HOST RESPONSE TO INFECTION

Priming of human oral epithelial cells by interferon- γ to secrete cytokines in response to lipopolysaccharides, lipoteichoic acids and peptidoglycans

A. UEHARA, S. SUGAWARA and H. TAKADA

Department of Microbiology and Immunology, Tohoku University School of Dentistry, Sendai 980-8575 Japan

An earlier study reported that human gingival epithelial cells in primary culture and oral epithelial cell lines KB and HSC-2 cells were devoid of membrane CD14 (mCD14) and did not show enhanced production of interleukin (IL)-8 or granulocyte macrophage-colony stimulating factor (GM-CSF) upon stimulation with bacterial cell-surface components such as lipopolysaccharide (LPS), lipoteichoic acid (LTA), peptidoglycan (PGN) and synthetic muramyl dipeptide (MDP) even in the presence of serum. The present study demonstrated that after treatment with interferon (IFN)- γ for 3 days, these cells secreted IL-8 and GM-CSF in response to the bacterial components. Treatment with IFN- γ enhanced Toll-like receptor (TLR) 2, TLR4, MD-2 and MyD88 mRNA expression as determined by reverse transcriptase PCR. Anti-TLR2 and anti-TLR4 monoclonal antibodies (MAbs) inhibited the IL-8 production induced by PGN and LTA as well as LPS, respectively, in IFN- γ -primed oral epithelial cells, whereas neither MAb inhibited IL-8 production induced by MDP. These findings suggested that IFN- γ primed oral epithelial cells to produce cytokines upon stimulation with various bacterial components by up-regulation of the TLR system.

Introduction

Oral epithelial cells are the first cells encountered by bacteria in the periodontal tissues. In addition to acting as a physical barrier against the invasion of pathogenic organisms, in inflamed sites oral (gingival) epithelial cells appear to produce several pro-inflammatory cytokines such as interleukin (IL)-1 β , IL-6, IL-8 and tumour necrosis factor (TNF) [1], indicating that the cells actively participate in the initiation and development of chronic oral inflammation. However, only a few studies [2, 3] have demonstrated cytokine production by human gingival epithelial cells and related cell lines derived from the oral cavity in response to several oral bacteria.

Recently, homologues of *Drosophila* Toll were found to participate in signalling of lipopolysaccharide (LPS) activity in man and mice, and were designated as Toll-

like receptors (TLRs). In the initial studies on human TLRs, TLR2, but not TLR4, was suggested to mediate LPS activity [4, 5]. In subsequent studies, the dominant role of TLR4 in LPS signalling was clearly demonstrated in mice by genetic studies on TLR4-knockout mice [6] as well as genetically LPS-non-responsive C3H/HeJ and C57BL/10ScCr mice [7–9]. More recent studies on human TLRs [10] supported the critical role of TLR4 in LPS signalling. In addition, MD-2 is necessary for LPS signalling of TLR4 in human and murine cells [11, 12]. In contrast, peptidoglycan (PGN)-induced cell activation is mediated by TLR2 [13–15]. Previous studies at this laboratory revealed the dominant role of TLR4 in lipoteichoic acid (LTA) signalling [14, 16], while other investigators suggested the involvement of TLR2 [13, 15, 17, 18]. MyD88 is an adaptor molecule for TLRs as well as IL-1 receptor (IL-1R) and IL-18R [19–21], and most signalling of bacterial components examined to date was mediated by MyD88, although recent studies showed that LPS also activated cells in a MyD88-independent manner [22–24].

Recently, this research group [25] reported that human

Received 3 Dec. 2001; revised version accepted 14 March 2002.

Corresponding author: Professor H. Takada (e-mail: dent-ht@mail.cc.tohoku.ac.jp).

oral epithelial cells did not respond to bacterial cell-surface components even in the presence of soluble CD14 (sCD14), in contrast to human colonic epithelial cells. Previous studies [26–28] showed that human gamma-interferon (IFN- γ) primed human gingival fibroblasts (HGF) to enhance production of inflammatory cytokines upon stimulation with the LPS fraction from black-pigmented bacteria (BPB) such as *Porphyromonas gingivalis* and *Prevotella intermedia*. Furthermore, IFN- γ primed HGF which highly expressed membrane CD14 (mCD14) to increase expression of mCD14 and mRNA for CD14, TLR4 and MyD88, resulting in production of higher levels of IL-8 than non-primed cells upon stimulation with purified *Salmonella* LPS [29].

This study examined the possible priming effect of IFN- γ on oral epithelial cells in relation to the production of cytokines upon stimulation with LPS, LTA and PGN and the possible regulation of the TLR-MyD88 signalling pathway by IFN- γ treatment in these cells.

