

Antigenic Activity of Bacterial Endodontic Contents from Primary Root Canal Infection with Periapical Lesions against Macrophage in the Release of Interleukin-1 β and Tumor Necrosis Factor α

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Abstract

Introduction: Periradicular tissue chronic stimulation by endotoxin may cause apical periodontitis. This study investigated the microbial profile and the levels of endotoxin found in primary root canal infection with apical periodontitis, determined their antigenicity against macrophages through the levels of interleukin (IL)-1 β and tumor necrosis factor α (TNF- α), and evaluated their relationship with clinical and radiographic findings. **Methods:** Samples were taken from 21 root canals with primary endodontic infection and apical periodontitis with paper points. PCR technique (16S rDNA) was used for the detection of the target bacteria. Limulus Amebocyte Lysate (LAL) was used to measure endotoxin. The amounts of IL-1 β /TNF- α in macrophages supernatants were measured by enzyme-linked immunosorbent assay – Duoset-kit (ELISA). **Results:** *Prevotella nigrescens* (13/21), *Porphyromonas endodontalis* (6/21), and *Treponema socranskii* (6/21) were the most frequently detected gram-negative bacterial species. The presence of the sinus tract (2/21) was related to the detection of *Filifactor alocis* ($p < 0.05$), whereas a tooth with a radiolucent area ≥ 2 mm was related to the detection of *Treponema denticola*. A correlation was found between the number of

gram-negative bacteria and the levels of IL-1 β /TNF- α ($p < 0.05$). Increased levels of endotoxin were followed by TNF- α release ($p < 0.05$). Higher levels of IL-1 β ($p < 0.05$) and endotoxin contents were related to the larger size of the radiolucent area.

Conclusion: The antigenicity of the endodontic contents is not only related to the amount of endotoxin found in the root canal but also to the number of different species of gram-negative bacteria involved in the infection. Moreover, a larger size (≥ 2 mm) of the radiolucent area was related to IL-1 β and endotoxin. (*J Endod* 2010;36:1467–1474)

Key Words

Antigenicity, bacteria, endodontic, endotoxin, macrophages

Primary endodontic infection is a polymicrobial infection caused predominantly by gram-negative anaerobic bacteria (1) that present lipopolysaccharide (LPS) on the outer layers of their cell walls. LPS is released during disintegration, multiplication, or bacterial death (2) and is capable of penetrating into the periradicular tissues (3), acting as endotoxin in the host organism (4) and leading to periradicular inflammation and bone destruction (5). The lipid A is the bioactive component of LPS responsible for the majority of the immunoresponse (3).

The accumulation of bacteria components in an infected area, particularly endotoxins (including lipoteichoic acid, peptidoglycan, lipopolysaccharide, and others), can stimulate the release of proinflammatory cytokines by different cell lines through TLR2 and -4 activation (5–7). The inflammatory tissue present in periradicular lesions is populated predominantly by macrophage (8, 9), which is the major source of interleukin-1 β (IL-1 β) (10), and almost the exclusive producer of tumor necrosis factor α (TNF- α) (11) in the presence of bacteria or LPS.

Clinical investigations of primary endodontic infection have elucidated the strong correlation between oral bacteria LPS and the presence of apical periodontitis (6, 12–15). Moreover, higher contents of endotoxins in root canals have been associated with spontaneous pain (6, 12, 16) and clinical signs/symptoms such as pain on palpation, tenderness to percussion, and exudation (6, 12, 14, 16).

Previous *in vitro* investigations (5–7) have shown that oral bacterial LPS extracted from bacteria commonly found in root canal infection induces a potent inflammatory response against different cell line cultures. IL-1 β and TNF- α have been detected in periapical tissues (3, 9, 17–19) and root canal exudates (20–23) from primary root canal infection in the presence apical periodontitis. Higher contents of IL-1 β have been detected in teeth with clinical signs/symptoms (3, 23) and larger size of radiolucent area corresponding to bone resorption (21, 22). However, studies correlating all these aspects have not yet been provided in the literature. Therefore, the aim of this clinical study was to investigate the microbial profile and the levels of endotoxin found in primary root canal infection with apical periodontitis and to determine their antigenicity against macrophages through the levels of IL-1 β /TNF- α , evaluating their relationship with clinical and radiographic findings.

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Material and Methods

Patient Selection

Twenty-one patients who attended the Piracicaba Dental School, Piracicaba, SP, Brazil, in need of endodontic treatment were included in this research. The age of the patients ranged from 13 to 73 years. Samples were collected from 21 root canals with pulp necrosis and showing radiographic evidence of apical periodontitis. The selected teeth showed absence of periodontal pockets more than 4 mm. The following clinical/radiographic features were found: pain on palpation (9/21), tenderness to percussion (8/21), exudation (12/21), and radiolucent area ≥ 2 mm (11/21) and < 2 mm (10/21). None of the patients reported spontaneous pain.

A detailed dental history was obtained from each patient. Patients who had received antibiotic treatment during the last 3 months or who had any general disease were excluded. The Human Volunteers Research and Ethics Committee of the Piracicaba Dental School approved the protocol describing specimen collection for this investigation, and previously all patients signed an informed consent document.

Sampling Procedures

All materials used in this study were heat sterilized at 200°C for 4 hours to become apyrogenic. The method followed for the disinfection of the operative field had been described previously (14, 15). Briefly, the teeth were isolated with a rubber dam. The crown and the surrounding structures were disinfected with 30% H₂O₂ for 30 seconds followed by 2.5% NaOCl for an additional 30 seconds. Subsequently, 5% sodium thiosulphate was used to inactivate the irrigant. The sterility of the of the external surfaces of the crown was checked by taking a swab sample from the crown surface and streaking it on blood agar plates that were incubated aerobically and anaerobically.

