Calcium hydroxide reduces lipopolysaccharide-stimulated osteoclast formation

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Objective. The purpose of this study was to determine the direct effects of lipopolysaccharide (LPS) on osteoclastogenesis and to assess the ability of calcium hydroxide—$Ca(OH)_2$—to inhibit the osteoclast formation stimulated by LPS.
Study design. RAW 264.7 cells were cultured with 50 ng/mL recombinant receptor activator of NF-κB ligand (RANKL) for 72 hours. RANKL was then removed, and the cells were treated with 0, 1, 10, or 100 ng/mL of LPS, $Ca(OH)_2$-treated LPS, or 50 ng/mL of RANKL as a positive control for an additional 48 hours. Cells were fixed and stained with fluorescein isothiocyanate–conjugated phalloidin to detect actin ring formation, and histochemistry was performed to detect multinucleated cells expressing tartrate-resistant acid phosphatase activity.
Results. LPS induced osteoclast-like cell (OCL) formation in a dose-dependent manner when osteoclast precursor RAW 264.7 cells were pretreated for 72 hours with RANKL. $Ca(OH)_2$ significantly inhibited the ability of LPS to stimulate OCL formation.
Conclusion. This study shows that LPS directly stimulates OCL formation. The detoxification of LPS by treatment with $Ca(OH)_2$ significantly reduced its ability to trigger the differentiation of OCLs.
(Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2003;95:348-54)

The development of apical periodontitis is associated with the presence of microorganisms in the root canal system.[1,2] It is well established that the predominant causative bacteria involved in endodontic infections are gram-negative anaerobic species,[3] which contain lipopolysaccharide (LPS) in the outer layers of their cell walls. LPS is released during the disintegration of bacteria after death. The presence of LPS has been reported in samples taken from necrotic pulp[4] and the pulpal dental wall of periapically involved teeth.[5] Furthermore, LPS can egress through the apical foramen into the periapex to initiate and sustain apical periodontitis.[6,7]

Calcium hydroxide, $Ca(OH)_2$, has been widely used in endodontics as an intracanal medication. $Ca(OH)_2$ may inactivate LPS within the pulp canal and adjacent dentinal tubule.[8,9] Safavi and Nichols[8,9] reported that $Ca(OH)_2$ hydrolyzes lipid A, which is the toxic component of LPS; they also found that after lipid A hydrolysis, this potent toxic agent is converted to fatty acids and amino sugars that are not toxic.

LPS was the first bacterial component shown to be capable of inducing bone resorption.[10] However, the mechanisms of action are not well understood. Specifically, it is not clear whether LPS enhances osteoclastogenesis directly by targeting osteoclast precursors or indirectly through intermediary cells such as osteoblasts. Osteoclasts are derived from the hematopoietic precursor cells of the monocyte/macrophage lineage. The precursors differentiate into multinucleated mature forms and activate to resorb bone in the specialized microenvironment of bone. As first noted in the late 1980s, the in vitro maturation of macrophages into osteoclasts requires the presence of marrow stromal cells or their osteoblast progeny.[11] Direct cell-to-cell contact is required for the induction of osteoclasts by cells in the osteoblastic lineage. Stromal cells and osteoblast precursors express a member of the tumor necrosis factor ligand family called *receptor activator of NF-κB-ligand* (RANKL).[12,13] This cell surface ligand stimulates osteoclastogenesis and osteoclast activity by binding to its cognate receptor, RANK, on the surfaces of osteoclast precursors.[14] It was recently reported that

Supported by NIH AR47959 to L. Shannon Holliday and Startup funding from the University of Florida to Jin Jiang.
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Received for publication Aug 15, 2002; returned for revision Sep 24, 2002; accepted for publication Sep 27, 2002.

1079-2104/2003/$30.00 + 0
doi:10.1067/moe.2003.18

the addition of soluble RANKL stimulates osteoclast formation in vitro.[15,16] This method provides means to examine the direct effects on osteoclasts independent of stromal cell elements. In the present study, we used the murine monocytic cell line RAW 264.7, which can be induced to differentiate into mature osteoclasts in the presence of recombinant RANKL,[16] to examine the direct effects of LPS on osteoclast differentiation and to assess the ability of $Ca(OH)_2$ to inhibit the osteoclast formation stimulated by LPS.