Materials and methods

Bacterial components and other reagents

Ultrapurified LPS from *Salmonella enterica* serovar Abortus-equi [30] was donated by C. Galanos (Max Plank Institut für Immunbiologie, Freiburg, Germany). LTA specimens prepared from *Staphylococcus aureus* and *Bacillus subtilis* were purchased from Sigma. These LTA specimens were slightly contaminated with extraneous LPS which was detected by a colorimetric *Limulus* test (Endospecy test; Seikagaku, Tokyo, Japan) as described previously [31]: 86.3 and 15.2 ng equivalent to the reference LPS of *Escherichia coli* O111:B4 (Difco) in 1 mg of *S. aureus* and *B. subtilis* LTA, respectively. Therefore, the repurified LTA prepared from the commercial *Streptococcus sanguis* specimen (Sigma) was also used as described previously [31]: *Limulus* activity of the LTA was <0.1 ng equivalent/mg. *S. aureus* PGN was purchased from Fluka Pharmaceutical (Bechs, Switzerland). A water-soluble PGN, SEPS, was prepared from the purified PGN of *S. epidermidis* ATCC 155 by cleaving pentapeptide cross-links between neighbouring stempeptide subunits with the PGN lytic endopeptidase SALE as described previously [32]. *S. aureus* PGN and SEPS were shown to be considerably contaminated with extraneous LPS on *Limulus* test: 369 and 120 ng equivalent to the reference LPS in 1 mg of *S. aureus* PGN and SEPS, respectively. *N*-Acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide, MDP) was supplied by Daiichi Pharmaceutical (Tokyo, Japan). *Limulus* activity of MDP was 0.3 ng equivalent to the reference LPS (in 1 mg of MDP). Anti-human TLR2 monoclonal antibody (MAb) TL2.1 (mouse immunoglobulin IgG2a; IgG2a) [33] was purchased from Cascade Bioscience (Winchester, MA, USA). Anti-human TLR4 MAb

HTA125 (mouse IgG2a) [11] was supplied by K. Miyake and S. Akashi (University of Tokyo, Tokyo, Japan). Control mouse IgG2a was purchased from Coulter (Miami, FL, USA) and dialysed against phosphate-buffered saline (PBS). Human natural IFN- γ (antiviral activity, 8.0×10^6 IU/mg of protein) was provided by Hayashibara Bioscience Institute (Okayama, Japan). All other reagents were obtained from Sigma unless otherwise indicated.

Cells and cell culture

Human gingival epithelial cells were prepared from the explants of normal human gingival tissues of a 7-year-old donor with her parents' informed consent. The explants were cut into pieces and cultured in tissue culture dishes (100 mm in diameter) (Falcon; Becton Dickinson Labware, Lincoln Park, NJ, USA) in keratinocyte serum-free medium (Gibco BRL, Grand Island, NY, USA) containing bovine pituitary extract 0.05% v/w and recombinant human epidermal growth factor ($820 \mu\text{M}$) supplemented with kanamycin (Meiji Seiyaku, Tokyo, Japan) $200 \mu\text{g/ml}$ with a medium change every 4 days for 15–20 days until subconfluent cell monolayers were formed. Human gingival epithelial cells obtained in this manner were characterised as keratinocytes on the basis of immunostaining with anti-cytokeratin type I and II antibodies (AE1+AE3 mouse IgG1 MAb, Progen Biotechnik GmbH, Germany) as described previously [30]. These procedures were approved by the Ethical Review Board of Tohoku University School of Dentistry. The human oral epidermoid carcinoma cell line (KB cells) was provided by the Health Science Research Resources Bank (Osaka, Japan). Human oral epithelial HSC-2 cells were provided by the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). KB cells were cultured in 100-mm dishes in alpha-modified minimal essential medium (α -MEM; Flow Laboratories, McLean, VA, USA) supplemented with fetal calf serum (FCS; Flow Laboratories) 10% with a medium change every 3 days. HSC-2 cells were cultured in 100-mm dishes in PRMI 1640 medium supplemented with FCS 10% with a medium change every 3 days.

As noted above, some bacterial components used in this study were contaminated with endotoxin. To rule out the possibility that the bioactivities of test materials were attributable to the endotoxin in the materials, IFN- γ -primed HSC-2 cells were also stimulated with representative test materials in the presence of polymyxin B sulphate (ICN Pharmaceuticals, Irvine, CA, USA).

RT-PCR assay

Total cellular RNA was extracted from 2.5×10^6 cells (one plate 100 mm diameter) by Isogen (Nippon Gene,

Tokyo, Japan) according to the manufacturer's instructions. Reverse transcription of the RNA samples to cDNA was performed with AMV XL (Life Science, St Petersburg, FL, USA) and random primer (Takara, Otsu, Japan). To transcribe the total RNA into cDNA, 1.0 µg of RNA, 50 pmol of random primer, 10× PCR buffer (Takara), 0.2 mM deoxynucleoside triphosphates and 5 U of AMV reverse transcriptase XL were added to a total volume of 20 µl. The reaction mixture was incubated first at 30°C for 10 min, next at 42°C for 30 min, then 99°C for 5 min to inactivate the reverse transcriptase, cooled on ice, and stored at -20°C. The primers used for PCR were as follows: CD14, forward 5'-CTCAACCTAGAGCCGTTTAT-3' and reverse 5'-CAGGATTGTCAGACAGGTCT-3'; human TLR2, forward 5'-GCCAAAGTCTTGATTGATTGG-3' and reverse 5'-TTGAAGTTCTCCAGCTCCTG-3'; TLR4, forward 5'-TGGATACGTTTCCTTATAAG-3' and reverse 5'-GAAATGGAGGCACCCCTTC-3'; MyD88, forward 5'-TAAGAAGGACCAGCAGAGCC-3' and reverse 5'-CATGTAGTCCAGCAACAGCC-3'; MD-2, forward 5'-GCACATTTTCTACATTCC-3' and reverse 5'-CCTTACATCTTTTACACG-3'; and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5'-TGAAGGTCGGAGTCAACGGATTGGT-3' and reverse 5'-CATGTGGGCCATGAGGTCCACCAC-3'. The primers for CD14, TLR2, TLR4, MD-2, MyD88 and GAPDH were constructed to generate fragments of 426, 368, 506, 157, 200 and 983 bp, respectively. The PCR mixtures contained 5 µl of the cDNA mixture, 2 µl of 10× PCR buffer, 0.2 mM deoxynucleoside triphosphates, 50 pmol of each primer, and 0.1 µl of Ex Taq DNA polymerase (Takara, Tokyo, Japan) in a total volume of 20 µl. Amplification was performed in a model MP TP3000 PCR thermal cycler (Takara) as follows: CD14, 25 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 1 min and extension at 72°C for 1 min; TLR2 and TLR4, 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 40 s and extension at 72°C for 2 min; MD-2, 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 1 min and extension at 72°C for 90 s; MyD88, 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 40 s and extension at 70°C for 2 min; GAPDH, 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min. Amplified samples were visualised on agarose 2.0% gels stained with ethidium bromide and photographed under UV light. The bands on the photographs were scanned and then analysed with an Image Master 1D (Pharmacia Biotech, Uppsala, Sweden). The results are expressed as relative mRNA accumulation corrected with reference to GAPDH mRNA as an internal standard.

