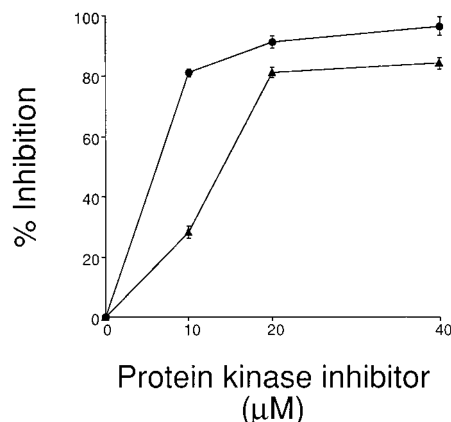

FIG. 6. Effects of PKC inhibitors on the formation of TRACP-positive multinucleated cells induced by Y4 capsular-like polysaccharide (2.5 μg/ml) in C3H/HeJ mouse bone marrow cultures. Mouse bone marrow cells (10^6 per well) were cultured with graded concentrations of H8 (●) or HA1004 (▲) in the presence of Y4 capsular-like polysaccharide (2.5 μg/ml). Percent inhibition was calculated as described in Materials and Methods. H8 and HA1004 (0 to 40 μM) had no effect on the viability of mouse marrow cells.

Moreover, polymyxin B sulfate (5 μg/ml) showed no effect on the formation of osteoclast-like multinucleated cells induced by Y4 capsular-like polysaccharide. These findings confirm that contamination with LPS from *A. actinomycetemcomitans* Y4 does not explain the ability of Y4 capsular-like polysaccharide to induce the formation of osteoclast-like multinucleated cells in mouse marrow cultures.

Osteoclasts are well known to be multinucleated cells formed by fusion of mononuclear precursors derived from hematopoietic progenitor cells (14). It is well known that the mouse TRACP-positive multinucleated cells have almost all of the characteristics of an authentic osteoclast (22). *A. actinomycetemcomitans* Y4 capsular-like polysaccharide strongly induced TRACP-positive mononuclear and multinucleated cells in mouse marrow cultures (Table 1). Takahashi et al. (22) reported that the addition of calcitonin to mouse marrow cultures in the presence of 1α,25-dihydroxyvitamin D_3 (10^{-8} M) decreased the number of TRACP-positive multinucleated cells but had no effect on the number of TRACP-positive mononuclear cell clusters, suggesting that calcitonin does not inhibit the differentiation of the precursor cells but selectively inhibits the fusion process of TRACP-positive mononuclear cells to form multinucleated cells. As shown in this study, salmon calcitonin almost completely inhibited the formation of TRACP-positive multinucleated cells induced by Y4 capsular-like polysaccharide. When mouse marrow cells were cultured on dentine slices in the presence of Y4 capsular-like polysaccharide, numerous resorption lacunae were formed on the surfaces (Fig. 1D). No resorption lacunae were detected on the dentine slice on which mouse bone marrow cells were cultured in the absence of Y4 capsular-like polysaccharide. The distribution of the resorption lacunae seemed to parallel the pattern of TRACP-positive multinucleated cells formed on the surface of the culture well. These findings satisfy the major criteria for osteoclasts, which act to resorb calcified bone by making Howship's lacunae (20, 26).

It is well recognized that networks of cytokines which immune cells produce may be important in the highly integrated control of normal bone remodeling. On the other hand, their excess production is linked to the bone destruction in inflammatory diseases such as rheumatoid arthritis and periodontal diseases (13). Several lines of evidence have suggested that IL-1 is a potent stimulator of bone resorption in vitro (6). It stimulates bone resorption at concentrations which are several orders of magnitude lower than that of any previously described inflammatory cytokine. Recently, we demonstrated that mouse IL-1ra purified from conditioned media of murine macrophage line $P388D_1$ cells blocks stimulation of bone resorption in organ cultures of newborn mouse calvaria and inhibits the osteoclast-like multinucleated cell formation in mouse bone marrow cultures, mediated by IL-1. The present study clearly demonstrates that anti-mouse rIL-1α serum and mouse IL-1ra inhibit the formation of osteoclast-like cells in the presence of Y4 capsular-like polysaccharide. In addition, we confirmed by RPMI 1788 assay and ELISA that the amount of IL-1α in culture supernatant was increased by adding Y4 capsular-like polysaccharide. When IL-1α and IL-1β were added to the bone marrow cultures in the presence of Y4 capsular-like polysaccharide, additional effects on the formation of osteoclast-like cells were found. These findings demonstrate that Y4 capsular-like polysaccharide may stimulate osteoclast recruitment by mechanisms involving IL-1α in mice. It has been shown that Y4 capsular-like polysaccharide can stimulate macrophage to secrete IL-1 (23). However, further work is needed to determine the source of the IL-1 activity in the presence of Y4 capsular-like polysaccharide in mouse marrow cultures.

