Signaling Pathways Activation by Primary Endodontic Infectious Contents and Production of Inflammatory Mediators

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Abstract

Introduction: This study investigated the bacterial community involved in primary endodontic diseases, evaluated its ability to activate the macrophage Toll-like receptor 4 receptor through p38 mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF-κB) signaling pathways, and determined the levels of endotoxins and interleukins (interleukin [IL]-6 and -10) produced by endodontic content-stimulated macrophages. **Methods:** Samples were taken from 21 root canals by using sterile/apyrogenic paper points. Raw 264.7 macrophages were stimulated with root canal contents. Checkerboard DNA-DNA hybridization was used for bacterial analysis and the limulus amebocyte lysate assay for endotoxin measurement; p38 MAPK and NF-κB activation was determined by Western blot analysis. IL-6 and IL-10 were measured using the enzyme-linked immunosorbent assay. **Results:** Bacteria and endotoxins were detected in 100% of the samples (21/21). The most frequently observed species were *Parvimonas micra* (16/21, 76%), *Fusobacterium nucleatum* ssp. *nucleatum* (15/21, 71%), and *Porphyromonas endodontalis* (14/21, 66%). Correlations were found between endotoxins and IL-6 and IL-10 ($P < .05$); p38 phosphorylation had a peak at 60 minutes, and NF-κB was quickly activated after 10 minutes of stimulation. **Conclusions:** It was concluded that the complex bacterial community was shown to be a potent activator of TLR-4 determined by the p38 MAPK and NF-κB signaling pathways, culminating in a high antigenicity against macrophages through the levels of IL-6 and IL-10, all significantly affected by endotoxin levels. *(J Endod 2014;40:484–489)*

Key Words

Endotoxin, interleukin, macrophage, root canal, signaling pathways

Bacterial infection of the dental pulp results in tissue destruction and, eventually, periapical bone resorption (1). Inflammatory cytokine production is induced in response to the infection, which is primarily produced by monocytes/macrophages that play a central role in cytokine production by modulating many aspects of the inflammatory response (2, 3).

It has long been known that primary endodontic infection has a polymicrobial etiology caused by both gram-positive and gram-negative anaerobic bacteria (4, 5). The latter have lipopolysaccharides (LPSs, known as endotoxins) located on the outer layers of bacterial cell walls (6) and are considered one of the major factors involved in the inflammation response.

LPSs have been shown to interact with Toll-like receptors (TLRs), both TLR-2 and TLR-4, but with greater affinity for TLR-4 (7, 8), which in turn recognizes the LPS molecule and activates multiple downstream signaling pathways (9, 10). The binding of LPSs to TLR-4 leads to the activation of p38 mitogen-activated protein kinase (p38 MAPK) (an upstream effector common to many inflammatory cytokines) and NF-κB transcription factor (central to several immune and inflammatory responses), which are responsible for proinflammatory cytokine production, such as interleukin (IL)-1 beta, tumor necrosis factor alpha, prostaglandin E2, and IL-6 and -10 (11–13).

The action of proinflammatory/bone resorptive mediators is potentially regulated by a network of different cytokines (7, 8). This network in endodontic infection is complex (7) and changes according to disease destruction and activity (9). The redundancy and overlapping of cytokines make it difficult to understand the inflammatory process (8, 10). Thus, the study of different signaling pathways involved in endodontic gene expression may allow us to gain an understanding of the modulation of the host response affecting the whole cytokine profile.

In order to better understand the immunobiology involved in primary endodontic diseases, the aims of this clinical study were to investigate the bacterial community involved, to evaluate its ability to activate macrophage TLR-4 receptor through p38

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MAPK and NF-κB signaling pathways, and to determine the levels of endotoxins and interleukins (IL-6 and -10) produced by endodontic content-stimulated macrophages.

Materials and Methods

Patient Selection

Twenty-one patients needing endodontic treatment who attended the Piracicaba Dental School, Piracicaba, São Paulo, Brazil, were included in this study. The age of the patients ranged from 13–73 years. Samples were collected from 21 root canals with pulp necrosis; they all showed radiographic evidence of apical periodontitis. The selected teeth showed the absence of periodontal pockets more than 4 mm in depth.

