# Sodium Hypochlorite Inactivates Lipoteichoic Acid of *Enterococcus faecalis* by Deacylation

*Sun Woong Hong, MS,* Jung Eun Baik, PhD,* Seok-Seong Kang, PhD,* Kee-Yeon Kum, DDS, PhD,† Cheol-Heui Yun, PhD,‡ and Seung Hyun Han, PhD**

## Abstract

**Introduction:** *Enterococcus faecalis* is a pathogenic gram-positive bacterium closely associated with apical periodontitis. Although sodium hypochlorite (NaOCl) has been used as a common endodontic irrigant to eradicate bacteria in the root canal, it has not been elucidated whether NaOCl attenuates the inflammatory response induced by the *E. faecalis* virulence factor lipoteichoic acid (EfLTA). **Methods:** Structurally intact EfLTA purified from *E. faecalis* was treated with NaOCl at various concentrations and time periods. Murine macrophage cell line RAW 264.7 was treated with interferon gamma followed by treatment with intact or NaOCl-treated EfLTA to determine the inducibility of inflammatory mediators such as nitric oxide, interferon gamma–inducible protein 10, and macrophage inflammatory protein-1$\alpha$. Reporter gene assays assessed by flow cytometry were used to examine the ability of intact or NaOCl-treated EfLTA to activate Toll-like receptor 2 (TLR2), which is known to recognize EfLTA on host cells. Structural damage of EfLTA by NaOCl was examined using silver staining and thin-layer chromatography. **Results:** NaOCl-treated EfLTA showed markedly less induction of nitric oxide, interferon gamma–inducible protein 10, and macrophage inflammatory protein-1$\alpha$ in RAW 264.7 cells compared with intact EfLTA. In contrast to intact EfLTA that potently stimulated TLR2 activation, NaOCl-treated EfLTA did not activate TLR2. Structural analysis showed that NaOCl damaged EfLTA structure by deacylation. **Conclusions:** NaOCl deacylates the glycolipid moiety of EfLTA, which fails to activate TLR2, leading to the reduced production of inflammatory mediators. *(J Endod 2016;■:1–6)*

## Key Words

Apical periodontitis, *Enterococcus faecalis*, lipoteichoic acid, sodium hypochlorite, Toll-like receptor 2

**Significance**

Sodium hypochlorite, which is a common endodontic irrigant to eradicate bacteria in the root canal, could effectively detoxify a major virulence factor of *Enterococcus faecalis*, lipoteichoic acid, leading to the reduced production of inflammatory mediators in macrophages.

Enterococci are gram-positive bacteria frequently found in mucosal tissues of the oral cavity, gastrointestinal tract, and genital tract (1). In addition to their role as a common nosocomial pathogen, the enterococci are a major etiologic agent of persistent apical periodontitis (1, 2). Of the enterococci, *Enterococcus faecalis* is a prevalent species in periradicular lesions of teeth after endodontic treatment and is also involved in persistent apical periodontitis (3, 4). *E. faecalis* is commonly resistant to disinfectants and antiseptics because it is known to survive in severe environmental conditions such as high alkalinity (5).

Virulence factors such as lipoteichoic acid (LTA), lytic enzymes, cytolysin, adhesins, aggregation substance, and pheromones of *E. faecalis* are known to be involved in pathogenicity (6). Among these virulence factors, LTA is considered a major etiologic agent based on the induction of the inflammatory response and tissue damage (7, 8). For example, it has been shown that *E. faecalis* LTA (EfLTA) induced several inflammatory mediators such as nitric oxide (NO), macrophage inflammatory protein-1$\alpha$ (MIP-1$\alpha$), and monocyte chemotactic protein-1 (7, 9). In addition, EfLTA is closely associated with bacterial adhesion and biofilm formation, which are responsible for resistance to disinfectants, antibiotics, and antimicrobial peptides (10).