MATERIAL AND METHODS

Preparation of LPS

LPS was prepared as previous described.[8,9] LPS (*Escherichia coli* O26:B6; Sigma, St Louis, Mo) was dissolved in pyrogen-free water to concentrations of 1 μg/mL, 10 μg/mL, and 100 μg/mL. $Ca(OH)_2$ powder (Sigma) was added to half of the samples for a final concentration of 25 mg/mL. All samples were vortexed for 20 seconds and incubated at 37°C for 7 days. After the incubation period, the insoluble $Ca(OH)_2$ was precipitated by centrifuging at 2000 rpm for 5 minutes, and the supernatants were collected. They were then neutralized to a pH of 7.5 by adding concentrated hydrochloric acid.

Cell culture

A recombinant protein composed of glutothione-S-transferase attached to amino acids 158 to 316 of mouse RANKL was constructed, expressed in bacteria, and isolated as described previously.[16] RAW 264.7 cells (American Type Culture Collection, Rockville, Md) were plated at a density of 20,000 cells/cm^2 in 24 well plates with cover slips in Dulbecco's modified eagle medium with 10% fetal bovine serum. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. The cells were cultured in the presence of 50 ng/mL of RANKL for the first 72 hours. After 72 hours, the medium was changed with various agents and further incubation was allowed to occur for another 48 hours to achieve osteoclast differentiation. Each group was run in triplicate and repeated twice.

Actin ring formation

Cells were fixed for 15 minutes at room temperature with 2% paraformaldehyde, washed with phosphate-buffered saline, and treated for 20 minutes with 0.2% Triton X-100 solution (Sigma, St. Louis, Mo) to permeabilize cell membranes. For the identification of F-actin, the cells were incubated with 1 μg/mL of fluorescein isothiocyanate–conjugated phalloidin (Sigma) for 20 minutes at room temperature. The cells were washed with phosphate-buffered saline, and the distribution of the actin ring was determined by using fluorescence microscopy as described in another study.[17]

TRAP assay

Cytochemical staining of tartrate-resistant acid phosphatase (TRAP)–positive cells was performed as described earlier.[18] TRAP-positive cells appeared dark red. Only TRAP-positive cells with more than 3 nuclei were counted. The values are expressed as means ± SE of triplicate cultures.

RESULTS

LPS induces osteoclast formation

To test whether LPS directly stimulates osteoclast formation, RAW 264.7 cells were cultured in the presence and absence of various concentrations of LPS. In 3 experiments, we found that RAW 264.7 cells did not form osteoclast-like cells (OCLs) in the presence of LPS (Fig 1).

Recently, Zou and Bar-Shavit[19] reported that although LPS could not directly stimulate osteoclast precursors to differentiate into osteoclasts, it could stimulate the later stages of differentiation if the early stages were induced by RANKL. To test this, osteoclast precursors were cultured with RANKL for 72 hours. At the end of this time, there were few multinucleated cells in the culture. Cultures were then stimulated for an additional 48 hours with RANKL (as a control), vehicle, or LPS. In the presence of LPS, these cultures generated many TRAP-positive multinucleated cells (Fig 2, *A*). When stained with fluorescein isothiocyanate-conjugated phalloidin, actin rings were detected in most multinucleated cells, which is consistent with the cells being osteoclasts (Fig 2, *B*). The morphologic features of LPS-induced OCLs were similar to those of RANKL-induced OCLs (Fig 2, *C* and *D*). In the vehicle controls, almost all of the cells were mononuclear and did not exhibit TRAP activity (Fig 2, *E* and *F*). As shown in Figure 3, the addition of 10 ng/mL and 100 ng/mL of LPS significantly increased TRAP-positive OCLs, and the stimulation was dose-dependent.