A detailed dental history was obtained from each patient. Those who had received antibiotic treatment during the past 3 months or who had any systemic disease were excluded. The Human Research Ethics Committee of the Piracicaba Dental School approved the protocol describing the sample collection for this investigation, and all volunteer patients signed an informed consent form before their participation in the study.

Sampling Procedures

All materials used in this study were heat sterilized at 200°C for 4 hours, thus becoming apyrogenic. The method followed for disinfection of the operative field was described previously (3, 4). The teeth were isolated with a rubber dam; the crown and surrounding structures were disinfected with 30% H_2O_2 for 30 seconds followed by 2.5% sodium hypochlorite for a further 30 seconds. Subsequently, 5% sodium thiosulfate was used to inactivate the irrigant. The sterility of the external surfaces of the crown was checked by taking a swab sample from the crown surface and streaking it onto blood agar plates, which were incubated aerobically and anaerobically.

A 2-stage access cavity preparation was made without the use of water spray but under manual irrigation with sterile/apyrogenic saline solution and by using a 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 according to the protocol described previously. 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 followed by irrigation of the root canal access with sterile apyrogenic water. The endotoxin sample was taken by 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 for 60 seconds. Next, the paper point was immediately placed on a pyrogen-free glass and frozen at −80°C for limulus amebocyte lysate (LAL) assay and cell culture stimulation. The procedure was repeated with 5 sterile paper points. The paper points were pooled in a sterile tube containing 1 mL VMGA III transport medium (Becton Dickinson Microbiology Systems, Cockeysville, MD) and then immediately processed for DNA extraction to detect the target bacteria using the checkerboard DNA-DNA technique.

Microbiological Assessment: Checkerboard DNA-DNA Hybridization

The presence, levels, and proportions of 40 bacterial species (Table 1) were determined in each sample by modifying the checkerboard DNA-DNA hybridization method as described by previous investigations (11, 14). Microbial DNA from endodontic samples and American Type Culture Collection bacterial strains (probes were extracted and purified with QIAamp DNA Mini Kit; Qiagen, Hilden, Germany) were obtained according to the manufacturer's instructions. The concentration of DNA (absorbance at 260 nm) was determined with a spectrophotometer (Nanodrop 2000; Thermo Scientific, Wilmington, DE).

The samples were boiled for 10 minutes and neutralized with 0.8 mL 5 mol/L ammonium acetate. The released DNA was then placed into extended slots of a Minislot 30 apparatus (Immunetics, Cambridge, MA), concentrated onto a 15 − 15 positively charged nylon membrane (Boehringer, Mannheim, Germany), and fixed to the membrane by incubation at 120°C for 20 minutes. A Miniblotter 45 (Immunetics) device was used to hybridize the 40 digoxigenin-labeled whole-genomic DNA probes at right angles to the lanes of the clinical samples. Bound probes were detected by using phosphatase-conjugated antibodies to digoxigenin and chemiluminescence (CDP-Star Detection Reagent; Amersham Biosciences, Chicago, IL). Signals were visually evaluated by comparison with 2 standards. These standards consisted of a mixture of 10^5 and 10^6 cells from each bacteria tested placed in the last 2 lanes of each membrane. The signals were coded in 6 different classes in relation to the following count levels:

0: Not detected
1: $<10^5$ cells
2: Nearly 10^5 cells
3: Between 10^5 and 10^6 cells
4: Approximately 10^6 cells
5: $>10^6$ cells

The sensitivity of this assay was adjusted to permit the detection of 10^4 cells of a given species by adjusting the concentration of each DNA probe.

Endotoxin Detection (LAL Assay)

Determination of Endotoxin Concentration (Turbidimetric Test and LAL Assay). The turbidimetric test (BioWhitaker Inc, Walkersville, MD) was used to measure endotoxin concentrations in root canals using the LAL technique. As a parameter for calculation of the amount of endotoxins in the root canal samples, a standard curve was plotted by using the endotoxins supplied by the kit with known concentrations (100 EU/mL) and dilutions, resulting in the following final concentrations according to the manufacturer's instructions: 0.01, 0.10, 1, and 10 EU/mL.

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

Calculation of Endotoxin Concentration. 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.

Activation of Signaling Pathways

P38 MAPK and NF-kB Signaling Pathways (Western Blot). For these short-term experiments, 3×10^5 RAW 264.7 macrophages were grown for 24 hours in each well of the 6-well plates and