A two-stage access cavity preparation was made without the use of water spray but under manual irrigation with sterile/apyrogenic saline solution and by using sterile/apyrogenic high-speed diamond bur. The first stage was performed to promote a major removal of contaminants. In the second stage, before entering the pulp chamber, the access cavity was disinfected following the protocol described earlier. The sterility of the internal surface of the access cavity was checked as previously described, and all procedures were performed aseptically. A new sterile and apyrogenic bur was used, accomplished by irrigation with sterile apyrogenic water to access the canal. The endotoxin sample was taken introducing sterile pyrogen-free paper points (size 15; Dentsply-Maillefer, Ballaigues, Switzerland) into the full length of the canal (determined radiographically) and retained in position during 60 seconds. Immediately, the paper point was placed in a pyrogen-free glass and frozen at -80°C for future Limulus amoebocyte lysate assay (LAL) and cell culture stimulation. The procedure was repeated with five sterile paper points. The paper points were pooled in a sterile tube containing 1 mL of VMGA (Viability Medium Göteborg Agar) III transport medium and immediately processed for DNA extraction for the detection of target bacteria by molecular method (16S ribosomal DNA).

Bacterial Detection (PCR 16S rDNA)

Reference bacteria strains used in this study were purchased from the American Type Culture Collection (ATCC) and are listed as follows: *Dialister pneumosintes* (ATCC 33048), *Prevotella intermedia* (ATCC 25611), *Prevotella nigrescens* (ATCC 33099), *Aggregatibacter actinomycetemcomitans* (ATCC 43718), *Porphyromonas gingivalis* (ATCC 33277), *Filifactor alocis* (ATCC 35896), *Tannerella forsythia*

(ATCC 43037), *Prevotella tanneriae* (ATCC 51259), *Treponema denticola* (ATCC 35405), *Porphyromonas endodontalis* (ATCC 35406), *Treponema socranskii* (35536), and *Parvimonas micra* (ATCC 33270). Bacterial selection criteria were performed based on the most commonly found species in primarily root canal infection.

DNA Extraction. Microbial DNA from endodontic samples as well as from ATCC bacteria were extracted and purified with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA concentration (absorbance at 260 nm) was determined using a spectrophotometer (Nanodrop 2000; Thermo Scientific, Wilmington, DE).

PCR Assay. The PCR reaction was performed in a thermocycler (MyCycler; Bio-Rad, Hercules, CA) thermocycler in a total volume of 25 μ L containing 2.5 μ L of 10X Taq buffer (1 \times) (MBI Fermentas, Mundolsheim, France), 0.5 μ L of dNTP mix (25 μ mol/L of each deoxyribonucleoside triphosphate—dATP, dCTP, dGTP, and dTTP) (MBI Fermentas, Hanover, MD), 1.25 μ L of 25 mmol/L MgCl₂, 0.25 μ L of forward and reversal universal primers (0.2 μ mol/L) (Invitrogen, Eugene, OR), 1.5 μ L of sample DNA (1 μ g/50 μ L), 1.5 μ L of Taq DNA polymerase (1 U) (MBI Fermentas), and 17.25 μ L of nuclease-free water. The primer sequences and PCR cycling parameters were previously optimized (13-15) and listed in Table 1.

Determination of Endotoxin Concentration (Turbidimetric Test and LAL Assay)

The turbidimetric test (BioWhittaker, Inc, Walkersville, MD) was used to measure endotoxin concentrations in the root canals using the LAL technique. First, as a parameter for the calculation of the amount of endotoxins in root canal samples, a standard curve was plotted using endotoxins supplied in the kit with a known concentration (100 EU/mL) and its dilutions with the following final concentrations (ie, 0.01, 0.10, 1, and 10 EU/mL) following the manufacturer's instructions.

Test Procedure. All reactions were accomplished in duplicate to validate the test. A 96-well microplate (Corning Costar, Cambridge, MA) was used in a heating block at 37°C and maintained at this temperature throughout the assay. First, the endotoxin samplings were suspended in 1 mL of LAL water supplied on the kit and agitated in vortex for 60 seconds and serially diluted to the 10⁻¹. Immediately, 100 μ L of the blank followed the standard endotoxin solutions in concentrations (ie, 0.01, 0.10, 1, and 10 EU/mL), and 100 μ L of the samples were added in duplicate in the 96-well microplate. The test procedure was performed following the manufacturer's instructions. The absorbencies of endotoxin were measured individually using an enzyme-linked immunosorbent assay plate reader (Ultramark, Bio-Rad Laboratories, Inc) at 340 nm.

Calculation of Endotoxin Concentrations. Because the mean absorbance value of the standards was directly proportional to the concentration of endotoxins present, the endotoxin concentration was determined from the standard curve.

Cell Culture and Cytokine Expression

Macrophages (RAW 264.7) were cultured in 100-mm culture plates in Dulbecco's modified Eagle minimal essential medium supplemented (DMEM) with 100 IU/mL of penicillin, 100 μ g/mL of streptomycin, and 10% heat-inactivated fetal bovine serum and maintained in a humidified atmosphere at 37°C and 5% CO₂ until 90% confluence. Unless noted otherwise, all tissue culture reagents were obtained from Invitrogen (Carlsbad, CA). Macrophages were released from 100-mm plates with 0.25% trypsin and counted in a Neubauer chamber; a total of 10⁴ macrophages were grown for 48 hours in each well of six-well

TABLE 1. PCR Primer Pairs and Cycling Parameters Used for Detection of Bacteria Species in Primary Root Canal Infection with Apical Periodontitis

