Porphyromonas gingivalis Lipopolysaccharide-Stimulated Bone Resorption via CD14 Is Inhibited by Broad-Spectrum Antibiotics

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In the present study, we examined mechanisms of Porphyromonas gingivalis lipopolysaccharide (P-LPS)-stimulated bone resorption via CD14, one of the lipopolysaccharide (LPS) receptors, and also assessed the inhibitory action of several kinds of antibiotics on the LPS-induced stimulation. First, we observed by using mouse embryonic calvarial cells that P-LPS stimulated bone resorption through the action of endogenous interleukin-1β (IL-1β) and interleukin-6 (IL-6) via CD14 because (i) P-LPS-stimulated expression of IL-1β and IL-6 genes in calvarial cells was inhibited by an anti-mouse CD14 antibody, (ii) stimulated bone resorption was markedly inhibited by both IL-1β and IL-6 antibodies, and (iii) P-LPS-stimulated bone resorption was clearly neutralized by an anti-mouse CD14 antibody. Next, we examined the effects of several kinds of antibiotics on P-LPS-stimulated bone resorption via CD14. Two of them, chloramphenicol and erythromycin, inhibited P-LPS-stimulated bone resorption in a dose-dependent manner. In addition, we observed that chloramphenicol clearly inhibited P-LPS-stimulated expression of the CD14, IL-1β, and IL-6 genes in calvarial cells. These results suggest that chloramphenicol might be a useful antibiotic as an anti-inflammatory agent against P-LPS-stimulated periodontal destruction occurring via CD14 in periodontal disease.

Porphyromonas gingivalis has been strongly implicated as one of the major causative agents of periodontal disease. P. gingivalis lipopolysaccharide (P-LPS) may play an important role in the pathogenic mechanism causing inflammation of the gingiva and alveolar bone loss in periodontal diseases because lipopolysaccharide (LPS) is a potent stimulator of inflammatory cytokine expression and bone resorption (3, 8, 12, 14, 15, 30, 31, 33). CD14 is one of the LPS receptors and is expressed predominantly on the surface of monocytes-macrophages and neutrophils (5, 19, 34, 35, 40, 41). Shapira et al. (29) showed that P-LPS stimulates human monocytes in a CD14-dependent manner. In addition, we recently (37) demonstrated that a signal transducing pathway of P-LPS in human gingival fibroblasts operates via CD14 as well. However, it is unknown whether P-LPS stimulates bone resorption via CD14. Therefore, it was of interest to us to examine the possible participation of CD14 in this stimulatory mechanism.

On the other hand, it is very important for us to develop a strategy for preventing periodontal disease. Several studies (1, 10, 11, 20, 32, 36) have shown that various kinds of antibiotics are effective for the treatment of chronic inflammatory disease. Most recently, an interesting study (32) showed that a macrolide antibiotic suppressed interleukin-6 (IL-6) expression in IL-1-stimulated human bronchial epithelial cells although the precise mechanism(s) of suppression has not been defined. This study suggested to us a possibility that some antibiotics may be able to prevent periodontal disease via inhibition of P-LPS-stimulated expression of inflammatory cytokines because inflammatory cytokines such IL-1 and IL-6 are involved in the pathogenesis of adult periodontal disease.

With the above points in mind, we carried out the present study to determine whether P-LPS-stimulated bone resorption occurs via CD14 and also to examine the inhibitory effects of several kinds of antibiotics on this resorption.

In this study, we demonstrated that P-LPS is able to stimulate bone resorption in mice through endogenous IL-1β and IL-6 induced via CD14 and also showed that wide-spectrum antibiotics inhibit the stimulated bone resorption by suppressing the expression of the IL-1β, IL-6, and CD14 genes.

