Lipopolysaccharides from Periodontopathic Bacteria

Porphyromonas gingivalis and Capnocytophaga ochracea Are Antagonists for Human Toll-Like Receptor 4

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Toll-like receptors (TLRs) 2 and 4 have recently been identified as possible signal transducers for various bacterial ligands. To investigate the roles of TLRs in the recognition of periodontopathic bacteria by the innate immune system, a Chinese hamster ovary (CHO) nuclear factor-κB (NF-κB)-dependent reporter cell line, 7.7, which is defective in both TLR2- and TLR4-dependent signaling pathways was transfected with human CD14 and TLRs. When the transfecants were exposed to freeze-dried periodontopathic bacteria, Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Capnocytophaga ochracea, and Fusobacterium nucleatum, and a non-oral bacterium, Escherichia coli, all species of the bacteria induced NF-κB-dependent CD25 expression in 7.7/huTLR2 cells. Although freeze-dried A. actinomycetemcomitans, F. nucleatum, and E. coli also induced CD25 expression in 7.7/huTLR4 cells, freeze-dried P. gingivalis did not. Similarly, lipopolysaccharides (LPS) extracted from A. actinomycetemcomitans, F. nucleatum, and E. coli induced CD25 expression in 7.7/huTLR4 cells, but LPS from P. gingivalis and C. ochracea did not. Furthermore, LPS from P. gingivalis and C. ochracea attenuated CD25 expression in 7.7/huTLR4 cells induced by repurified LPS from E. coli. LPS from P. gingivalis and C. ochracea also induced the secretion of interleukin-6 (IL-6) from U373 cells, the secretion of IL-1β from human peripheral blood mononuclear cells, and ICAM-1 expression in human gingival fibroblasts induced by repurified LPS from E. coli. These findings indicated that LPS from P. gingivalis and C. ochracea worked as antagonists for human TLR4. The antagonistic activity of LPS from these periodontopathic bacteria may be associated with the etiology of periodontal diseases.

It is generally accepted that most periodontal diseases are caused by bacteria in dental plaque (10). More than 300 species of bacteria colonize in the subgingival area, and their cell wall components can trigger immune activation (17). Those compounds induce a series of proinflammatory cytokines from host tissue (40), which induce alveolar bone resorption and destruction of gingival connective tissue (9, 27).

Recently, two members of the Toll-like receptor (TLR) family, TLR2 and TLR4, have been identified as possible signaling receptors for bacterial cell wall components. The expression of TLR2 in Chinese hamster ovary (CHO) fibroblasts or human embryonic kidney cells (HEK293), which are TLR2 deficient (6, 14), conferred responsiveness to various bacterial components, such as peptidoglycan, lipoprotein, and lipoarabinomannan (19, 21, 31, 41). tlr4 cloned from lipopolysaccharide (LPS)-resistant C3H/HeJ mice harbored a point mutation that rendered it nonfunctional (28). Subsequently, in vivo roles of TLR2 and TLR4 were investigated (33). While TLR4-deficient mice were unresponsive to LPS, macrophages from TLR2-deficient mice lacked the response to gram-positive bacterial cell wall, strengthening the hypothesis that TLR4 is a principal signal transducer for LPS and TLR2 is a signal transducer for other bacterial components, such as peptidoglycan and lipoprotein.

In the present study, we investigated the roles of human TLR2 and TLR4 in the recognition of periodontopathic bacteria using a mutant CHO/CD14 reporter cell line, 7.7, which has a defect in its LPS-signaling pathway (2). As CHO cells do not express a functional transcript for TLR2 (6), 7.7 has a defect in both TLR2- and TLR4-dependent signaling pathways. Although the exact defect in signal transduction in 7.7 remains unknown, 7.7 transfected with human TLR4 (7.7/huTLR4) responds to high concentrations of LPS, and 7.7 transfected with human TLR2 (7.7/huTLR2) was as sensitive to CHO/CD14/huTLR2 to bacterial lipoprotein (19). These transfecants that expressed human TLRs were exposed to the following freeze-dried periodontopathic bacteria: Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans, Capnocytophaga ochracea, and Fusobacterium nucleatum (11, 12). A non-oral bacterium, Escherichia coli, was used as a control. The results indicated that human TLR2 expression in 7.7 enhanced the response to each freeze-dried bacterium but that human TLR4 expression failed to enhance the response to freeze-dried P. gingivalis and C. ochracea, as determined by nuclear factor-κB (NF-κB)-dependent CD25 expression. As LPS is a potent TLR4 ligand, we extracted LPS from each of the bacteria and exposed 7.7/huTLR4 cells to each of the LPS. The results revealed a unique ability of LPS from P. gingivalis and...
C. orihavea to act as antagonists for human TLR4. The antagonistic activities of those LPS were also analyzed in the U373 human astrocytoma cell line, freshly isolated human peripheral blood mononuclear cells (PBMC), and human gingival fibroblasts.