Target bacteria	Primer pairs (5'- 3')	Amplicon size	Cycles
Universal (16s rDNA)	Forward: TCC TAC GGG AGG CAG CAG T Reverse: GGA CTA CCA GGG TAT CTA ATC CTG TT	466 bp	Initial denaturation at 95°C for 10 min and 40 cycles of 95°C for 10 s, 60°C for 10 s, and a final extension step at 72°C for 25 s
<i>Dialister pneumosintes</i>	Forward: TTC TAA GCA TCG CAT GGT GC Reverse: GAT TTC GCT TCT CTT TGT TG	1105 bp	Initial denaturation at 95°C for 2 min and 36 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 2 min, and a final step 72°C for 2 min
<i>Prevotella intermedia</i>	Forward: TTT GTT GGG GAG TAA AGC GGG Reverse: TCA ACA TCT CTG TAT CCT GCG T	575 bp	Initial denaturation at 95°C for 2 min and 36 cycles of 94°C for 30 s, 58°C for 1 min, 72°C for 2 min, and a final step 72°C for 10 min
<i>Prevotella nigrescens</i>	Forward: ATG AAA CAA AGG TTT TCC GGT AAG Reverse: CCC ACG TCT CTG TGG GCT GCG A	804 bp	Initial denaturation at 95°C for 2 min and 36 cycles of 94°C for 30 s, 58°C for 1 min, 72°C for 2 min, and a final step 72°C for 10 min
<i>Aggregatibacter actinomycetemcomitans</i>	Forward: AAA CCC ATC TCT GAG TTC TTC TTC Reverse: ATG CCA ACT TGA CGT TAA AT	557 bp	Initial denaturation at 94°C for 30 s and 36 cycles of 95°C for 30 s, 55°C for 1 min, 72°C for 2 min, and a final step 72°C for 10 min
<i>Porphyromonas gingivalis</i>	Forward: AGG CAG CTT GCC ATA CTG CG Reverse: ACT GTT AGC AAC TAC CGA TGT	404 bp	Initial denaturation at 95°C for 2 min and 36 cycles of 94°C for 30 s, 60°C for 1 min, 72°C for 2 min, and a final step 72°C for 2 min
<i>Filifactor alocis</i>	Forward: CAG GTG GTT TAA CAA GTT AGT GG Reverse: CTA AGT TGT CCT TAG CTG TCT CG	594 bp	Initial denaturation at 95°C for 2 min and 26 cycles of 95°C for 30 s, 58°C for 1 min, 72°C for 1 min, and a final step 72°C for 2 min
<i>Tannerella forsythia</i>	Forward: GCG TAT GTA ACC TGC CCG CA Reverse: TGC TTC AGT GTC AGT TAT ACC T	641 bp	Initial denaturation at 95°C for 1 min and 36 cycles of 95°C for 30 s, 60°C for 1 min, 72°C for 1 min, and a final step 72°C for 2 min
<i>Prevotella tannerae</i>	Forward: CTT AGC TTG CTA AGT ATG CCG Reverse: CAG CTG ACT TAT ACT CCC G	550 bp	Initial denaturation at 95°C for 2 min and 36 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 2 min, and a final step 72°C for 10 min
<i>Treponema denticola</i>	Forward: TAA TAC CGA ATG TGC TCA TTT ACA T Reverse: TCA AAG AAG CAT TCC CTC TTC TTC TTA	316 bp	Initial denaturation at 95°C for 2 min and 36 cycles of 94°C for 30 s, 60°C for 1 min, 72°C for 2 min, and a final step 72°C for 10 min
<i>Porphyromonas endodontalis</i>	Forward: GCT GCA GCT CAA CTG TAG TC Reverse: CCG CTT CAT GTC ACC ATG TC	672 bp	Initial denaturation at 95°C for 2 min and 36 cycles of 94°C for 30 s, 58°C for 1 min, 72°C for 2 min, and a final step 72°C for 10 min
<i>Treponema socranskii</i>	Forward: GAT CAC TGT ATA CGG AAG GTA GAC A Reverse: TAC ACT TAT TCC TCG GAC AG	288 bp	Initial denaturation at 95°C for 2 min and 36 cycles of 94°C for 30 s, 56°C for 1 min, 72°C for 2 min, and a final step 72°C for 10 min
<i>Parvimonas micra</i>	Forward: AGA GTT TGA TCC TGG CTC AG Reverse: ATA TCA TGC GAT TCT GTG GTC TC	207 bp	Initial denaturation at 95°C for 2 min and 36 cycles of 94°C for 30 s, 60°C for 1 min, 72°C for 2 min, and a final step 72°C for 10 min

plates, deinduced by incubation for 8 hours in culture medium (DMEM) containing 0.3% fetal bovine serum, and stimulated with 60 µL of root canal contents during 24 hours in order to quantify the total amount of protein released in the culture media, IL-1β, and TNF-α protein. The supernatants were collected and stored at -80°C until protein evaluation.

IL-1β and TNF-α Messenger RNA Expression

The macrophage cell viability was tested in the present study by its capacity to express IL-1β and TNF-α messenger RNA after 24 hours of root canal contents stimulation. A total of 10⁴ macrophages were grown for 48 hours in each well of six-well plates, deinduced by incubation for

8 hours in culture medium (DMEM) containing 0.3% fetal bovine serum, and stimulated with 60 µL of primary infection contents for 24 hours for IL-1β and TNF-α messenger RNA expression. Total RNA was isolated from cells using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The quantity and purity of total RNA were determined on a Biomate 3 spectrophotometer (Thermo-Spectronic, Rochester, NY). Complementary DNA was synthesized by reverse transcription of 500 ng of total RNA using 2.5 µmol/L Oligo (dT)₁₂₋₁₈ primers and 1.25 U/µL Moloney murine leukemia virus reverse transcriptase in the presence of 3 mmol/L MgCl₂, 2 mmol/L dNTPs, and 0.8 U/µL RNase inhibitor according to the manufacturer's protocol (Improm II; Promega, Madison, WI, USA). The PCR reaction was performed in a MyCycler (Bio-Rad) thermocycler using 2 µL of the