MATERIALS AND METHODS

Reagents. Chloramphenicol, tetracycline-HCl, streptomycin sulfate, erythromycin, and ampicillin A were purchased from Wako Pure Chemicals (Osaka, Japan). 5'-[3H]-[32P]dCTP and a multiprimer DNA labeling system were purchased from Amersham Japan (Tokyo, Japan). Anti-mouse IL-1β and IL-6 monoclonal antibodies were from R&D Systems, Inc. (Minneapolis, Minn.). Horseradish peroxidase-conjugated anti-rat immunoglobulin G (IgG) antibody was obtained from Bio-Rad Japan (Tokyo, Japan). Anti-mouse CD14 antibody was kindly provided by S. Yamamoto (Ohita Medical University, Ohita, Japan).

P-LPS. P-LPS was provided by T. Umemoto (Kanagawa Dental College, Kanagawa, Japan). The procedures used for LPS preparation were described previously (21). Briefly, the LPS was extracted from acetone-dried cells by the phenol-water method and then purified by ultracentrifugation six times at 105,000 × g for 16 h each time and RNase treatment.

Preparation of mouse embryonic calvarial cells. Pregnant ICR mice carrying 14-day-old embryos were purchased from CLEA Japan (Tokyo, Japan). The calvarial cells were isolated from the embryos as described previously (2). Briefly, the embryonic calvariae were dissected out and digested at room temperature for 30 min in 10 ml of phosphate-buffered saline (pH 7.2) containing 0.1% bacterial collagenase (Sigma, St. Louis, Mo.), 0.05% trypsin (Difco Laboratories, Detroit, Mich.), and 4 mM EDTA. The cells liberated by digestion were harvested and then washed with α-MEM (Flow Laboratories, McLean, Va.). This washed calvarial cell population was used in subsequent experiments.

Bone resorption assay. Bone resorption was assayed as described previously (2). Embryonic calvarial cells at a density of 5 × 10^5/15 μl were incubated for 60 min on a dentine slice (4 by 4 mm) in each well of a 24-well flat-type Falcon plastic plate containing 10% fetal calf serum (FCS; Flow Laboratories) supplemented α-MEM. The medium covering the cells on the dentine slice was then removed.
and the cells were cultured in 1 ml of 10% FCS containing α-MEM with or without test samples. At selected times thereafter, the calvarial cells were scraped off the slice with a rubber policeman to enable visualization of the dentine surface. After having been rinsed with distilled water, the dentine slices were dehydrated with ethanol and sputter coated with gold. For evidence of osteoclastic bone resorption, the dentine slices were examined with a T200 scanning electron microscope (SEM; Japan Electronics Co., Tokyo, Japan). The excavations (pits) in the dentine slices were also counted. The results were expressed as the mean ± the standard error (SE) of quadruplicate cultures. Significance of differences was analyzed by Student’s t test.

Immunohistochemistry. Calvarial cells were or were not treated with P-LPS at 10 μg/ml. Immunostaining was performed on acetone-fixed smears of calvarial cells at 12 h after the initiation of LPS treatment. Binding of rat anti-mouse CD14 antibody to the cells was detected with horseradish peroxidase-conjugated anti-rat IgG antibody. Smears incubated in the presence of normal rat IgG were included as negative controls.

Preparation of total RNA and Northern blot analysis. Embryonic calvarial cells (10⁵ cells) in culture dishes were or were not treated with test samples. Thereafter, total cellular RNA was extracted by the guanidine isothiocyanate procedure as described previously (4). Also, Northern blotting assays were performed as detailed earlier (16, 22). In brief, the RNA was subjected to 1% agarose electrophoresis and blotted onto a nylon membrane (MSI Magnagraph, Westboro, Mass.). The membranes were then baked, prehybridized, and hybridized to mouse IL-1β cDNA (provided by T. Hamilton, Cleveland Clinic Foundation, Cleveland, Ohio), mouse IL-6 cDNA (American Type Culture Collection, Rockville, Md.), mouse CD14 cDNA (provided by S. Yamamoto), and β-actin cDNA (Japanese Cancer Research Resources Bank [JCRB], Tokyo, Japan) probes labeled with 5'-[α-32P]dCTP by use of a multiprime DNA labeling system. After hybridization, the membrane was washed, dried, and exposed to X-Omat film (Eastman Kodak Co., Rochester, N.Y.) at −70°C. β-Actin was used as an internal standard for quantification of total mRNA in each lane of the gel.