Measurement of cytokines

Confluent epithelial cells were collected by trypsinisation and washed three times in PBS. The cells (5×10^4 cells/200 µl) were seeded in culture medium

in the wells of 96-well plates (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ, USA). After incubation for 1 day at 37°C in a CO₂ 5% incubator, cells were cultured with IFN-γ 1000 IU/ml for 3 days. After washing three times with PBS, the cells were stimulated with various test materials in 200 µl of medium without serum for 24 h. Cultivation was performed in triplicate, and IL-8 and granulocyte macrophage-colony stimulating factor (GM-CSF) levels in the supernates were measured by enzyme-linked immunosorbent assay (ELISA) kits (PharMingen, San Diego, CA, and Biosource, Camarillo, CA, USA, respectively). The concentrations of the cytokines in the supernates were determined with the Softmax data analysis programme (Molecular Devices Corp., Menlo Park, CA, USA).

Data analysis

All experiments were performed at least three times to confirm the reproducibility of the results, and representative results are shown. In most experiments, values are presented as means and SD of triplicate assays. The statistical significance of differences between the two means was evaluated by one-way analysis of variance (ANOVA), by the Bonferroni or Dunn method, and p values <0.05 were considered significant.

Results

Effects of IFN-γ on production of IL-8 and GM-CSF by oral epithelial cells

First, the study examined whether oral epithelial cells produced IL-8 in response to *Salmonella* LPS, *S. aureus* and *B. subtilis* LTA, *S. aureus* PGN, a water-soluble PGN, SEPS, and MDP. In accordance with a previous report [25], primary gingival epithelial cells, oral epithelial HSC-2 and KB cells did not secrete IL-8 in response to the bacterial components even in the presence of FCS 10% (Fig. 1b). Under the same conditions, treatment with IFN-γ (1000 IU/ml) for 3 days made the oral epithelial cells responsive to stimulation with bacterial cell-surface components, resulting in the production of IL-8 (Fig. 1a). Clear dose-dependency of IL-8 secretion by IFN-γ-primed HSC-2 cells was observed upon stimulation with bacterial cell-surface components (Fig. 2). Then, the study examined the optimum concentration and duration of IFN-γ treatment required for the priming of HSC-2 cells. Marked IL-8 release in response to LPS was observed when the cells were treated with IFN-γ 1000 and 10 000 IU/ml for 3 days, whereas IFN-γ at 10 and 100 IU/ml showed only a marginal effect in this respect (Fig. 3a). As shown in Fig. 3b, treatment with IFN-γ for 1 and 2 days was not sufficient to prime oral epithelial cells, whereas after IFN-γ priming for 3 days, LPS significantly activated the cells to release IL-8.

Fig. 1. IFN- γ priming of human oral epithelial cells to secrete IL-8 in response to bacterial cell-surface components. Human oral epithelial cells (primary gingival epithelial, HSC-2 and KB cells) were incubated with (a) or without (b) IFN- γ (1000 IU/ml) for 3 days, then stimulated with *Salmonella* LPS (0.1 μ g/ml), *S. aureus* LTA (Sa LTA; 100 μ g/ml), *B. subtilis* LTA (B LTA; 100 μ g/ml), *S. aureus* PGN (100 μ g/ml), SEPS (100 μ g/ml) or MDP (100 μ g/ml) for 24 h in triplicate. Concentrations of IL-8 in the culture supernates were determined by ELISA. Bars indicate SD. * $p < 0.05$, ** $p < 0.01$: significantly different from the respective control (medium alone). Results are representative of three different experiments.

Next, the study examined whether IFN- γ -primed oral epithelial cells also secreted another inflammatory cytokine, GM-CSF, in response to these bacterial cell-surface components. The IFN- γ -primed HSC-2 cells markedly secreted GM-CSF upon stimulation with bacterial cell-surface components, whereas non-treated cells did not show enhanced secretion of GM-CSF (Fig. 4).