reverse transcription reaction product on a 20- μ L total volume PCR reaction mix (GoTaq Flexi, Promega) in the presence of 100 pmol/ μ L of each gene's primers (50 pmol/ μ L of sense and antisense primers) for IL-1 β , TNF- α , and GAPDH genes yielding products of 494, 451, and 418 bp, respectively. The primer pair used for IL-1 β (accession no.: NM031512) was sense 5'-GACCTGTTCTTTGAGGCTGA-3', antisense 5'-CGTTGCTTGTCTCTCCTTGT-3'; TNF- α (accession no.: NM012675) sense 5'-GGAGAACAGCACTCCAGAA-3', and antisense 5'-TCTTTGAGATCCATGCCATT-3' and for GAPDH (accession no.: BC083065) sense 5'-CACCATGGAGAAGCCGGGG-3', and antisense 5'-GACGGACACATTGGGGTAG-3'. The optimized cycling conditions used for TNF- α and IL-1 β were initial denaturation at 95°C for 2 minutes and 35 cycles of 95°C for 1 minute, 58°C for 1 minute, 72°C for 2 minutes, and a final extension step at 72°C for 7 minutes in the presence of 1.5 mmol/L MgCl₂. For GAPDH, conditions were as follows: initial denaturation at 95°C for 2 minutes and 25 cycles of 95°C for 1 minute, 52°C for 1 minute, 72°C for 1 minute, and a final extension step at 72°C for 10 minutes in the presence of 1.5 mmol/L MgCl₂. PCR products were resolved by electrophoresis on 1.5% (w/v) agarose gels containing ethidium bromide (0.5 μ g/mL). The amplified DNA bands were analyzed densitometrically after digital imaging capture (Image Quant 100; GE Healthcare, Sunnyvale, CA) using ImageJ 1.32j software (National Institute of Health, <http://rsb.info.nih.gov/ij/>; Bethesda, MD). The density of the bands corresponding to TNF- α and IL-1 β messenger RNA in each sample was normalized to the quantity of the housekeeping gene GAPDH and expressed as fold change over unstimulated control.

Measurements of Total Protein Levels Released to the Culture Media

The total amount of protein released in the culture media following root canal contents stimulation was measured by the Coomassie (Bradford) Protein Assay kit (Rockford, IL). As a parameter for calculation of the amount of protein released to the culture media, a standard curve was plotted using bovine serum albumin standard supplied in the kit with a known concentration (2.0 mg/mL) with a series bovine serum albumin concentration (ie, 0, 25, 125, 250, 500, 750, 1,000, 1,500, and 2,000 μ g/mL). The protein assay was performed following the manufacturer's instructions.

Calculation of Protein Concentration

The protein standard and sample solutions were measured individually using an enzyme-linked immunosorbent assay plate reader (Ultramark) at 595 nm. Because this absorbance value was directly proportional to the concentration of protein, the protein concentration from the samples solutions was determined from the standard curve.

Measurements of IL-1 β and TNF- α Levels

The amounts of IL-1 β and TNF- α released to the culture media following root canal contents stimulation of macrophages were measured by enzyme-linked immunosorbent assay (Duoset kit; R&D, Minneapolis, MN). A medium of unstimulated macrophage culture was used as a negative control. Briefly, standard, control, or sample solution was added to the enzyme-linked immunosorbent assay well plate, which had been precoated with specific monoclonal capture antibody. After shaking gently for 3 hours at room temperature, polyclonal anti-TNF- α and IL-1 β antibody, conjugated with horseradish peroxidase, was added to the solution, respectively, and incubated for 1 hour at room temperature. A substrate solution containing hydrogen peroxidase and chromogen was added and allowed to react for 20 minutes. The levels of cytokines were assessed by a micro-enzyme-

linked immunosorbent assay reader at 450 nm and normalized with an abundance of standard solution. Each densitometric value was expressed as mean \pm standard deviation and was obtained from three independent experiments.

Statistical Analysis

The data collected for each case (clinical features and the bacteria isolated) were typed onto a spreadsheet and statistically analyzed using SPSS for Windows (SPSS, Inc., Chicago, IL). The Pearson chi-square test or the one-sided Fisher exact test, as appropriate, was chosen to test the null hypothesis that there was no relationship between bacteria species such as endodontic clinical signs/symptoms and the presence of a specific group of bacteria in the root canal samples. The Pearson coefficient was used to correlate the amount of LPS, IL-1 β , and TNF- α levels with the size of the radiolucent area and the number of gram-negative bacteria present in root canals with apical periodontitis. The correlation between the presence of clinical/radiographic findings with the median levels of LPS, IL-1 β , and TNF- α was analyzed using the Student *t* test or the Mann-Whitney *U* test; $p < 0.05$ was considered statistically significant.

Results

Bacterial Detection (16 rDNA)

Bacterial DNA was detected in all root canal samples (21/21). The maximum of five species was detected in the root canal samples. At least 1 gram-negative species was detected in 18 of 21 root canals (Table 2). *Prevotella nigrescens* (13/21), *Porphyromonas endodontalis* (6/21), and *Treponema socranskii* (6/21) were the three most frequently target gram-negative bacterial species detected. A combination of two or more gram-negative target species was detected in 8 of 21 root canals (Table 2). The *Parvimonas micra* positive samples (6/21) were associated in 100% with at least one gram-negative target bacterial species. Positive associations were found between *P. endodontalis* and *Treponema denticola* ($p = 0.003$, Odds Ratio = 2.000, Confidence Bound = 0.899-4.452) and *P. micra* and *Filifactor alocis* ($p = 0.008$, OR = 1.667, CB = 0.815-3.409) in primary root canal infection. Teeth with sinus tract (2/21) were related to the presence of *F. alocis* ($p = 0.040$, OR = 18.000, CB = 0.585-553.586). A radiolucent area ≥ 2 mm was associated with the presence of *T. denticola* ($p = 0.012$, OR = 10.000, CB = 2.685-37.239). A correlation between the number of different gram-negative bacterial species and the levels of IL-1 β ($p < 0.05$, Pearson $r = 0.124$) (Fig. 1A) and TNF- α ($p < 0.05$, Pearson $r = 0.173$) (Fig. 1B) was found.