MATERIALS AND METHODS

Reagents. Phosphate-buffered saline (PBS), Ham’s F-12, RPMI 1640, Dulbecco’s modified Eagle’s medium (DMEM), α-MEM, penicillin-streptomycin, G418, and trypsin-EDTA were obtained from Gibco BRL (Rockville, Md.). Fetal bovine serum (FBS) was obtained from Biological Industry (Kibbutz Beit Haemek, Israel). Hygromycin B was obtained from Calbiochem (San Diego, Calif.). Anti-CD25 monoclonal antibody (MAb) conjugated with fluorescein isothiocyanate (FITC) was obtained from Becton Dickinson (Bedford, Mass.). Anti-ICAM-1 (CD54) MAb conjugated with FITC was obtained from Beckman Coulter (Fullerton, Calif.). Freeze-dried E. coli K12 and LPS from E. coli O111:B4 were obtained from Sigma (St. Louis, Mo.). Ficol-Paque was obtained from Pharmacia (Uppsala, Sweden). An enzyme-linked immunosorbent assay (ELISA) kit, Cytoselect, for interleukin-1β (IL-1β) and IL-6 was obtained from Biosource (Camarillo, Calif.). An enhanced colloidal gold kit was obtained from BioCell (Herz, Calif.).

Bacterial strains and growth conditions. P. gingivalis, C. orihavea, and F. nucleatum ATCC 10953 cells were grown in GAM broth supplemented with vitamin K₃ (5 µg/ml) and hemin (5 µg/ml) at 37°C for 3 days under anaerobic conditions (10% H₂, 10% CO₂, 80% N₂) and A. actinomycetemcomitans Y4 cells were grown in Todd-Hewitt broth with 1% yeast extract under the same conditions (4, 35). The organisms were harvested by centrifugation, washed three times with distilled water, and freeze dried. Died LPS was obtained from the freeze-dried periodontopathic bacteria and freeze-dried E. coli cells were used in experiments, and the remaining bacteria were used to prepare LPS. Staphylococcus aureus IID671 (a gift from N. Obara, Department of Oral Bacteriology, Nagasaki University) was grown in LPS-free α-MEM. The cells were washed twice with PBS, and the cell density was determined by limiting dilution. Bacteria were resuspended in PBS, killed by incubation at 95°C for 20 min, and stored at −20°C until use.

Preparations of LPS. LPS was purified according to the procedure described by Koga et al. (15) unless otherwise mentioned. Briefly, LPS was extracted from five species of microorganisms using the hot-phenol water procedure and then ultracentrifuged, treated with pronase and nuclease P1, and heated at 100°C for 5 min. To ensure the cells were activated by LPS but not contaminated protein, the remaining bacteria were used to prepare LPS.

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expression levels in 7.7/huTLR4 cells were no higher than those in 7.7 cells when stimulated with freeze-dried C. ochracea.

Activation of 7.7/huTLR4 cells by LPS from E. coli, A. actinomycetemcomitans, and F. nucleatum but not by LPS from P. gingivalis and C. ochracea. As LPS is the most potent inducer of TLR4-mediated activation, we exposed 7.7 and 7.7/huTLR4 cells to LPS extracted from the five species of bacteria. While none of the LPS from the five species of bacteria activated the NF-κB reporter construct in 7.7 cells (data not shown), the expression of human TLR4 in 7.7 cells conferred responsiveness to LPS from E. coli, A. actinomycetemcomitans, and F. nucleatum (Fig. 2). However, it failed to confer responsiveness to LPS from P. gingivalis and C. ochracea. No CD25 expression was observed in 7.7/huTLR4 cells when stimulated with up to 100 μg of LPS/ml from P. gingivalis and C. ochracea (data not shown). These results indicated that LPS was at least one of the components that determined the responsiveness of 7.7/huTLR4 cells to the microorganisms.