Effect of polymyxin B on activities of LTA, PGN, SEPS and MDP

To examine the possible involvement of contaminating endotoxin in the bioactivities of the test materials, IL-8-inducing abilities of representative bacterial cell-surface components on IFN- γ -primed HSC-2 were also examined in the presence of polymyxin B. The activities of *S. aureus* LTA, PGN, SEPS and MDP were not inhibited but rather slightly increased by polymyxin B, whereas the activity of LPS was completely inhibited (Fig. 5). These findings clearly

Fig. 2. IFN- γ primed human oral epithelial cells to secrete IL-8 upon stimulation with bacterial cell-surface components in a dose-dependent manner. HSC-2 cells were stimulated with IFN- γ (1000 IU/ml) for 3 days, then stimulated with *Salmonella* LPS (0.1–100 ng/ml), *S. aureus* LTA (0.1–100 μ g/ml), *B. subtilis* LTA (0.1–100 μ g/ml), *S. aureus* PGN (0.1–100 μ g/ml), SEPS (0.1–100 μ g/ml) or MDP (0.1–100 μ g/ml) for 24 h in triplicate. Concentrations of IL-8 in the culture supernates were determined by ELISA. Bars indicate SD. ** $p < 0.01$: significantly different from the respective control (medium alone). Results are representative of three different experiments.

indicated that bioactivities of test materials in this study are not attributable to the contaminating endotoxin.

Effects of IFN- γ on TLR2, TLR4, MD-2 and MyD88 mRNA expression in oral epithelial cells

HSC-2 cells were treated with IFN- γ (1000 IU/ml) for 3 days, then RT-PCR was performed to detect levels of CD14, TLR2, TLR4, MD-2 or MyD88 mRNA expression. As shown in Fig. 6, mRNA expression by the molecules of the TLR-MyD88 system were significantly up-regulated by IFN- γ . In contrast, the cells did not express CD14 mRNA even after treatment with IFN- γ .

Effects of anti-TLR2 and anti-TLR4 MAbs on IL-8 secretion by IFN- γ -primed HSC-2 cells in response to bacterial cell-surface components

To elucidate whether the responses of IFN- γ -primed oral epithelial cells to bacterial cell-surface components are mediated by the TLR-MyD88 signalling pathway, IFN- γ -primed HSC-2 cells were pre-incubated with anti-TLR2 MAb (10 μ g/ml), anti-TLR4 MAb (10 μ g/ml) or isotype-matched control antibody (IgG2a) (10 μ g/ml) for 30 min, then stimulated with various bacterial components. LPS-induced IL-8 secretion in IFN- γ -primed HSC-2 cells was inhibited by anti-TLR4 MAb to the control (medium alone) level, but not by anti-TLR2 MAb (Fig. 7). The activities of *S. aureus*

Fig. 3. Dose and time kinetics of IFN- γ -priming of human oral epithelial cells to secrete IL-8 in response to LPS. (a) HSC-2 cells were incubated with IFN- γ (0, 10, 100, 1000 and 10000 IU/ml) for 3 days, then stimulated with *Salmonella* LPS (100 ng/ml) for 24 h in triplicate. (b) HSC-2 cells were incubated with IFN- γ (1000 IU/ml) for 0, 1, 2 or 3 days, then stimulated with *Salmonella* LPS (100 ng/ml) for 24 h in triplicate. Concentrations of IL-8 in the culture supernates were determined by ELISA. Bars indicate SD. ** $p < 0.01$: significantly different from the respective control (medium alone). Results are representative of three different experiments.

Fig. 4. IFN- γ primed human oral epithelial HSC-2 cells to secrete GM-CSF upon stimulation with various bacterial cell-surface components. HSC-2 cells were incubated with (a) or without (b) IFN- γ (1000 IU/ml) for 3 days, then stimulated with *Salmonella* LPS (100 ng/ml), *S. aureus* LTA (100 μ g/ml), *B. subtilis* LTA (100 μ g/ml), *S. aureus* PGN (100 μ g/ml), SEPS (100 μ g/ml) and MDP (100 μ g/ml) for 24 h in triplicate. Concentrations of GM-CSF in the culture supernates were determined by ELISA. Bars indicate SD. ** $p < 0.01$: significantly different from the respective control (medium alone). Results are representative of three different experiments.

Fig. 5. Influences of polymyxin B (PB) on IL-8-inducing activity of LTA, PGN, SEPS and MDP and the reference LPS. Human oral epithelial HSC-2 cells were stimulated with IFN- γ (1000 IU/ml) for 3 days, then were incubated with *Salmonella* LPS (0.1 μ g/ml, ■), *S. aureus* LTA (100 μ g/ml, ●), *S. aureus* PGN (100 μ g/ml, ▲), SEPS (100 μ g/ml, ▼) and MDP (100 μ g/ml, ◆) in the presence or absence of 1 or 10 μ g/ml PB for 24 h in triplicate. Concentrations of IL-8 in the culture supernates were determined by ELISA. The activities are expressed as percentages, where 100% and 0% indicate the IL-8 levels induced by the respective stimulant without PB and the negative control with medium alone or indicated concentrations of PB alone, respectively. ** $p < 0.01$: significantly different from respective positive control (stimulant alone). Bars indicate SD. Results are representative of three different experiments.

Fig. 6. IFN- γ up-regulated TLR2, TLR4, MD-2 and MyD88 mRNA expression by human oral epithelial cells. HSC-2 cells were cultured with or without IFN- γ (1000 IU/ml) for 3 days. Total cellular RNA was extracted from these cells and RT-PCR was performed to amplify specific signals of CD14, TLR2, TLR4, MD2 and MyD88 mRNA with the respective specific primer. The resulting PCR products were electrophoresed on agarose 2% gels and visualised by staining with ethidium bromide (a). Bands were further quantified by means of an imaging analyser. The results are expressed relative to GAPDH mRNA as an internal standard (b). Three additional experiments gave results similar to those shown here.