LTA is an amphiphilic molecule consisting of a glycolipid anchor linked with polyglycerolphosphate or polyribitolphosphate backbones (11). Most gram-positive bacteria including *E. faecalis* contain polyglycerolphosphate-type LTA, whereas a few gram-positive bacteria such as *Streptococcus pneumoniae* express polyribitolphosphate-type LTA (12, 13). LTA exclusively activates Toll-like receptor 2 (TLR2), which leads to the production of various proinflammatory chemokines and cytokines (14). It has been well described that the glycolipid moiety of the LTA structure is critical for its immunostimulating potential. Delipidation of LTA completely abolishes the immunostimulating potential (15). On the other hand, LTA with more acyl chains shows higher immunostimulating activity (16). Therefore, the glycolipid moiety of the LTA structure can be a determinant to elicit immune responses including the production of inflammatory mediators.

Endodontic irrigants are used to eliminate causative bacteria including *E. faecalis* in the infected root canal. Sodium hypochlorite (NaOCl) has been widely used as an

From the *Department of Oral Microbiology and Immunology, Dental Research Institute, and BK21 Plus Program, School of Dentistry, †Department of Conservative Dentistry and Dental Research Institute, School of Dentistry, and ‡Department of Agricultural Biotechnology and Research Institute for Agriculture and Life Sciences, Seoul National University, Seoul, Republic of Korea.

Address requests for reprints to Dr Seung Hyun Han, Department of Oral Microbiology and Immunology, Dental Research Institute, and BK21 Plus Program, School of Dentistry, Seoul National University, Building 86 (Room 304), 1 Gwanak-ro, Gwanak-gu, Seoul 08826, Republic of Korea. E-mail address: shhan-mi@snu.ac.kr

0099-2399/$ - see front matter



http://dx.doi.org/10.1016/j.joen.2016.06.018

endodontic irrigant for effective bactericidal and nonspecific proteolytic activity (17) and is strongly alkaline and hypertonic (18). In addition, it is known to dissolve organic tissues containing fatty acids and lipids via a saponification reaction (19). Although the antibacterial effect of NaOCl is well recognized, it is poorly understood if NaOCl detoxifies a major virulence factor of gram-positive bacteria, LTA. Therefore, the aim of the study was to investigate whether NaOCl inactivates EfLTA, leading to the reduction of the inflammatory response.

## Materials and Methods

### Bacteria, Reagents, and Chemicals

*E. faecalis* ATCC29212 was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Brain-heart infusion medium was purchased from BD Biosciences (Franklin, NJ). NaOCl was purchased from Duksan Pure Chemicals (Ansan, Korea). All other reagents were obtained from Sigma-Aldrich (St Louis, MO) unless otherwise stated. Fluorescein isothiocyanate–conjugated mouse monoclonal antihuman TLR2 antibody was purchased from Biolegend (San Diego, CA).

### EfLTA Preparation and Treatment with NaOCl

*E. faecalis* was cultured in brain-heart infusion medium at 37°C, and the highly pure and structurally intact EfLTA was prepared as described previously (7, 20, 21). Unwanted biological molecules including endotoxin, nucleic acids, and proteins in the preparation were not detectable (data not shown). NaOCl-treated EfLTA was subjected to a PD-10 desalting column (GE Healthcare Life Sciences, Buckinghamshire, UK) to remove NaOCl and debris that may have formed by structural damage. After lyophilization, the concentration of EfLTA was determined using phosphate assay as previously described (22).

### Culture of RAW 264.7 Cells

The murine macrophage cell line RAW 264.7 obtained from the ATCC was maintained in Dulbecco's Modified Eagle's Medium (HyClone, Logan, UT) supplemented with 10% fetal bovine serum (Gibco, Waltham, MA), 100 U/mL penicillin, and 100 $\mu$g/mL streptomycin at 37°C in a humidified incubator with 5% $CO_2$ (23).

### Measurement of NO and Proinflammatory Cytokines

RAW 264.7 cells ($1 \times 10^6$ cells/mL) were treated with EfLTA or NaOCl- or sodium hydroxide (NaOH)-treated EfLTA for 24 hours. Then, the culture supernatants were collected, and the production of NO was measured as described previously (24, 25). The secretion of interferon-$\gamma$-inducible protein-10 (IP-10) and MIP-1$\alpha$ was determined using commercial enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instruction.