Inhibitory effects of LPS from P. gingivalis and C. ochracea on activation of 7.7/huTLR4 cells but not 7.7/huTLR2 cells. It is known that lipid IVa, a lipid A precursor, and Rhodobacter sphaeroides lipid A lack the ability to stimulate human cells and work as potent LPS antagonists (18, 29). We examined whether LPS from P. gingivalis and C. ochracea can act similarly as LPS antagonists. When 7.7/huTLR4 cells were stimulated with LPS from E. coli, A. actinomycetemcomitans, and F. nucleatum (1 μg/ml), the addition of LPS from P. gingivalis or C. ochracea (10 μg/ml) attenuated the expression of CD25 (Fig. 3A). To ensure that the cells were activated by LPS but not a small amount of contaminated protein, 7.7/huTLR4 cells were also exposed to repuriﬁed LPS from E. coli (1 μg/ml). The addition of LPS from P. gingivalis or C. ochracea (10 μg/ml) attenuated the expression of CD25 in 7.7/huTLR4 cells stimulated with repuriﬁed LPS from E. coli.

Next, we examined whether LPS from P. gingivalis and C. ochracea could inhibit other signaling pathways. When 7.7/huTLR2 cells were exposed to heat-killed S. aureus (1 × 10⁸ to

FIG. 1. TLR2 and/or TLR4 mediated cellular activation by freeze-dried periodontopathic bacteria. (A) LPS-nonresponsive mutant CHO/CD14, 7.7, 7.7/huTLR2, and 7.7/huTLR4 cells were exposed to 100 μg of freeze-dried bacteria/ml for 18 h. The cells were stained with FITC-labeled anti-CD25 MAb and subjected to flow cytometric analysis for NF-κB-driven CD25 expression. (B to D) 7.7 (B), 7.7/huTLR2 (C), and 7.7/huTLR4 (D) cells were exposed to indicated doses of freeze-dried bacteria for 18 h. The cells were stained with FITC-labeled anti-CD25 MAb and subjected to flow cytometric analysis for NF-κB-driven CD25 expression. Activation is expressed as the fold induction of mean channel fluorescence in comparison to unstimulated cells. Representative results of one of three experiments performed are shown. E.c., freeze-dried E. coli; P.g., freeze-dried P. gingivalis; A.a., freeze-dried A. actinomycetemcomitans; C.o., freeze-dried C. ochracea; F.n., freeze-dried F. nucleatum.
2 × 10^8 CFU/ml (equivalent to 18.5 to 37 μg/ml), four- to fivefold higher levels of activation were induced as reported previously (19). Although LPS from *P. gingivalis* or *C. ochracea* themselves (10 μg/ml) induced the expression of CD25 in 7.7/huTLR2 cells, the addition of those LPS did not inhibit the activation in 7.7/huTLR2 cells induced by heat-killed *S. aureus* (Fig. 3B). Similarly, LPS from *P. gingivalis* or *C. ochracea* did not inhibit the expression of CD25 in 7.7/huTLR2 cells induced by freeze-dried *P. gingivalis*, indicating that LPS from *P. gingivalis* and *C. ochracea* did not inhibit TLR2-mediated signal transduction.

**Inhibitory effects of LPS from *P. gingivalis* and *C. ochracea* on LPS-induced secretion of IL-6 from U373 cells.** In order to determine whether our findings in CHO reporter cell lines reflect the signal transduction systems used by other physiological cell types, we stimulated a human astrocytoma cell line, 7.7/huTLR4 cells were exposed to either PBS (thin line) or LPS from indicated species of bacteria (dotted line, 1 μg/ml; thick line, 10 μg/ml). After 18 h of incubation, the cells were stained with FITC-labeled anti-CD25 MAb and subjected to flow cytometric analysis to determine transgene expression. Representative results of one of three experiments performed are shown. *E. coli; P.g., P. gingivalis; A.a., A. actinomycetemcomitans; C.o., C. ochracea; F.n., F. nucleatum.*
U373, which has been reported to express TLR4 but not TLR2 (7, 36). As shown in Fig. 4A, repurified LPS from E. coli (50 ng/ml) induced a significant level of IL-6 release from U373 cells, while LPS from P. gingivalis and C. ochracea induced only minimal levels of it. The addition of LPS from P. gingivalis or C. ochracea (100 and 1,000 ng/ml) to the culture evidently attenuated the IL-6 secretion by U373 cells stimulated with repurified LPS from E. coli. As shown in Fig. 4B, IL-6 secretion by U373 cells stimulated with LPS from E. coli (10 ng/ml), A. actinomycetemcomitans (10 ng/ml), or F. nucleatum (100 ng/ml) was also attenuated by the addition of LPS from P. gingivalis or C. ochracea (1,000 ng/ml), indicating that LPS from P. gingivalis and C. ochracea worked as antagonists in U373 cells.