Determination of Endotoxin Concentration (Turbidimetric Test and LAL Assay)

The LAL assay (turbidimetric test) indicated that endotoxin was present in 100% of the root canals samples (21/21). The median value of endotoxin contents was 7,490 pg/mL in root canals with periradicular lesions. A higher median value of endotoxin contents was detected in teeth with the presence of a radiolucent area ≥ 2 mm (9,190 pg/mL; range, 257-212,000 pg/mL) than in teeth with a radiolucent area < 2 mm (3,480 pg/mL; range, 27-289,000 pg/mL). Teeth with exudation presented higher median levels of endotoxins (9,190 pg/mL; range, 355-289,000 pg/mL) than teeth with no exudation (2,620 pg/mL; range, 27-112,000 pg/mL). The median value of endotoxins in the presence of pain on palpation was 5,580 pg/mL (range, 27-269,000 pg/mL), and in its absence it was 35,200 pg/mL (range, 59-289,000 pg/mL). Moreover, in the presence of tenderness to percussion, the median value was 3,480 pg/mL (range, 27-289,000 pg/mL), and in the absence of tenderness to percussion, it was 9190 pg/mL (range, 59-232,000 pg/mL). Table 3 shows the median concentration of

TABLE 2. PCR Detection of Bacteria Species in 21 Root Canals with Primary Root Canal Infection and Apical Periodontitis

Target bacteria	ATCC	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	
Universal (16 rDNA)																							
<i>Dialister pneumosintes</i>		+	+																				
<i>Prevotella intermedia</i>	+																						
<i>Prevotella nigrescens</i>	+																						
<i>Aggregatibacter actinomycetemcomitans</i>	+																						
<i>Porphyromonas gingivalis</i>	+																						
<i>Filifactor alocis</i>	+																						
<i>Tannerella forsythia</i>	+																						
<i>Prevotella tanneræ</i>	+																						
<i>Treponema denticola</i>	+																						
<i>Porphyromonas endodontalis</i>	+																						
<i>Treponema socranskii</i>	+																						
<i>Parvimonas micra</i>	+																						

S, samples; +, positive detection.

endotoxin according to the clinical findings and the size of the radiolucent area. A correlation was found between endotoxin contents and the levels of TNF- α released in the culture media ($p < 0.05$, Pearson $r = 0.740$) (Fig. 1C).

Cell Culture and Cytokine Expression

IL-1 β and TNF- α Messenger RNA Expression. The macrophage cell viability after 24 hours of root canal contents stimulation was confirmed in the present study tested by its capacity to express IL-1 β and TNF- α messenger RNA.

Measurements of IL-1 β and TNF- α Levels. IL-1 β and TNF- α were detected in all culture media after stimulation with root canal contents. The median level of IL-1 β (24.835 pg/mL) was present in almost 90-fold higher than TNF- α (0.2830 pg/mL). A higher median level of IL-1 β was detected when one of the following clinical symptoms/radiographic findings was present: pain on palpation (25.528 pg/mL), tenderness to percussion (25.528 pg/mL), or size of the radiolucent area ≥ 2 mm (25.291 pg/mL) was present (Table 3). A higher level of TNF- α was found in teeth with exudation (0.2870 pg/mL) than in its absence (0.2340 pg/mL). A correlation between the levels of IL-1 β released on the culture media and the size of radiolucent area was found in this study ($p < 0.05$, Pearson $r = 0.028$) (Fig. 1D). The median concentration of endotoxin, IL-1 β , and TNF- α according to clinical findings and the size of radiolucent area is shown in Table 3.

Discussion

Data obtained in the present study revealed that a wide variety of gram-negative bacterial species do play a role in primary root canal infection with apical periodontitis, detecting at least 1 of 11 gram-negative target bacterial species in 18 of 21 root canals investigated, with a predominance of *P. nigrescens*, *P. endodontalis*, and *T. socranskii*.

The high frequency of *P. nigrescens* in primary endodontic infection with apical periodontitis seems to be related to its LPS potential in causing bone resorption (24). The almost exclusive presence of *P. endodontalis* in endodontic infections suggests a specific association with bone resorption and activation of macrophages cells (5, 25). Moreover, *P. endodontalis* LPS has been detected in a very high percentage in severe endodontic infection (26).

The combination of two or more gram-negative bacterial species found in 8 of 21 root canals investigated indicates that different bacterial LPS with different toxicity structure (lipid A) (27) can be involved in the root canal infection, enhancing or even inhibiting each other's antigenicity activity over periradicular tissues. For instance, *P. endodontalis* seems to enhance the *Fusobacterium nucleatum* LPS toxicity (5), whereas *Porphyromonas* (27) and *Bacteroides fragilis* (28) are limited in the presence of *Escherichia coli* LPS.