and *Str. sanguis* LTA were also inhibited by anti-TLR4 MAb, but not by anti-TLR2 MAb. In contrast, *B. subtilis* LTA-induced IL-8 secretion was inhibited by both anti-TLR2 and anti-TLR4 MAb. PGN- and SEPS-induced IL-8 secretion was significantly inhibited by anti-TLR2 MAb but not by anti-TLR4 MAb. However, MDP-induced IL-8 release was suppressed by neither anti-TLR2 nor anti-TLR4 MAb. These findings suggested that IFN- γ -primed HSC-2 cells produced IL-8 in

Fig. 7. Effects of anti-TLR2 and anti-TLR4 MABs on IL-8 secretion by IFN- γ -primed human oral epithelial cells in response to bacterial cell-surface components. HSC-2 cells were incubated with IFN- γ (1000 IU/ml) for 3 days, then pre-incubated with anti-TLR2 MAB TL2.1 (10 μ g/ml), anti-TLR4 MAB HTA125 (10 μ g/ml), or isotype-matched IgG2a Ab (10 μ g/ml) for 30 min, then stimulated with *Salmonella* LPS (0.1 ng/ml), *S. aureus* LTA (0.1 μ g/ml), *Str. sanguis* LTA (0.1 μ g/ml), *B. subtilis* LTA (0.1 μ g/ml), *S. aureus* PGN (0.1 μ g/ml), SEPS (0.1 μ g/ml) and MDP (0.1 μ g/ml) for 24 h in triplicate. Concentrations of IL-8 in the culture supernates were determined by ELISA. Bars indicate SD. ** $p < 0.01$: significantly different from the respective control (stimulant alone). Results are representative of three different experiments. □, Stimulant alone; ▨, + α -TLR2; ▩, + α -TLR4; ■, +IgG2a.

response to LTA as well as LPS via TLR4 and to PGN-related materials except MDP via TLR2. IL-8 production induced by these bacterial cell-surface components in IFN- γ -primed HSC-2 cell cultures was not inhibited by anti-CD14 MAB (data not shown).

Discussion

This study demonstrated that treatment with IFN- γ made human oral epithelium cells responsive to bacterial cell-surface components, whereas non-treated cells showed no response even in the presence of sCD14, as reported previously [25]. There is accumulating evidence that sCD14 in serum makes mCD14-defective cells responsive to LPS [34]. This is the case in some human colonic epithelial cell lines such as SW620 and HT29 cells [25, 35, 36] but not in others such as T84 and CaCo2 cells [36, 37]. The mechanism underlying the lack of responsiveness of these epithelial cells is not clear at present.

IFN- γ exhibits various regulatory effects in different cell types [38, 39]. This group has demonstrated the priming effect of IFN- γ on human gingival fibroblasts in culture to produce inflammatory cytokines, including IL-8, upon stimulation with LPS from BPB, but not from Enterobacteriaceae [26–28]. Stříž *et al.* [40] reported that IFN- γ enhanced secretion of IL-8 and expression of HLA-DR and ICAM-1 by human bronchial epithelial cells. The mechanism of the priming effect of IFN- γ on oral epithelial cells to respond to bacterial cell-surface components is not clear at present. One possible explanation might be an increased number or affinity of receptor(s) other than mCD14 on oral epithelial cells as suggested by Triantafyllou *et al.* [41]. In this context, Wan *et al.* [42] suggested that a potential mechanism of LPS

synergy with cytokines such as IFN- γ may be attributable to up-regulation of putative LPS receptors on cells by cytokines. With regard to the possible involvement of mCD14, Funda *et al.* [43] reported that human intestinal epithelial cells expressed mCD14 and released sCD14, in contrast to many previous reports. However, in the present study, human oral epithelial cells did not express CD14 mRNA as determined by RT-PCR (Fig. 5) and mCD14 determined by flow cytometric analysis, even after treatment with IFN- γ (data not shown). Furthermore, IL-8 induced by bacterial cell-surface components on IFN- γ -primed HSC-2 cells was not inhibited by anti-CD14 MAB even in the presence of serum (data not shown). Alternatively, the priming activity of IFN- γ may be due to augmentation of the TLR-MyD88 signalling pathway, which mediates signalling not only of gram-negative but also of gram-positive bacterial cell-surface components, irrespective of CD14 [44–46]. In this context, this group confirmed that IFN- γ up-regulated mCD14 expression and MyD88 mRNA expression by human gingival fibroblasts, and resulted in enhanced production of IL-8 by the cells in response to *Salmonella* LPS [29]. Faure *et al.* reported that IFN- γ induced TLR2 and TLR4 expression in human endothelial cells. In this study, oral epithelial cells expressed TLR2, TLR4, MD-2 and MyD88 mRNA, and IFN- γ up-regulated mRNA of all the molecules to a similar extent. However, cell surface expression of these molecules could not be detected by FACS (data not shown).

To elucidate the involvement of TLRs in the response of IFN- γ -primed oral epithelial cells to bacterial components, the study examined whether the responses of IFN- γ -primed HSC-2 cells to bacterial cell-surface components were inhibited by anti-TLR2 MAB or anti-TLR4 MAB. The dominant role of TLR4 in LPS

signalling was clearly demonstrated, in agreement with previous reports [7–10].