Inhibitory effects of LPS from P. gingivalis and C. ochracea on LPS-induced secretion of IL-1β by PBMC and on LPS-induced ICAM-1 expression in human gingival fibroblasts. Next, we stimulated freshly isolated human PBMC with LPS from periodontopathic bacteria. In contrast to U373 cells, PBMC express both TLR2 and TLR4, and they secrete IL-1β when stimulated with high concentrations of LPS from P. gingivalis or C. ochracea (Fig. 5A). Nevertheless, when the cells were stimulated with a low concentration of repurified LPS from E. coli (5 ng/ml), the addition of LPS from P. gingivalis or C. ochracea (10 and 100 ng/ml) inhibited the IL-1β production from PBMC dose dependently. Similarly, LPS from P. gingivalis or C. ochracea also inhibited IL-1β production from PBMC induced by LPS from E. coli, A. actinomycetemcomitans, or F. nucleatum (Fig. 5B).

We also examined the effects of LPS from P. gingivalis or C. ochracea using a human gingival fibroblast cell line. As observed for PBMC, those cells expressed ICAM-1 when stimu-
Plated at a density of 10^5 cells/well in 24-well dishes. The next day, cells were exposed to repurified LPS from *E. coli* (100 ng/ml) with or without indicated concentrations of LPS from *P. gingivalis* or *C. ochracea*. Some of the cells were exposed to LPS from *P. gingivalis* or *C. ochracea* alone. (B) Human gingival fibroblasts were exposed to LPS from *E. coli*, *A. actinomycetemcomitans*, or *F. nucleatum* (10 ng/ml) with or without LPS from *P. gingivalis* or *C. ochracea* (10 µg/ml). After incubation for 18 h, the cells were stained with FITC-labeled anti-CD25 MAb and subjected to flow cytometric analysis. Activation is expressed as the fold induction of mean channel fluorescence in comparison to unstimulated cells. Representative results of one of three experiments performed are shown. *E. coli*, *P. gingivalis*; *A. a.* *A. actinomycetemcomitans*; *C. ochracea*, *F. nucleatum*.

Species-specific recognition of LPS from *P. gingivalis* and *C. ochracea*. Although our present results strongly indicated that LPS from *P. gingivalis* and *C. ochracea* are antagonists for human TLR4, it has been reported that a synthetic lipid A of *P. gingivalis* activated C3H/HeJ macrophages but not C3H/HeJ macrophages, indicating that mouse TLR4 played an essential role in the response to this compound (24). To address the question of whether the antagonistic activities of LPS from *P. gingivalis* and *C. ochracea* are species specific, CHO/CD14 cells which express a functional hamster TLR4 transcript were transfected with human TLR4. Although LPS from *P. gingivalis* (10 µg/ml) induced CD25 expression in CHO/CD14 cells, the overexpression of human TLR4 resulted in the loss of sensitivity (Fig. 7). Similarly, LPS from *C. ochracea* (10 µg/ml) induced CD25 expression in CHO/CD14 cells, but the expression of human TLR4 resulted in the loss of sensitivity (data not shown). In contrast, LPS from *A. actinomycetemcomitans*, *E. coli*, and *F. nucleatum* induced CD25 expression in both CHO/CD14 and CHO/CD14/huTLR4 cells (Fig. 7 and data not shown).

**DISCUSSION**

When 7.7/huTLR2 cells were exposed to freeze-dried bacteria, NF-κB was activated dose dependently in response to all

![Image](http://iai.asm.org/)
species of freeze-dried microorganisms used in this study (Fig. 1C), indicating that human TLR2 played an important role in the recognition of these bacteria. Although the expression of human TLR4 in 7.7 cells also conferred responsiveness to freeze-dried E. coli, A. actinomycetemcomitans, and F. nucleatum, it did not affect the response to freeze-dried P. gingivalis and C. ochracea, indicating two possibilities: (i) the whole bacterial components of P. gingivalis and C. ochracea were not recognized by human TLR4, or (ii) some microbial components present in these bacteria inhibited TLR4-mediated activation. LPS is known to be one of the most potent bacterial components in inducing TLR4-mediated signaling. In view of our findings that stable expression of human TLR4 in mutant 7.7 led to a bypass reversion of the LPS-nonresponder phenotype, we hypothesized that the unresponsiveness of 7.7/huTLR4 cells to freeze-dried P. gingivalis and C. ochracea was due to the unique characteristics of their LPS. In fact, LPS from P. gingivalis and C. ochracea did not induce human TLR4-mediated signaling. Conversely, those LPS inhibited the activation of 7.7/huTLR4 cells induced by LPS from the other bacteria, indicating that those LPS worked as antagonists for human TLR4. Although it remains unknown whether P. gingivalis and C. ochracea possess any component to induce TLR4-mediated signaling, it has become clear that their LPS contributed to the unresponsiveness of 7.7/huTLR4 cells to these organisms.