The association of *Parvimonas micra* (gram-positive bacteria) with at least 1 gram-negative target bacteria (eg, *F. alocis*) found in the present study turns endodontic contents even more complex and immunogenic to the immune system. Peptidoglycan (PGN), which is present in a significant amount in the gram-positive bacterial cells, plays a synergistic effect on LPS antigenic activity when they activate TLR-2 and -4, respectively (29). In this case, more macrophages differentiate into osteoclast-like cells through the Receptor Activator for Nuclear Factor κ B Ligand (RANKL):Osteoprotegerin (OPG) ratio increase (29). Yoshioka et al (30) reported that LPS can bind to *P. micra* cells conferring to this gram-positive bacteria the capacity to induce a strong TNF- α response in macrophage-like cells. Such critical finding stresses the importance of considering gram-positive non-LPS components

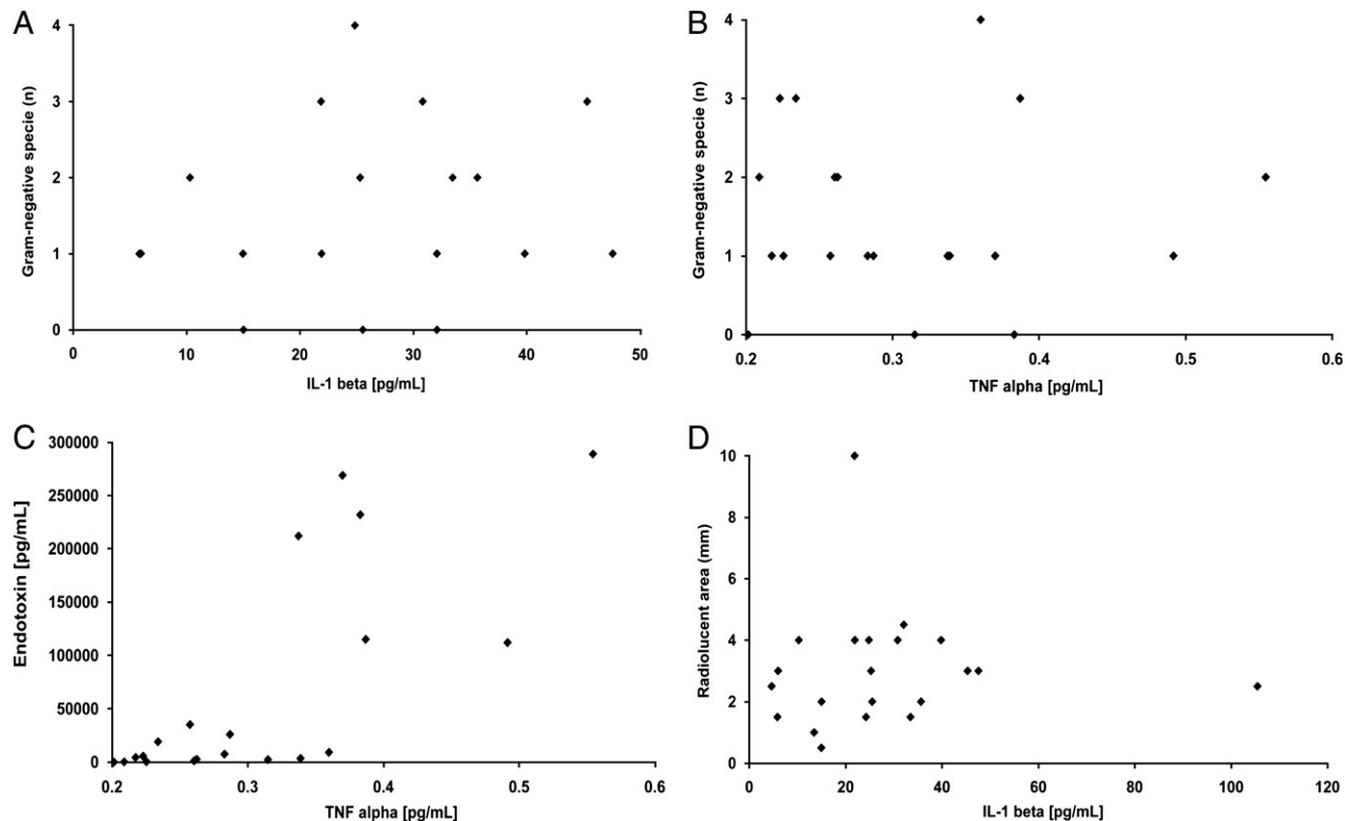


Figure 1. The correlation between the number of gram-negative species, endotoxins, and the size of the radiolucent area (mm) and the levels of cytokines. (A) The correlation between the number of gram-negative bacteria (n) and the levels of IL-1 β detected in the macrophage supernatant (pg/mL). (B) The correlation between the number of gram-negative bacteria (n) and the levels of TNF- α detected in the macrophage supernatant (pg/mL). (C) The correlation between the endotoxin concentration (pg/mL) and the levels of TNF- α detected in the macrophage supernatant (pg/mL). (D) The correlation between the size of the radiographic area (mm) and the levels of IL-1 β in the macrophage supernatant (pg/mL).

when interpreting findings on the expression of proinflammatory cytokines.

Previous *in vitro* investigations have attempted to extract LPS in order to determine the antigenic activity of the endodontic contents (5–7). However, the clinical significance of these investigations is unclear considering the complexity of the antigens involved in endodontic infection described earlier. Moreover, *in vitro* models, using bacterial growth culture media, fail in reproducing the infection environment, particularly regarding the hemin concentration in the infection site (31). Hemin concentration (in the hemoglobin form) varies considerably depending on the inflammatory response and blood vessels integrity, and it might modulate the lipid A structure in the LPS molecule, which is responsible for the majority of IL-1 induction (27).

To address these points, the present study stimulated macrophage cells with material individually isolated from 21 infected root canals presenting primary endodontic infection and apical periodontitis, concomitantly investigating the microbiota and the endotoxin content of each infected root canal. The analysis revealed that the increase in the number of gram-negative bacteria was significantly followed by an increase in the IL-1 β and TNF- α levels. These findings suggest that the presence of clinical signs/symptoms such as bone destruction involved in apical periodontitis evoked by the immune system in response to LPS is not only associated with the amount of endotoxin elucidated by previous clinical investigations (12, 14–15) but also with the presence of a different number and heterogeneity of gram-negative bacteria, which acting synergistically can lead to a stronger immune response in periapical tissues.