Preparation of antisense and sense CD14 oligonucleotides. Antisense or sense 25-mer phosphorothioated oligonucleotides including the translation initiation region were synthesized and purified by Sci-Media Ltd. (Tokyo, Japan). The sequences were as follows: antisense oligomer, 5'-AAGCCCATGGTCGGTAG-3'; sense oligomer, 5'-CTACCGACATTGAGCCTGTGCTT-3'.

RESULTS

P-LPS stimulates bone resorption via endogenous IL-1β and IL-6 in mouse embryonic calvarial cells. Although it is well known that P-LPS is a potent stimulator of bone resorption, the stimulatory mechanism has not been demonstrated in detail. Therefore, we first investigated, by using mouse calvarial cells, whether LPS-stimulated bone resorption is mediated by inflammatory cytokines such as IL-1 and IL-6 because LPS is a potent inducer of both of these cytokines, which are well-known stimulators of bone resorption. As shown in Fig. 1, although LPS stimulated bone resorption in a dose-dependent manner, the stimulatory effect was markedly neutralized by either anti-IL-1β or anti-IL-6 antibodies (Fig. 2A and B). These results strongly suggest a functional role of CD14 in P-LPS-stimulated bone resorption. To verify this suggestion conclusively, we investigated whether its LPS-induced expression would be inhibited by an antisense CD14 oligonucleotide. LPS-induced expression of the IL-1β gene was inhibited by an antisense CD14 oligonucleotide. However, no significant inhibition was observed in sense CD14 oligonucleotide-treated cells (Fig. 6C). These results strongly suggest a functional role of CD14 in P-LPS-stimulated bone resorption via endogenous IL-1β and IL-6. To confirm the functional role of CD14 in P-LPS-induced expression of the IL-1β cytokine gene, we investigated whether its LPS-induced expression would be inhibited by an anti-CD14 antibody. As shown in Fig. 7, LPS-stimulated bone resorption was markedly inhibited by the CD14 antibody at 1/500 dilution. Therefore, a functional role for CD14 in P-LPS-stimulated bone resorption via endogenous IL-1β and IL-6 is evidenced.

Wide-spectrum antibiotics inhibit P-LPS-stimulated bone resorption. It is very important to design a strategy for regulating or inhibiting the alveolar bone loss that occurs in adult periodontal disease. A recent study (32) showed that erythromycin is able to inhibit the expression of inflammatory cytokines markedly. This observation suggested the possibility that some kinds of antibiotics may be able to inhibit P-LPS-stimulated bone resorption by suppressing expression of IL-1β and IL-6. Therefore, we examined the effects of several kinds of antibiotics on P-LPS-stimulated bone resorption. As shown in Fig. 8, chloramphenicol and erythromycin at 10 μM markedly inhibited LPS-stimulated bone resorption. However, no significant inhibition by ampicillin, tetracycline, or streptomycin was observed at the doses tested.
To confirm the inhibitory action of chloramphenicol, we examined the kinetics of chloramphenicol-mediated inhibition of P-LPS-stimulated bone resorption. As shown in Fig. 9, although P-LPS stimulated bone resorption in a treatment time-dependent manner, the antibiotic drastically inhibited it as early as 3 days after the initiation of antibiotic treatment and the antibiotic maintained its strong inhibitory effect for at least 7 days. The inhibitory effect did not result from any cytotoxic action of the antibiotic toward calvarial cells because, based on the results of a trypan blue dye exclusion assay, significant cytotoxicity was not observed even when the drug was used at 10 μM (data not shown).