### Determination of TLR2 Activation

CHO/CD14/TLR2 cells are Chinese hamster ovary cells constitutively expressing human CD14 and TLR2 that are stably transfected with a nuclear factor kappa B–dependent reporter plasmid to express membrane-bound CD25 in proportion to TLR2 activation (26). For the determination of TLR2 activation, the cells ($3 \times 10^5$ cells/mL) were stimulated with EfLTA (0, 1, 3, 10, or 30 $\mu$g/mL); NaOCl-treated EfLTA (0, 1, 3, 10, or 30 $\mu$g/mL); or $Pam_2CSK_4$, a synthetic lipopeptide mimicking gram-positive bacterial lipoproteins that is widely used as a TLR2 ligand (27) (0.1 $\mu$g/mL), for 24 hours. To determine TLR2 activation, the cells were stained with FITC-conjugated mouse monoclonal antihuman TLR2, and then the expression was analyzed by flow cytometry using a FACSCalibur flow cytometer with CellQuest software (BD Biosciences).

### Thin-layer Chromatographic Separation and Silver Staining

Thin-layer chromatographic (TLC) separation was performed on a silica gel TLC plate (Silica gel 60; Merck, Whitehouse Station, NJ) (28), and sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by silver staining was performed as described previously (29).

### Statistical Analysis

All experiments were conducted in triplicate, and treatment groups with EfLTA or NaOCl-treated EfLTA were compared with the nontreatment group; statistical significance was determined using the Student $t$ test. An asterisk indicates a significant difference ($P < .05$) when compared with the nontreatment group.

## Results

### When Compared with EfLTA, NaOCl-treated EfLTA Showed No or Little Immunostimulating Activities

We previously showed that EfLTA induced the production of inflammatory mediators such as NO, IP-10, and MIP-1$\alpha$ in murine macrophages (9). To investigate whether the NaOCl treatment abolishes the immunostimulating activity of EfLTA, RAW 264.7 cells were treated with EfLTA or NaOCl-treated EfLTA for 24 hours. EfLTA induced a significant amount of NO, IP-10, and MIP-1$\alpha$ (Fig. 1*A–C*, respectively). However, NaOCl-treated EfLTA failed to induce the respective mediators, suggesting that NaOCl treatment completely abolished EfLTA-induced inflammatory responses. In addition, as a positive control, NaOH-treated EfLTA showed significantly less induction of NO, IP-10, and MIP-1$\alpha$ compared with EfLTA.

### NaOCl Reduced Ability of EfLTA to Induce NO Production at Low Concentrations and at Early Time Points

To identify the optimal concentration and duration of NaOCl needed for inactivation, EfLTA was pretreated with $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, or $10^{-5}$ % NaOCl for 1 hour. Then, RAW 264.7 cells were incubated with NaOCl-treated EfLTA for 24 hours. As shown in Figure 2*A*, the production of NO was not induced by EfLTA treated with $10^{-1}$ to $10^{-4}$ % (v/v) NaOCl. However, it was noted that $10^{-5}$ % NaOCl–treated EfLTA slightly increased the production of NO. This concentration of NaOCl appears to be insufficient to completely inactivate EfLTA and thus it might induce the expression of inducible nitric oxide synthase resulting in NO production (30). Furthermore, when EfLTA was treated with 5.25% NaOCl for various time points, a complete reduction of NO was observed as early as 0.5 minutes (Fig. 2*B*).

### Compared with EfLTA, NaOCl-treated EfLTA Fails to Activate TLR2

We previously observed that EfLTA-induced inflammatory responses were through TLR2 activation (7). To examine whether the reduction of inflammatory responses was caused by impaired TLR2 activation, CHO/CD14/TLR2 cells were incubated with NaOCl-treated EfLTA to examine its activation. EfLTA increased TLR2 activation in a dose-dependent manner, as previously shown (7). However, NaOCl-treated EfLTA did not increase TLR2 activation (Fig. 3*A* and *B*), indicating that NaOCl treatment impaired TLR2 activation and that the reduction of inflammatory responses by NaOCl treatment is attributable to impaired TLR2 activation.