P. nigrescens contains a very potent LPS molecule for prostaglandin E₂ stimulation (24) in inflamed pulp tissue (32) and in acutely inflamed periapical tissue (17). Additionally, IL-1 β and TNF- α released from macrophages supernatants treated with infectious material derived from teeth with exudation are potent stimuli for prostaglandin E₂ release (33). Our results agree with Ataoglu et al (22) who reported no association between teeth with exudates and higher levels of IL-1 β and are inconsistent with Kuo et al (23). Furthermore, higher significantly levels of endotoxin were found in root canal exudation in this current study corroborating with previous clinical investigations (6, 12, 16).

Even though higher levels of endotoxins were expected particularly in pain on palpation and tenderness to percussion, it was not found in the present study. These particular data might be explained by the characteristics of the samples investigated in which a greater number of cases in the absence of these clinical features were found, contributing to such findings.

Teeth with a larger size of periradicular lesions (≥ 2 mm) were related to the detection of *T. denticola* in the root canal positively associated with *P. endodontalis* exhibiting a potent biological activity in cell culture (5) and with chronic bone resorption (29). Higher levels of endotoxin were found in those teeth, which is in agreement with Schein and Schilder (16) who reported that the endotoxin contents of teeth with radiolucent area were five times greater than in teeth without such area.

The larger size of periradicular lesions (≥ 2 mm) was also correlated with higher levels of IL-1 β in accordance to Tani-Ishii et al (34).

TABLE 3. The Median Concentration of Endotoxin, IL-1 β , and TNF- α in pg/mL According to the Clinical Findings and Size of the Radiolucent Area

	Total Amount		Pain on palpation		Tenderness to percussion		Exudation		Size of radiolucent area	
	n = 21		Present n = 9	Absent n = 12	Present n = 8	Absent n = 13	Present n = 12	Absent n = 9	≥ 2 mm n = 11	<2 mm n = 10
Endotoxin (pg/mL)	7,490	5,580	5,580	35,200	3,480	9,190	9,190	2,620	9,190	3,480
IL-1 β (pg/mL)	24.835	25.528	25.528	24.835	25.528	24.835	24.835	25.291	25.291*	15.007*
TNF- α (pg/mL)	0.2830	0.2605	0.2605	0.3150	0.2340	0.2870	0.2870	0.2340	0.2575	0.2870

*Statistically significant difference (p < 0.05).

This finding might be related to the PGN present in gram-positive and -negative bacteria, which inhibits the differentiation of monocytes/macrophages into mature cells. Besides this inhibition, PGN promotes cytokine production by undifferentiated precursors (29), consequently increasing localized osteolysis particularly at sites with larger number of macrophages (35).

More studies that analyze the mechanisms involved in the production of cytokines and bone resorption are important for the development of new therapies. The interference in toll-like receptors and interleukin-1 receptor downstream signaling pathways should be considered to control the development and progression of endodontic lesions. In the future, the use of the RNA interference can reduce the differentiation of macrophages into osteoclasts; the induction of cytosolic phospholipase-A (cPLA) and beta-defensin2; and, consequently, the release of TNF- α , IL-1 β , and IL-6 (36–38). Another strategy is the superexpression of specific regulator proteins such as SOCS3, interferon-gamma, and interleukin-10 through local delivery of DNA vectors (39).

Overall, the present study suggested that the antigenicity of the endodontic contents against macrophages (IL- α and IL-1 β release) is not only associated with the amount of endotoxin but also with the number of gram-negative bacteria involved in the infection. Further investigations should be performed in order to assess the effect of antimicrobial agents over different lipid A structures isolated from species commonly found in endodontic infections and their mechanism of action in different cell types.

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References

1. Fabricius L, Dahlén G, Holm SE, et al. Influence of combinations of oral bacteria on periapical tissues of monkeys. *Scand J Dent Res* 1982;90:200–6.
2. Nair PNR. Pathogenesis of apical periodontitis and the causes of endodontic failures. *Crit Rev Oral Biol Med* 2004;15:348–81.
3. Lim GC, Torabinejad M, Kettering J, et al. Interleukin 1 β in symptomatic and asymptomatic human periradicular lesions. *J Endod* 1994;20:225–7.
4. Rietschel ET, Brade H. Bacterial endotoxins. *Sci Am* 1992;267:54–61.
5. Hong CY, Lin SK, Kok SH, et al. The role of lipopolysaccharide in infectious bone resorption of periapical lesion. *J Oral Pathol Med* 2004;33:162–9.
6. Horiba N, Maekawa Y, Abe Y, et al. Cytotoxicity against various cell lines of lipopolysaccharides purified from bacteroides, fusobacterium, and veillonella isolated from infected root canals. *J Endod* 1989;15:530–4.
7. Hosoya S, Matsushima K. Stimulation of interleukin-1 beta production of human dental pulp cells by *Porphyromonas endodontalis* lipopolysaccharide. *J Endod* 1997;23:39–42.
8. Matsuo T, Ebisu S, Shimabukuro Y, et al. Quantitative analysis of immunocompetent cells in human periapical lesions: correlations with clinical findings of the involved teeth. *J Endod* 1992;18:497–500.
9. Artese L, Piattelli A, Quaranta M, et al. Immunoreactivity for interleukin 1-beta and tumor necrosis factor-alpha and ultrastructural features of monocytes/macrophages in periapical granulomas. *J Endod* 1991;17:483–7.
10. Stashenko P, Dewhirst FE, Rooney ML, et al. Interleukin-1 beta is a potent inhibitor of bone formation *in vitro*. *J Bone Miner Res* 1987;2:559–65.
11. Beutler B, Cerami A. Cachectin and tumour necrosis factor as two sides of the same biological coin. *Nature* 1986;320:584–8.
12. Jacinto RC, Gomes BPFA, Shah HN, et al. Quantification of endotoxins in necrotic root canals from symptomatic and asymptomatic teeth. *J Med Microbiol* 2005;54:777–83.
13. Vianna ME, Hartz HP, Conrads G, et al. Effect of root canal procedures on endotoxins and endodontic pathogens. *Oral Microbiol Immunol* 2007;22:1–8.
14. Martinho FC, Gomes BP. Quantification of endotoxins and cultivable bacteria in root canal infection before and after chemomechanical preparation with 2.5% sodium hypochlorite. *J Endod* 2008;34:268–72.