Chloramphenicol inhibits expression of the CD14 gene in mouse embryonic calvarial cells. As described above, since CD14 plays a central role in the stimulatory action of P-LPS on bone resorption, it is very important for understanding the inhibitory mechanism of chloramphenicol in P-LPS-stimulated bone resorption to examine whether the antibiotic affects the expression of the CD14 gene in calvarial cells. As shown in Fig. 10, the antibiotic at 10 μM clearly inhibited P-LPS-stimulated expression of the CD14 gene in calvarial cells. This inhibition did not result from some nonspecific effect, because expression of the β-actin gene, the internal control, was not affected by the antibiotic. Therefore, the antibiotic inhibition is specific for the CD14 gene.

Chloramphenicol inhibits expression of IL-1β and IL-6 genes involved in P-LPS-stimulated bone resorption in mouse embryonic calvarial cells. As demonstrated above, since P-LPS-stimulated bone resorption is mediated by endogenous IL-1β and IL-6 via CD14, these observations suggested the possibility that chloramphenicol-mediated inhibition of P-LPS-stimulated bone resorption may result from the suppression of expression of both of these cytokine genes by the antibiotic. Therefore, we investigated whether the antibiotic could inhibit the expression of the IL-1β and IL-6 genes in P-LPS-treated calvarial cells. Figure 11 shows that the antibiotic was able to inhibit the expression of both cytokine genes in calvarial cells.

**DISCUSSION**

It is well known that enterobacterial LPS induces many of its biological actions via CD14, one of the LPS receptors. However, it has not been demonstrated whether CD14 plays an important role in the stimulatory action of P-LPS on bone resorption. In the present study, we demonstrated, by using a mouse embryonic calvarial cell population, that P-LPS stimulates bone resorption through endogenous IL-1β and IL-6 induced via CD14.

P-LPS-stimulated bone resorption in our assay was mediated by endogenous IL-1β and IL-6 activity on calvarial cells of osteoclastic lineage because the stimulated bone resorption was significantly inhibited by treatment with antibody against...
either cytokine. This stimulatory action of IL-1β and IL-6 is supported by many recent studies (9, 23–26, 39) showing that several inflammatory cytokines are able to stimulate bone resorption. In addition, interestingly, the expression of P-LPS-stimulated IL-1β and IL-6 genes was clearly inhibited by anti-CD14 antibody and antisense CD14 oligonucleotide treatments and the anti-CD14 antibody also significantly inhibited LPS-stimulated bone resorption. These findings strongly suggested an important role of CD14 in P-LPS-stimulated bone resorption. Several recent studies (7, 17, 18, 27) have demonstrated the presence of two forms of CD14, i.e., membrane-bound CD14 and soluble CD14 in serum. In general, membrane-bound CD14-bearing cells, such as monocytes-macrophages, respond to LPS via CD14. On the other hand, cells lacking membrane-bound CD14 respond to complexes of LPS and soluble CD14. We know that the calvarial cells in our assay consisted of several heterogeneous cell populations, including osteoclast progenitors, monocytes-macrophages, osteoblastic cells, and fibroblastic cells. To understand the precise role of CD14 in P-LPS-stimulated bone resorption, we need to know what cell type(s) in the calvarial cell population is CD14 positive. Therefore, we examined the total population immunohistochemically for the presence of CD14-positive cells. As a result, we found that almost all of the calvarial cells were CD14 positive. Since the bone resorption assay was carried out with 10% FCS-containing culture medium, the possibility is raised that soluble CD14 is also involved in part in stimulated bone resorption. However, we believe that membrane-bound CD14 may play a central role in stimulated bone resorption because (i) the LPS response in monocytes-macrophages is triggered via membrane-bound CD14 (13, 38, 39), (ii) Fcαm et al. (6) showed high-level expression of the CD14 gene in epithelial tissues of several mouse organs, and (iii) interestingly, we (37) very recently also demonstrated that human fibroblasts constitutively express CD14 and, furthermore, that P-LPS induced monocyte chemoattractant protein 1 (MCP-1) via CD14 under culture conditions without serum. In addition, we observed constitutive expression of CD14 in mouse osteo-