$Ca(OH)_2$ inhibited LPS-induced osteoclast formation

$Ca(OH)_2$ directly inactivates LPS.[8,9] To investigate whether the stimulatory effect of LPS on osteoclast formation could be reduced by $Ca(OH)_2$ treatment, the LPS solution was pretreated with $Ca(OH)_2$. OCL formation was significantly reduced in groups in which 10 and 100 ng/mL of LPS were pretreated with $Ca(OH)_2$ compared with those without $Ca(OH)_2$

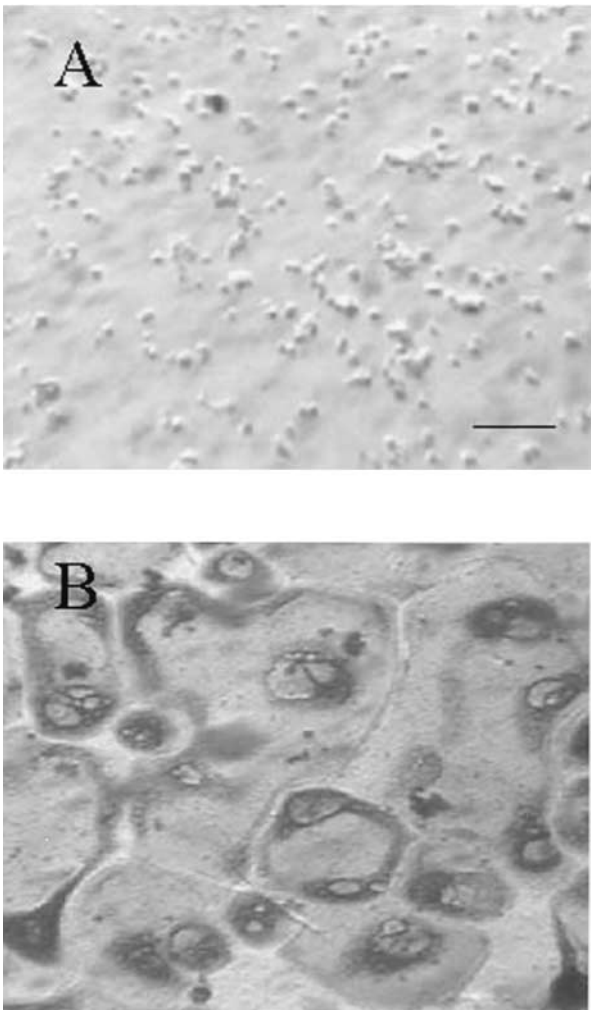

Fig 1. Lipopolysaccharide (LPS) alone did not induce RAW 264.7 cells to differentiate into osteoclasts. RAW 264.7 cells were incubated for 5 days with 100 ng/mL of LPS (**A**) and 50 ng/mL of receptor activator of NF-κB ligand. After incubation, cells were stained for tartrate-resistant acid phosphatase activity as described in "Material and Methods." *Bar* = 100 μm.

pretreatment. Figure 4 shows that the maximal stimulatory effect of 100 ng/mL of LPS was reduced 78% with $Ca(OH)_2$ pretreatment.

DISCUSSION

Gram-negative bacteria predominantly cause apical periodontitis. Microorganisms are generally limited to the root canal system. It is their products and not the intact microorganisms that prompt bone resorption.[20] LPS, a cell wall component of gram-negative bacteria, is abundant in the necrotic pulp and dentin wall. Dahlen et al[6] experimentally applied bacterial LPS into the root canals of monkeys. After 3 and 7 months, the periapical tissues showed signs of resorptive bone loss and inflammatory reactions. These data provide strong evidence that LPS is essential to the formation of periapical lesions. However, the mechanism by which LPS triggers bone resorption is not clear.

Several lines of evidence have established the concept that LPS stimulates osteoblasts and surrounding cells to secrete PGE2 and cytokines, including TNF-α and IL-1.[21] It has been recently reported that LPS directly stimulates osteoblasts to express RANKL, resulting in the induction of osteoclastogenesis.[22] Compared with the wealth of data supporting the indirect action of LPS on osteoclast formation, relatively few studies have addressed the potential direct effects of LPS on osteoclasts (and the results have been varied). Suda et al[23] reported that LPS supports the survival and fusion of osteoclasts independent of TNF-α, IL-1, and RANKL. Abu-Amer et al[24] showed that LPS directly targets osteoclast precursors, prompting their commitment to the osteoclast phenotype. In contrast, Takami et al[25] showed that LPS inhibits the differentiation of osteoclast precursors into mature osteoclasts. Zou and Bar-Shavit[19] found that LPS does not induce bone marrow mononuclear cells to differentiate into mature osteoclast without RANKL stimulation during early differentiation but does induce the later stages of osteoclastogenesis independent of RANKL. Likewise, we found that LPS did not support osteoclastogenesis entirely independent of RANKL but that it did independently support late differentiation.