LPS from P. gingivalis has been studied by many investigators, while little is known about LPS from C. ochracea. Ogawa et al. and Tanamoto et al. analyzed the chemical structure of P. gingivalis lipid A and reported that the absence of ester-linked phosphate at the 4’ position of glucosamine disaccharide and the presence of branched and relatively longer fatty acids are unique features (16, 23). Tanamoto et al. also demonstrated that P. gingivalis lipid A induced TNF-α release from C3H/HeJ macrophages to the same extent as that from C3H/HeN macrophages (34), indicating that the induction of TNF-α release was irrelevant to the thr4 mutation in the C3H/HeJ mouse. Using repuriﬁed LPS from P. gingivalis, Kirikae et al. conﬁrmed that it could induce TNF-α release from C3H/HeJ macrophages (13), and Hirschfeld et al. showed that repuriﬁed P. gingivalis LPS induced IL-6 production from U87 cells transfected with human TLR2 but not TLR4, indicating that it does not signal through human TLR4 (8). Furthermore, Ogawa et al. demonstrated clear antagonistic effects of P. gingivalis lipid A and LPS against IL-1β production by PBMC stimulated with E. coli LPS or synthetic E. coli-type lipid A (compound 506) (26). In addition, Darveau et al. demonstrated that LPS from P. gingivalis blocked E. coli LPS-induced E-selectin expression in human umbilical cord vein endothelial cells (HUVEC) (1), which strongly express TLR4 but only very weakly express TLR2 (3). In accordance with the reported unique features of P. gingivalis LPS, our present results using a genetically engineered 7.7/huTLR4 cell line clearly demonstrated that LPS from P. gingivalis and C. ochracea inhibit TLR4-mediated signaling but not TLR2-mediated signaling. Furthermore, the inhibitory effect on the secretion of IL-6 by U373 cells strongly supports the hypothesis that LPS from P. gingivalis and C. ochracea work as antagonists for TLR4 in physiological human cells.

In contrast to 7.7/huTLR4 and U373 cells, human PBMC and gingival fibroblasts were weakly activated by LPS from P. gingivalis and C. ochracea, consistent with previous reports by other investigators (25, 32, 37). Since those cells express both TLR2 and TLR4 (22, 39), the TLR2-signaling complex might be activated in these cells, as observed in 7.7/huTLR2 cells (Fig. 3B). Although we could not detect any protein in our P. gingivalis and C. ochracea LPS preparation with colloidal gold staining, we cannot be certain what molecule is responsible for TLR2-mediated activation. Like LPS from Leptospira interrogans, which has recently been reported to act as a TLR2 agonist (38), LPS from P. gingivalis and C. ochracea may act in a similar way. However, very small amounts of contaminants may have remained in P. gingivalis and C. ochracea LPS preparations. These contaminants might activate human PBMC and gingival fibroblasts through TLR2.

Recently, P. gingivalis lipid A was synthesized and characterized (24). There was a slight difference between the natural P. gingivalis lipid A and the synthetic compound in cytokine-induced activity, and synthetic P. gingivalis lipid A induced cytokine production in C3H/HeN macrophages but not in C3H/HeJ macrophages, indicating that functional mouse TLR4 was necessary for the response to this compound (24). However, we demonstrated in the present study that the overexpression of human TLR4 in CHO/CD14 cells, which express a functional hamster TLR4, abrogated the sensitivity to LPS from P. gingivalis and C. ochracea, indicating that these LPS are antagonists for human TLR4 but not for hamster TLR4. These pharmacological behaviors are quite similar to those of a lipid A analogue, lipid IVα, and R. sphaeroides lipid A (18, 29). Investigators have suggested that the species-dependent discrimination of lipid A substructures is fully attributable to the species origin of TLR4, consistent with our results. The species-specific activity of P. gingivalis LPS might be related to the sensitivity of the animals to periodontal diseases.

In the present study, LPS from two of four periodontopathogenic bacteria worked as antagonists for human TLR4. Although the precise effect of the antagonistic LPS on bacterial growth in the gingival sulcus and the prevalence of these unique bacteria in patients remain to be elucidated, the antagonistic activity would be a great advantage for the microorganisms to escape from the innate immune system. In spite of the potent proinflammatory activity of LPS, gram-negative bacteria predomi-

nate in moderate to severe periodontal lesions (11). The an-

tagonistic LPS may play a role in this paradoxical situation and may be associated with the progression of periodontal diseases.

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