Recently, we found that the amount of PGE_2 in culture supernatant is greatly increased by adding Y4 capsular-like polysaccharide at the latter term of culture for 9 days and that PGE_2 added at day 6, the latter term of 9-day culture, markedly increases the number of osteoclast-like multinucleated cells (27), suggesting that the bone-resorbing activity of Y4 capsular-like polysaccharide might be explained by its recruiting of new osteoclasts induced by PGE_2 at the latter term of culture. Furthermore, it is highly likely from the following evidence that IL-1α at the initial term of culture is an important key mediator of the osteoclast-like multinucleated cell formation mediated by capsular-like polysaccharide from *A. actinomycetemcomitans* Y4. First, the amount of bioactive IL-1 in culture supernatant was greatly increased by Y4 capsular-like polysaccharide at day 3, the initial term of 9-day culture. Second, when mouse marrow cells were cultured with Y4 capsular-like polysaccharide in the presence of anti-mouse rIL-1α serum, the amount of PGE_2 was strongly decreased, to 21.7 ± 3.0 ng/ml. Akatsu et al. reported that IL-1 stimulates osteoclast-like cell formation and PGE_2 production in mouse marrow cultures (2). This finding is in accord with our results wherein anti-mouse rIL-1α serum significantly suppressed the production of PGE_2 in mouse marrow cultures.

Ishimi et al. reported that bone-resorbing agents such as IL-1α, IL-1β, TNF-α, and LPS induce IL-6 mRNA expression in osteoblasts and that IL-6 stimulates the release of ^{45}Ca from prelabeled fetal mouse calvaria (9). In this study, anti-mouse rIL-6 serum showed no effect on the formation of osteoclast-like multinucleated cells. Recently, Tamura et al. reported that neither mouse rIL-6 nor mouse soluble IL-6 receptor induced osteoclast-like multinucleated cell formation in mouse marrow cultures when they were added separately (24). The osteoclast-like cell formation induced by both mouse rIL-6 and mouse soluble IL-6 receptor was dose dependently inhibited by adding monoclonal anti-mouse IL-6 receptor antibody, indicating that soluble IL-6 receptor triggers osteoclast formation in the presence of IL-6 (24). These findings suggest that IL-6 may be a nonessential mediator for the formation of osteoclast-like multinucleated cells induced by Y4 capsular-like polysaccha-

ride in the absence of soluble IL-6 receptor in culture supernatants of mouse marrow cells.

A role for cAMP in osteoclast-like multinucleated cell formation has been documented in mouse bone marrow cultures in the presence of PGE_2 (1). To study the effects of cAMP, mouse bone marrow cells were cultured with Y4 capsular-like polysaccharide in the presence or absence of H8 and HA1004, specific inhibitors of cAMP-dependent protein kinases. H8, which inhibits cAMP-dependent protein kinases more effectively than other types of protein kinase, has an inhibition constant (K_i) of 1.2 μM for cAMP-dependent protein kinases. HA1004 has a K_i of 2.3 μM for cAMP-dependent protein kinases. The inhibitory action of HA1004 against PKC is the weakest among the isoquinolinesulfonamide derivatives (7). H8 and HA1004 significantly inhibited the formation of osteoclast-like multinucleated cells induced by Y4 capsular-like polysaccharide in mouse marrow cultures. Recently, Loh et al. indicated that U937 cells, human monocytic leukemic cells, have PGE_2 receptors which are linked to the adenylate cyclase system (11). These findings are considered to confirm that the activity of Y4 capsular-like polysaccharide in osteoclast-like cell formation is mediated mainly by a mechanism involving PGE_2. In conclusion, the bone-resorbing activity of *A. actinomycetemcomitans* Y4 capsular-like polysaccharide might be explained by its recruiting of new osteoclasts mediated by IL-1α at the initial stage of culture. Furthermore, the stimulating effect of *A. actinomycetemcomitans* Y4 capsular-like polysaccharide on mouse osteoclast-like multinucleated cell formation appears to occur by a mechanism involving PGE_2 produced by IL-1α at the later stages of the process of osteoclast formation. These findings might give insight into the network of inflammatory mediators in bone destruction in periodontal diseases.

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