![Image](https://example.com/image1)

**FIG. 5.** Immunohistochemical detection of CD14 in embryonic mouse calvarial cells. Calvarial cells prepared from 14-day-old mouse embryos were inoculated onto plastic plates and then incubated for 12 h in 10% FCS-containing α-MEM in the presence of P-LPS at 10 μg/ml. Acetone-fixed cells were incubated in the presence of normal rat Ig (A) or rat anti-mouse CD14 antibody (B) and then reacted with horseradish peroxidase-conjugated anti-rat IgG antibody.

![Image](https://example.com/image2)

**FIG. 6.** Functional role of CD14 in P-LPS-induced expression of IL-1β and IL-6 genes in embryonic mouse calvarial cells. (A) Calvarial cells prepared from 14-day-old mouse embryos were inoculated onto plastic plates and then pre-treated for 1 h with anti-CD14 antibody or not pretreated. Thereafter, the cells were cultured in 10% FCS-containing α-MEM in the presence or absence of P-LPS at 10 μg/ml. Total RNA was prepared at 6 h after the initiation of LPS treatment. Northern blot analysis was performed with IL-1β, IL-6, and β-actin cDNAs as probes. (B) Quantification of the cytokine gene expression in A was done by densitometry and expressed as a percentage of the maximum. (C) Calvarial cells were pretreated for 2 h with an antisense or a sense CD14 oligonucleotide in α-MEM or not pretreated and then cultured in 10% FCS-containing α-MEM supplemented with LPS (10 μg/ml) or not supplemented. Total RNA was prepared at 6 h after the initiation of LPS treatment. Northern blot analysis was performed with IL-1β and β-actin cDNAs as probes.
blastic MC3T3-E1 cells by using Northern and Western blotting assays (unpublished data). In general, it has been recognized widely that development of osteoclastic cells and bone resorption by osteoclastic cells are mediated by the action of cytokines produced by stromal cells such as osteoblastic cells and fibroblastic cells. Therefore, these observations support our belief that membrane-bound CD14 functions as an initiator of P-LPS-stimulated bone resorption.

On the other hand, as shown in our present study, P-LPS-stimulated bone resorption was not completely neutralized by an anti-CD14 antibody. This phenomenon suggested the possibility that another pathway(s), in addition to the CD14 pathway, is involved in stimulated bone resorption because LPS is also able to bind receptor substances other than CD14. However, we do not know by what mechanism the residual bone resorption occurs after anti-CD14 antibody treatment.

It is important clinically to develop drugs that can prevent the alveolar bone loss associated with periodontal disease. Golub et al. (10, 11, 28) suggested a nonantimicrobial action of tetracycline that inhibits gingival matrix metalloproteinase and bone loss in P. gingivalis-induced periodontitis in rats. They also proposed that tetracycline can affect parameters of osteoclast function, as the drug decreased the ruffled border area and inhibited acid production. On the other hand, a recent study showed that tetracycline can inhibit LPS-stimulated osteoclast formation in vitro.

FIG. 7. P-LPS-stimulated bone resorption by embryonic mouse calvarial cells via CD14. Calvarial cells were pretreated for 1 h with anti-CD14 antibody or not pretreated, and then \(5 \times 10^5\) cells were inoculated onto a dentine slice and incubated in 10% FCS-containing α-MEM in the presence or absence of P-LPS at 10 μg/ml. After 7 days, the resorption pits were counted. The results are expressed as the mean ± SE of quadruplicate cultures. *, \(P < 0.01\) for anti-CD14 antibody treatment versus no antibody treatment.