15. Gomes BP, Martinho FC, Vianna ME. Comparison of 2.5% sodium hypochlorite and 2% chlorhexidine gel on oral bacterial lipopolysaccharide reduction from primarily infected root canals. *J Endod* 2009;35:1350–3.
16. Schein B, Schilder H. Endotoxin content in endodontically involved teeth. *J Endod* 1975;1:19–21.
17. McNicholas S, Torabinejad M, Blankenship J, et al. The concentration of prostaglandin E2 in human periradicular lesions. *J Endod* 1991;17:97–100.
18. Shimauchi H, Takayama S, Miki Y, et al. The change of periapical exudate prostaglandin E2 levels during root canal treatment. *J Endod* 1997;23:755–8.
19. Barkhordar RA, Hussain MZ, Hayashi C. Detection of interleukin-1 beta in human periapical lesions. *Oral Surg Oral Med Oral Pathol* 1992;73:334–6.
20. Safavi KE, Rossomando EF. Tumor necrosis factor identified in periapical tissue exudates of teeth with apical periodontitis. *J Endod* 1991;17:12–4.
21. Matsuo T, Ebisu S, Nakanishi T, et al. Interleukin-1 alpha and interleukin-1 beta periapical exudates of infected root canals: correlations with the clinical findings of the involved teeth. *J Endod* 1994;20:432–5.
22. Ataoglu T, Ungör M, Serpek B, et al. Interleukin-1beta and tumour necrosis factor-alpha levels in periapical exudates. *Int Endod J* 2002;35:181–5.
23. Kuo ML, Lamster IB, Hasselgren G. Host mediators in endodontic exudates. I. Indicators of inflammation and humoral immunity. *J Endod* 1998;24:598–603.
24. Chung YH, Chang EJ, Kim SJ, et al. Lipopolysaccharide from *Prevotella nigrescens* stimulates osteoclastogenesis in cocultures of bone marrow mononuclear cells and primary osteoblasts. *J Periodontol Res* 2006;41:288–96.
25. van Winkelhoff AJ, van Steenberghe TJ, de Graaff J. *Porphyromonas (Bacteroides) endodontalis*: its role in endodontal infections. *J Endod* 1992;18:431–4.
26. Hanazawa S, Sagiya T, Kitami H, et al. Monoclonal antibody against *Porphyromonas (Bacteroides) endodontalis* lipopolysaccharide and application of the antibody for direct identification of the species. *J Clin Microbiol* 1991;29:2550–3.
27. Dixon DR, Darveau RP. Lipopolysaccharide heterogeneity: innate host responses to bacterial modification of lipid A structure. *J Dent Res* 2005;84:584–95.
28. Magnuson DK, Weintraub A, Pohlman TH, et al. Human endothelial cell adhesiveness for neutrophils, induced by *Escherichia coli* lipopolysaccharide in vitro, is inhibited by *Bacteroides fragilis* lipopolysaccharide. *J Immunol* 1989;143:3025–30.
29. Jiang J, Zuo J, Hurst IR, et al. The synergistic effect of peptidoglycan and lipopolysaccharide on osteoclast formation. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2003;96:738–43.
30. Yoshioka M, Grenier D, Mayrand D. Binding of *Actinobacillus actinomycetemcomitans* lipopolysaccharides to *Peptostreptococcus micros* stimulates tumor necrosis factor alpha production by macrophage-like cells. *Oral Microbiol Immunol* 2005;20:118–21.
31. Al-Qutub MN, Braham PH, Karimi-Naser LM, et al. Hemin-dependent modulation of the lipid A structure of *Porphyromonas gingivalis* lipopolysaccharide. *Infect Immun* 2006;74:4474–85.
32. Cohen JS, Reader A, Fertel R, et al. A radioimmunoassay determination of the concentrations of prostaglandins E2 and F2 alpha in painful and asymptomatic human dental pulps. *J Endod* 1985;11:330–5.
33. Richards D, Rutherford RB. The effects of interleukin 1 on collagenolytic activity and prostaglandin-E secretion by human periodontal-ligament and gingival fibroblast. *Arch Oral Biol* 1988;33:237–43.
34. Tani-Ishii N, Wang CY, Stashenko P. Immunolocalization of bone-resorptive cytokines in rat pulp and periapical lesions following surgical pulp exposure. *Oral Microbiol Immunol* 1995;10:213–9.
35. Gowen M, Wood DD, Ihrle EJ, et al. An interleukin 1 like factor stimulates bone resorption in vitro. *Nature* 1983;306:378–80.
36. Fahid FS, Jiang J, Zhu Q, et al. Application of small interfering RNA for inhibition of lipopolysaccharide-induced osteoclast formation and cytokine stimulation. *J Endod* 2008;34:563–9.
37. Fukushima A, Kajiya H, Izumi T, et al. Pro-inflammatory cytokines induce suppressor of cytokine signaling-3 in human periodontal ligament cells. *J Endod* 2010;36:1004–8.
38. Kim YS, Min KS, Lee HD, et al. Effect of cytosolic phospholipase A(2) on proinflammatory cytokine-induced bone resorptive genes including receptor activator of nuclear factor kappa B ligand in human dental pulp cells. *J Endod* 2010;36:636–41.
39. Kim YS, Min KS, Lee SI, et al. Effect of proinflammatory cytokines on the expression and regulation of human beta-defensin 2 in human dental pulp cells. *J Endod* 2010;36:64–9.