Model development whereby osteoclasts can be generated in culture has provided a vital tool to study the mechanisms of osteoclastogenesis. Until recently, bone resorptive osteoclasts could only be reliably differentiated in culture by using primary bone marrow. The marrow cultures contain osteoclast precursors and stromal cells/osteoblasts. Bone stimulatory agents such as calcitriol or parathyroid hormone induce osteoblasts in the cultures to indirectly support osteoclast differentiation and activity by stimulating them to express RANKL.[26] In these heterogenous systems, determining whether exogenously added agents affect osteoclasts directly or indirectly is problematic.

To analyze the direct effects of LPS on osteoclast formation, we used RANKL-stimulated RAW 264.7 cells. This hematopoietic cell line expresses RANK and differentiates into osteoclasts in response to the addition of recombinant RANKL.[14,16] The differentiated cells express genes typical of mammalian osteoclasts, including TRAP, cathepsin K, integrin $\alpha_V\beta_3$, *c-src*, and calcitonin receptor. Morphologically, they are TRAP-positive multinucleated cells

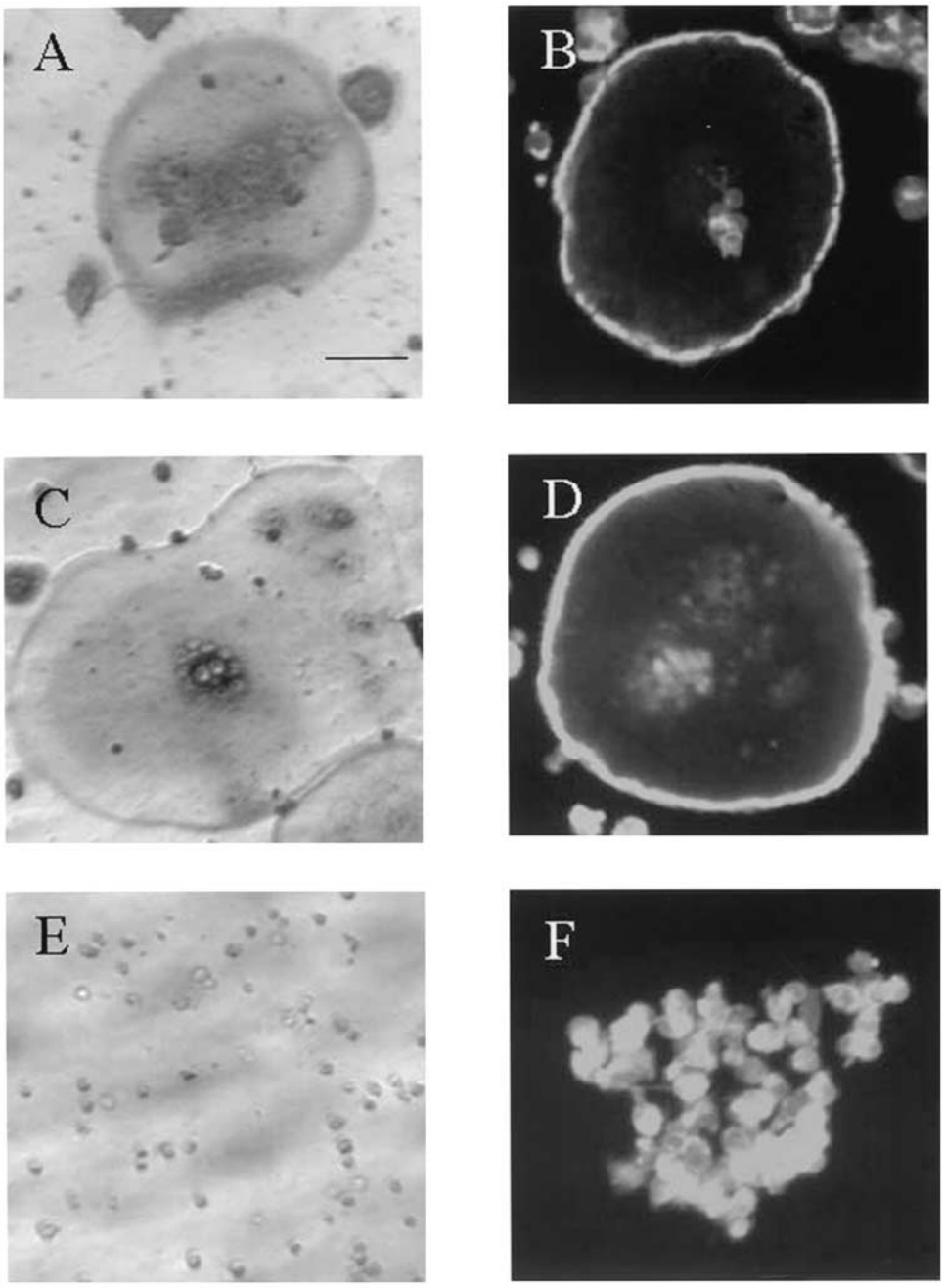