As described above, it has been suggested that both TLR2 and TLR4 are involved in LTA signalling. This group first reported that commercial LTA preparations were considerably contaminated with endotoxin and peptidoglycan, and prepared a purified LTA with bioactivity from the commercial *Str. sanguis* LTA by hydrophobic interaction chromatography, which was practically free from contamination with endotoxin and peptidoglycan [31]. On the other hand, Kusunoki *et al.* [48] prepared a non-hydrophobic bioactive fraction different from LTA from the commercial *S. aureus* LTA. With the purified LTA prepared from *S. aureus* and *Str. sanguis* by the same method, Takeuchi *et al.* [14] demonstrated the essential role of TLR4 in LTA signalling in macrophage cultures from TLR4-knockout mice, although several investigators [13, 15, 17, 18] reported that TLR2 was a signal transducer for LTA. In this study, IL-8 secretion induced by the purified LTA from *Str. sanguis* as well as the commercial LTA from *S. aureus* in IFN- γ -primed HSC-2 cells were significantly inhibited by anti-TLR4 MAb but not by anti-TLR2 MAb. However, *B. subtilis* LTA-induced IL-8 secretion was inhibited by both anti-TLR2 and anti-TLR4 MAbs. These findings suggested that some LTA preparations such as *B. subtilis* LTA used in this study exerted TLR2-mediated activity that might be attributable to other material(s) co-existing with LTA. Recently, Gao *et al.* [49] reported that major activities of commercial LTA from *S. aureus*, *Str. sanguis* and *B. subtilis* were attributable to contaminated endotoxin in the preparations. The possibility that the bioactivities of test LTA preparations shown in this study were also attributable to endotoxin contamination of the materials was clearly ruled out for the following reasons: (i) the activity of the purified LTA from *Str. sanguis*, which was practically free from endotoxin contamination, was dependent on TLR4 (Fig. 7); (ii) the activity of *S. aureus* LTA was not inhibited by polymyxin B sulphate (Fig. 5) [25].

S. aureus PGN- and soluble PGN, SEPS-induced IL-8 secretion was clearly inhibited by anti-TLR2 MAb, in accordance with previous reports [13–15, 50]. Although MDP is the minimal structure essential for the bioactivities of PGN, its signalling in relation to TLRs has not been elucidated. Recently, this group reported that MDP activated OCT (an analogue of the active form of vitamin D₃)-differentiated human monocytic U937 cells, which were devoid of TLR2 on their surface [16]. In accordance with these findings, the present study found that MDP activated human oral epithelial cells in a TLR2- and TLR4-independent manner (Fig. 6).

Three days were required for priming oral epithelial cells by IFN- γ – a 1–2-day period was not sufficient – perhaps suggesting that the priming effect of IFN- γ

was an indirect effect *via* other mediator(s) such as inflammatory cytokines and nitric oxide (NO). In fact, it was found that IL-1 β and TNF- α also primed oral epithelial cells to respond to bacterial components (Uehara *et al.*, unpublished observations). However, we confirmed that IL-1 β and TNF- α were not detected in the culture supernate of IFN- γ -primed cells by ELISA, and anti-NO antibody did not suppress the response of IFN- γ -primed cells to bacterial cell-surface components (Uehara *et al.*, unpublished observations). Therefore, treatment with IFN- γ might have directly modulated intracellular function(s) of oral epithelial cells, which in turn endowed the cells with responsiveness to bacterial components, although the intracellular events are not clear at present.

Oral epithelial cells might be exposed to various inflammatory mediators in the inflamed lesions. Excessive production of IFN- γ is characteristic of immune responses mediated predominantly by Th1 lymphocytes [51]. In fact, numerous Th1 cells infiltrate into the gingival epithelium and lamina propria in inflamed gingival tissues [52]. The intraepithelial $\gamma\delta$ T cells in chronically inflamed gingival tissues express IFN- γ [1]. Therefore, it is conceivable that in the inflamed gingival tissues, oral epithelial cells are primed with IFN- γ produced by these lymphoid cells and secrete various inflammatory cytokines in response to bacterial components such as LPS, LTA and PGN, as shown in this study, which in turn may augment periodontal inflammation.

We thank Dr C. Galanos (Max Plank Institute für Immunbiologie, Freiburg, Germany) and Dr K. Miyake and S. Akashi (University of Tokyo, Tokyo, Japan) for providing the purified *Salmonella* LPS and anti-TLR4 MAb HTA125, respectively. We are indebted to Drs H. Tamura and J. Aketagawa (Seikagaku Kogyo, Co.) for performing the *Limulus* test. We also thank D. Mrozek (Medical English Service, Kyoto, Japan) for reviewing this paper. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture, Japan (no. 10470378) and from the Japan Society for the Promotion Science (nos 12470380 and 13671894).

References

1. Lundqvist C, Baranov V, Teglund S, Hammarström S, Hammarström M-L. Cytokine profile and ultrastructure of intraepithelial $\gamma\delta$ T cells in chronically inflamed human gingiva suggest a cytotoxic effector function. *J Immunol* 1994; **153**: 2302–2312.
2. Huang GT-J, Haake SK, Kim J-W, Park N-H. Differential expression of interleukin-8 and intercellular adhesion molecule-1 by human gingival epithelial cells in response to *Actinobacillus actinomycetemcomitans* or *Porphyromonas gingivalis* infection. *Oral Microbiol Immunol* 1998; **13**: 301–309.
3. Yumoto H, Nakae H, Fujinaka K, Ebisu S, Matsuo T. Interleukin-6 (IL-6) and IL-8 are induced in human oral epithelial cells in response to exposure to periodontopathic *Eikenella corrodens*. *Infect Immun* 1999; **67**: 384–394.
4. Kirschning CJ, Wesche H, Ayres TM, Rothe M. Human Toll-like receptor 2 confers responsiveness to bacterial lipopolysaccharide. *J Exp Med* 1998; **188**: 2091–2097.
5. Yang R-B, Mark MR, Gray A *et al.* Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. *Nature* 1998; **395**: 284–288.
6. Hoshino K, Takeuchi O, Kawai T *et al.* Cutting edge: Toll-like

- receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the *Lps* gene product. *J Immunol* 1999; **162**: 3749–3752.
7. Poltorak A, He X, Smirnova I *et al.* Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in *Tlr4* gene. *Science* 1998; **282**: 2085–2088.
 8. Qureshi ST, Larivière L, Leveque G *et al.* Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (*Tlr4*). *J Exp Med* 1999; **189**: 615–625.
 9. Vogel SN, Johnson D, Perera P-Y *et al.* Cutting edge: Functional characterization of the effect of the C3H/HeJ defect in mice that lack an *Lps*ⁿ gene: in vivo evidence for a dominant negative mutation. *J Immunol* 1999; **162**: 5666–5670.
 10. Arbour NC, Lorenz E, Schutte BC *et al.* *TLR4* mutations are associated with endotoxin hyporesponsiveness in humans. *Nat Genet* 2000; **25**: 187–191.
 11. Shimazu R, Akashi S, Ogata H *et al.* MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J Exp Med* 1999; **189**: 1777–1782.
 12. Akashi S, Shimazu R, Ogata H *et al.* Cutting edge: Cell surface expression and lipopolysaccharide signaling via the Toll-like receptor 4-MD-2 complex on mouse peritoneal macrophages. *J Immunol* 2000; **164**: 3471–3475.
 13. Schwandner R, Dziarski R, Wesche H, Rothe M, Kirschning CJ. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by Toll-like receptor 2. *J Biol Chem* 1999; **274**: 17406–17409.
 14. Takeuchi O, Hoshino K, Kawai T *et al.* Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity* 1999; **11**: 443–451.
 15. Yoshimura A, Lien E, Ingalls RR, Tuomanen E, Dziarski R, Golenbock D. Cutting edge: Recognition of gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. *J Immunol* 1999; **163**: 1–5.
 16. Yang S, Tamai R, Akashi S *et al.* Synergistic effect of muramyl dipeptide with lipopolysaccharide or lipoteichoic acid to induce inflammatory cytokines in human monocytic cells in culture. *Infect Immun* 2001; **69**: 2045–2053.
 17. Lehner DL, Morath S, Michelsen KS, Schumann RR, Hartung T. Induction of cross-tolerance by lipopolysaccharide and highly purified lipoteichoic acid via different toll-like receptors independent of paracrine mediators. *J Immunol* 2001; **166**: 5161–5167.
 18. Opitz B, Schröder NWJ, Spreitzer I *et al.* Toll-like receptor-2 mediates *Treponema* glycolipid and lipoteichoic acid-induced NF- κ B translocation. *J Biol Chem* 2001; **276**: 22041–22047.
 19. Adachi O, Kawai T, Takeda K *et al.* Targeted disruption of the *MyD88* gene results in loss of IL-1- and IL-18-mediated function. *Immunity* 1998; **9**: 143–150.
 20. Kawai T, Adachi O, Ogawa T, Takeda K, Akira S. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* 1999; **11**: 115–122.
 21. Takeuchi O, Takeda K, Hoshino K, Adachi O, Ogawa T, Akira S. Cellular responses to bacterial cell wall components are mediated through MyD88-dependent signaling cascades. *Int Immunol* 2000; **12**: 113–117.
 22. Inohara N, Ogura Y, Chen FF, Muto A, Nuñez G. Human Nod1 confers responsiveness to bacterial lipopolysaccharides. *J Biol Chem* 2001; **276**: 2551–2554.
 23. Kaisho T, Akira S. Dendritic-cell function in Toll-like receptor- and MyD88-knockout mice. *Trends Immunol* 2001; **22**: 78–83.
 24. Seki E, Tsutsui H, Nakano H *et al.* Lipopolysaccharide-induced IL-18 secretion from murine Kupffer cells independently of myeloid differentiation factor 88 that is critically involved in induction of production of IL-12 and IL-1 β . *J Immunol* 2001; **166**: 2651–2657.
 25. Uehara A, Sugawara S, Tamai R, Takada H. Contrasting responses of human gingival and colonic epithelial cells to lipopolysaccharide, lipoteichoic acids and peptidoglycans in the presence of soluble CD14. *Med Microbiol Immunol* 2001; **189**: 185–192.
 26. Takada H, Mihara J, Morisaki I, Hamada S. Induction of interleukin-1 and -6 in human gingival fibroblast cultures stimulated with *Bacteroides* lipopolysaccharides. *Infect Immun* 1991; **59**: 295–301.
 27. Tamura M, Tokuda M, Nagaoka S, Takada H. Lipopolysaccharides of *Bacteroides intermedius* (*Prevotella intermedia*) and *Bacteroides* (*Porphyromonas*) *gingivalis* induce interleukin-8 gene expression in human gingival fibroblast cultures. *Infect Immun* 1992; **60**: 4932–4937.
 28. Sakuta T, Tokuda M, Tamura M *et al.* Dual regulatory effects of interferon- α , - β , and - γ on interleukin-8 gene expression by human gingival fibroblasts in culture upon stimulation with lipopolysaccharide from *Prevotella intermedia*, interleukin-1 α , or tumor necrosis factor- α . *J Dent Res* 1998; **77**: 1597–1605.