FIG. 8. Effects of several kinds of antibiotics on P-LPS-stimulated bone resorption by embryonic mouse calvarial cells. Calvarial cells (\(5 \times 10^5\) / dentine slice) prepared from 14-day-old mouse embryos were inoculated onto dentine slices, pretreated for 24 h with an antibiotic in 10% FCS-containing α-MEM or not pretreated; and then incubated in the presence or absence of P-LPS at 10 μg/ml. After 7 days, the resorption pits were counted. The results are expressed as the mean ± SE of quadruplicate cultures. *, \(P < 0.01\) for each antibiotic treatment versus no treatment.

FIG. 9. Kinetics of chloramphenicol inhibition of P-LPS-stimulated bone resorption by embryonic mouse calvarial cells. Calvarial cells (\(5 \times 10^5\) / dentine slice) prepared from 14-day-old mouse embryos were inoculated onto plastic plates, pretreated for 24 h with chloramphenicol (10 μM) in 10% FCS-containing α-MEM or not pretreated, and then incubated in the presence or absence of P-LPS at 10 μg/ml. After the times shown, the resorption pits were counted. The results are expressed as the mean ± SE of quadruplicate cultures. *, \(P < 0.01\) for chloramphenicol treatment versus no treatment.

FIG. 10. Chloramphenicol inhibits LPS-stimulated CD14 gene expression in embryonic mouse calvarial cells. Calvarial cells prepared from 14-day-old mouse embryos were inoculated onto plastic plates and pretreated for 24 h with chloramphenicol or not pretreated. Thereafter, the cells were incubated in 10% FCS-containing α-MEM in the presence or absence of P-LPS at 10 μg/ml. Total RNA was prepared at 3 h after the initiation of LPS treatment, and Northern blot analysis was performed with mouse CD14 and β-actin cDNAs as probes.
interesting study (32) showed that erythromycin, a macrolide antibiotic, suppressed IL-6 expression in human bronchial epithelial cells, although the mechanism of suppression was not well explained. This observation suggests the possibility that erythromycin is an anti-inflammatory agent. In fact, erythromycin and chloramphenicol have been shown to be effective in the treatment of chronic inflammatory disease. Since inflammatory cytokines such as IL-1, IL-6, and tumor necrosis factor alpha are potent stimulators of bone resorption, these cytokines actually play an important role in inflammatory reactions that take place in periodontal tissues, such as in periodontal disease. Therefore, the suppressive effect of a macrolide antibiotic on the expression of IL-1 and IL-6 as described above suggested the possibility that wide-spectrum antibiotics may function as potent inhibitors of the P-LPS-stimulated bone resorption that is mediated by these inflammatory cytokines via CD14. Therefore, we explored what kinds of antibiotics might be able to inhibit P-LPS-stimulated bone resorption as described above. We showed here that chloramphenicol and erythromycin function as potent inhibitors of P-LPS-stimulated bone resorption in mouse calvarial cell cultures, although such an inhibitory effect was not observed for streptomycin, tetracycline, or ampicillin.

In line with the fact that IL-1β and IL-6 play the predominant role in P-LPS-stimulated bone resorption in our assay system, we observed a significant inhibitory effect of chloramphenicol on P-LPS-stimulated expression of the IL-1β and IL-6 genes in calvarial cells. Although we are not able to explain the inhibitory mechanism of the expression of these genes, it will be very important to do so in the future for application of the antibiotic as an anti-inflammatory drug. The inhibition effected by chloramphenicol could not be based on its cytotoxic effect on osteoclastic cells, because neither calvarial cell viability nor β-actin gene expression, as an internal control, was affected by chloramphenicol at 10 μM. These results suggest that chloramphenicol inhibits P-LPS-stimulated bone resorption through inhibition of the expression of IL-1β and IL-6. Interestingly, we observed that the antibiotic did not inhibit the bone resorption function of mature mouse osteoclasts (unpublished data).

In conclusion, we provide evidence here that P-LPS through binding with CD14, stimulates bone resorption by the action of endogenous IL-1β and IL-6 and show that wide-spectrum antibiotics such as chloramphenicol and erythromycin are potent inhibitors of P-LPS-stimulated bone resorption.

REFERENCES