Fig 2. LPS induced RAW 264.7 cells to differentiate into osteoclast-like cells after a 72-hour pretreatment with RANKL. RAW 264.7 cells were cultured in the presence of 50 ng/mL of RANKL for 72 hours. RANKL was then removed and cells were treated with 100 ng/mL of LPS (**A and B**), 50 ng/mL of RANKL (**C and D**), or vehicle (**E and F**) for an additional 48 hours. Cells were fixed, and TRAP staining was performed to identify TRAP-positive multinucleated cells (**A, C, and E**). Cells were stained with fluorescein isothiocyanate-conjugated phalloidin to detect actin ring formation (**B, D, and F**). *Bar* = 100 μm.

with the capability of resorbing bone.[14] By using RAW 264.7 cells, we could examine the direct effects of LPS on osteoclast differentiation in the absence of osteoblastic/stromal cells.

We found that LPS did not induce osteoclast formation without RANKL stimulation. This result is in line with those of other recent studies.[19,25] However, when RAW 264.7 cells were treated with RANKL for 72 hours, then stimulated with LPS, TRAP-positive multinucleated cells formed that possessed actin rings,

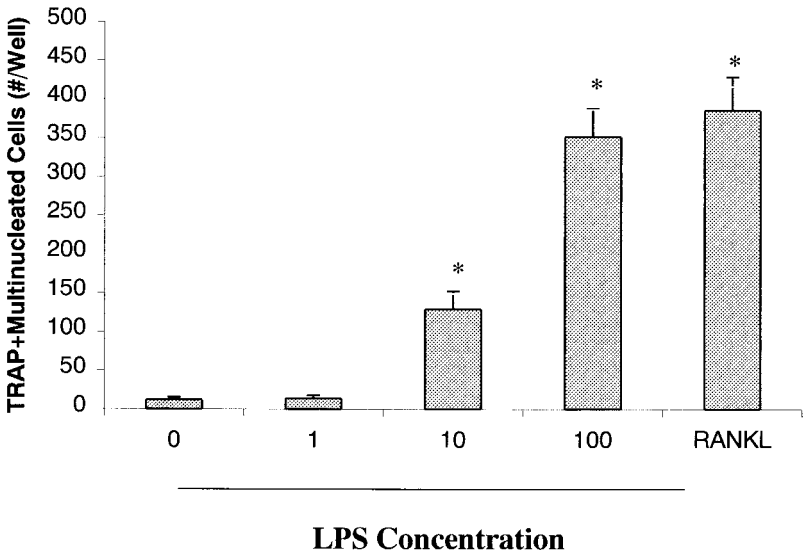

Fig 3. LPS induced osteoclast-like cell formation from RANKL-pretreated RAW 264.7 cells in a dose-dependent fashion. The number of TRAP-positive multinucleated cells in cultures was counted. Values are expressed as mean ± SE of the triplicate samples. *$P < .01$ versus the vehicle group.