 29. Tamai R, Sakuta T, Matsushita K *et al.* Human gingival CD14⁺ fibroblasts primed with gamma interferon increase production of interleukin-8 in response to lipopolysaccharide through up-regulation of membrane CD14 and MyD88 mRNA expression. *Infect Immun* 2002; **70**: 1272–1278.
 30. Galanos C, Lüderitz O, Westphal O. Preparation and properties of a standardized lipopolysaccharide from *Salmonella abortus equi* (Novo-Pyrexal). *Zentralbl Bakteriol Orig A* 1979; **243**: 226–244.
 31. Sugiyama A, Arakaki R, Ohnishi T, Arakaki N, Daikuhara Y, Takada H. Lipoteichoic acid and interleukin 1 stimulate synergistically production of hepatocyte growth factor (scatter factor) in human gingival fibroblasts in culture. *Infect Immun* 1996; **64**: 1426–1431.
 32. Harada K, Kotani S, Takada H *et al.* Liberation of serotonin from rabbit blood platelets by bacterial cell walls and related compounds. *Infect Immun* 1982; **37**: 1181–1190.
 33. Flo TH, Halaas Ø, Lien E *et al.* Human Toll-like receptor 2 mediates monocyte activation by *Listeria monocytogenes*, but not by group B streptococci or lipopolysaccharide. *J Immunol* 2000; **164**: 2064–2069.
 34. Ulevitch RJ, Tobias PS. Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. *Annu Rev Immunol* 1995; **13**: 437–457.
 35. Pugin J, Schürer-Maly C-C, Leturcq D, Moriarty A, Ulevitch RJ, Tobias PS. Lipopolysaccharide activation of human endothelial and epithelial cells is mediated by lipopolysaccharide-binding protein and soluble CD14. *Proc Natl Acad Sci USA* 1993; **90**: 2744–2748.
 36. Schuerer-Maly C-C, Eckmann L, Kagnoff MF, Falco MT, Maly F-E. Colonic epithelial cell lines as a source of interleukin-8: stimulation by inflammatory cytokines and bacterial lipopolysaccharide. *Immunology* 1994; **81**: 85–91.
 37. Eckmann L, Kagnoff MF, Fierer J. Epithelial cells secrete the chemokine interleukin-8 in response to bacterial entry. *Infect Immun* 1993; **61**: 4569–4574.
 38. Sen GC, Lengyel P. The interferon system: a bird's eye view of its biochemistry. *J Biol Chem* 1992; **267**: 5017–5020.
 39. De Maeyer E, De Maeyer-Guignard J. Interferons. In: Thomson AW (ed) *The cytokine handbook*, 3rd edn. San Diego, Academic Press. 1998: 491–516.
 40. Striz I, Mio T, Adachi Y, Carnevali S, Romberger DJ, Rennard SI. Effects of interferons alpha and gamma on cytokine production and phenotypic pattern of human bronchial epithelial cells. *Int J Immunopharmacol* 2000; **22**: 573–585.
 41. Triantafyllou K, Triantafyllou M, Dedrick RL. A CD14-independent LPS receptor cluster. *Nat Immunol* 2001; **2**: 338–345.
 42. Wan Y, Freeswick PD, Khemlani LS *et al.* Role of lipopolysaccharide (LPS), interleukin-1, interleukin-6, tumor necrosis factor, and dexamethasone in regulation of LPS-binding protein expression in normal hepatocytes and hepatocytes from LPS-treated rats. *Infect Immun* 1995; **63**: 2435–2442.
 43. Funda DP, Tucková L, Farré MA, Iwase T, Moro I, Tlaskalová-Hogenová H. CD14 is expressed and released as soluble CD14 by human intestinal epithelial cells in vitro: lipopolysaccharide activation of epithelial cells revisited. *Infect Immun* 2001; **69**: 3772–3781.
 44. Aderem A, Ulevitch RJ. Toll-like receptors in the induction of the innate immune response. *Nature* 2000; **406**: 782–787.
 45. Kaisho T, Akira S. Critical roles of Toll-like receptors in host defense. *Crit Rev Immunol* 2000; **20**: 393–405.
 46. Means TK, Golenbock DT, Fenton MJ. Structure and function of Toll-like receptors. *Cytokine Growth Factor Rev* 2000; **11**: 219–232.
 47. Faure E, Thomas L, Xu H, Medvedev AE, Equils O, Arditi M. Bacterial lipopolysaccharide and IFN- γ induce Toll-like receptor 2 and Toll-like receptor 4 expression in human endothelial cells: role of NF- κ B activation. *J Immunol* 2001; **166**: 2018–2024.
 48. Kusunoki T, Hailman E, Juan TS-C, Lichenstein HS, Wright

- SD. Molecules from *Staphylococcus aureus* that bind CD14 and stimulate innate immune responses. *J Exp Med* 1995; **182**: 1673–1682.
49. Gao JJ, Xue Q, Zuvanich EG, Haghi KR, Morrison DC. Commercial preparations of lipoteichoic acid contain endotoxin that contributes to activation of mouse macrophages in vitro. *Infect Immun* 2001; **69**: 751–757.
50. Yoshimura A, Takada H, Kaneko T, Kato I, Golenbock D, Hara Y. Structural requirements of muramylpeptides for induction of Toll-like receptor 2-mediated NF- κ B activation in CHO cells. *J Endotoxin Res* 2000; **6**: 407–410.
51. Del Prete G. The concept of type-1 and type-2 helper T cells and their cytokines in humans. *Int Rev Immunol* 1998; **16**: 427–455.
52. Séguier S, Godeau G, Leborgne M, Pivert G, Brousse N. Immunohistologic and morphometric analysis of cytotoxic T lymphocytes in gingivitis. *J Periodontol* 1999; **70**: 1383–1391.