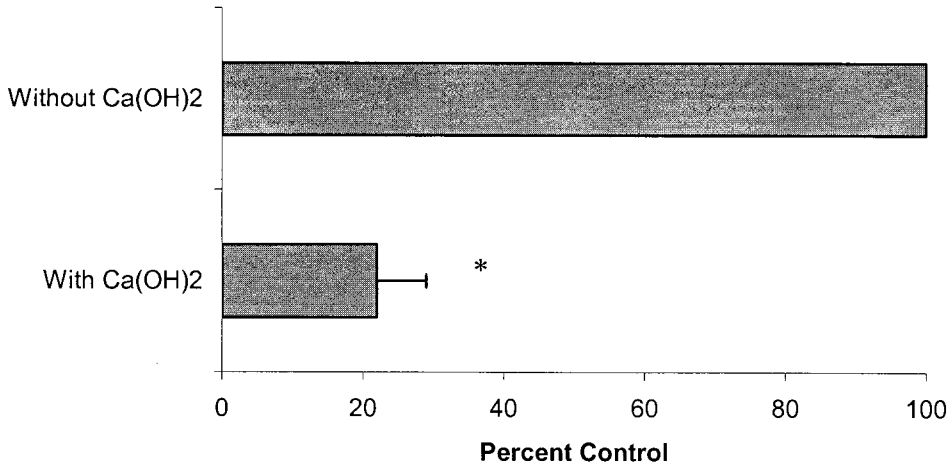

Fig 4. Calcium hydroxide, $Ca(OH)_2$, inactivation of LPS greatly reduced its capacity to stimulate osteoclast-like cell formation. RAW 264.7 cells were treated for 72 hours with RANKL and for an additional 48 hours with 100 ng/mL of LPS (control) or 100 ng/mL of $Ca(OH)_2$-inactivated LPS. The numbers of osteoclasts in the control cultures were considered 100%. Values are expressed as mean ± SE of the triplicate samples. *$P < .01$ versus LPS without $Ca(OH)_2$ pretreatment.

or dotlike aggregations of actin that cluster in a ring around the osteoclast periphery. Actin ring formation is one of the typical characteristics of osteoclasts.[27] The number and morphologic features of LPS-induced OCLs were similar to those of RANKL-induced OCLs. This result suggests that LPS can support the late stages of osteoclast formation.

LPS can induce the activation of NF-κB and upregulate the expression levels of TNF-α. NF-κB activation in osteoclast precursors has been implicated in the successful differentiation of precursors to mature osteoclasts. TNF-α can also enhance the differentiation and survival of osteoclasts. The mechanism by which LPS enhances osteoclast formation in our system will be determined during future examination. We hypothesize that in chronic apical lesions, LPS from necrotic pulp stimulates macrophages, leukocytes, and osteoblasts to synthesize and secrete cytokines and RANKL.

Consequently, RANKL induces osteoclast precursors to mature into active osteoclasts and to resorb bone. LPS, in turn, stimulates RANKL-primed cells to form active osteoclasts in the absence of a continuous supply of RANKL.

The lipid A portion of LPS is the greatest source of bioactivity.[28] Lipid A has been found to be required for LPS-induced osteoclast formation in bone marrow culture.[29] Safavi and Nichols[8,9] showed that a slight variation in the structure of lipid A renders it biologically inactive. Thus, a change in lipid chains might detoxify LPS.[8,9] $Ca(OH)_2$, which is quite basic, can break down ester bonds within the lipid A moiety of LPS by means of alkaline hydrolysis. In this study, $Ca(OH)_2$ was incubated with LPS for 7 days, and then the hydroxide ions were neutralized with hydrochloric acid. We demonstrated that $Ca(OH)_2$ can significantly reduce the stimulatory effect of LPS on osteoclast formation. Barthel et al[30] showed that $Ca(OH)_2$ treatment of *E coli* LPS prevented the stimulation of TNF-α production in peripheral blood monocytes. Destruction of the lipid A side chain by $Ca(OH)_2$ might account for the inability of LPS to stimulate osteoclast formation.

We tested LPS from *E coli*, a standard LPS in osteoclast research internationally, although LPS from black-pigmented bacteria reportedly have distinct chemical and biologic properties.[31] However, they have a common structure in the lipid A portion. Ito et al[29] found that there was no difference in the induction of osteoclast formation between LPS from *Porphyromonas gingivalis* and LPS from *E coli* in mouse bone marrow cell culture. However, it would be very interesting to determine whether LPS from endogenous pathogens, especially those of black-pigmented bacteria, have similar activities in the RAW 264.7 osteoclast culture system.

In conclusion, the results of this study show that LPS has direct stimulatory effects on the later stages of RANKL-initiated osteoclast formation. The detoxification of LPS with $Ca(OH)_2$ significantly inhibited osteoclast formation. This might be one of the mechanisms by which interappointment $Ca(OH)_2$ dressings create a favorable environment for periapical bone healing.

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