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Innate immune responses of gingival epithelial cells to nonperiodontopathic and periodontopathic bacteria

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Background and Objective: We have previously reported different susceptibilities of periodontopathic and nonperiodontopathic bacteria to antimicrobial peptides and phagocytosis by neutrophils. Differences between the two groups of bacteria may exist also in their ability to induce immune responses from the host. Therefore, we evaluated the effects of various oral bacteria on innate immune responses by gingival epithelial cells.

Material and Methods: HOK-16B cells were cocultured with live or lysed nonperiodontopathic ($n = 3$) and periodontopathic ($n = 5$) bacterial species. The levels of human beta defensin-1, -2 and -3, and of the cathelicidin, LL-37, were examined by real-time reverse transcription-polymerase chain reaction, and the accumulated interleukin-8 and interleukin-1α were measured by enzyme-linked immunosorbent assay.

Results: Nonperiodontopathic bacteria up-regulated some antimicrobial peptides without affecting the levels of cytokines. In the periodontopathic group, the orange-complex bacteria induced antimicrobial peptides and interleukin-8 efficiently, but the red-complex bacteria often demonstrated suppressive effects. In contrast to live bacteria, bacterial lysates had no suppressive effects. In addition, some bacterial lysates demonstrated a reduced ability to induce antimicrobial peptides compared with live bacteria.

Conclusion: The nonperiodontopathic, the orange-complex, and the red-complex bacteria had different effects on the innate immune responses from gingival epithelial cells, which may affect the outcome of their host–microbial interaction in gingival sulcus.

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The pathogenesis of periodontitis involves the interplay of microbiota present in the subgingival plaque and the subsequent host responses (1). During the transition from health to disease, a shift in microbial composition from gram-positive facultative to gram-negative anaerobic microorganisms has been established for decades (2). In particular, 10–20 species are known to be periodontopathic, based on their predominance in diseased sites (3,4). Although the virulence factors of periodontopathic bacteria have been studied extensively, why nonperiodontopathic bacteria are compatible with periodontal health has been explored in less detail. Features that differentiate periodontopathic from nonperiodontopathic bacteria may provide new insights into the pathogenesis of periodontitis. We previously reported that bacteria resistant both to antimicrobial

peptides and to phagocytosis by neutrophils are more common in the periodontopathic group than in the nonperiodontopathic group (5). Differences between the two groups of bacteria may exist also in their ability to induce immune responses in the host.

The gingival epithelium is the tissue first encountered by subgingival plaque-associated bacteria. In general, mucosal epithelia not only form a protective barrier, but also initiate immune responses by secreting various cytokines and chemokines (6). The barrier function of epithelia is attributed to the unique architectural integrity and production of antimicrobial peptides, such as human beta defensins and a cathelicidin, LL-37 (6). These peptides have broad-spectrum antimicrobial activity against gram-positive and gram-negative bacteria, as well as fungi and some viruses (6). The striking importance of antimicrobial peptides has been shown in patients with Kostmann syndrome, who lack LL-37 in their saliva and develop severe periodontitis in young adulthood (7). The epithelia of many body sites express human beta defensin-1 constitutively, but express human beta defensin-2 and -3 only under conditions of infection or inflammation (8). By contrast, clinically healthy gingival epithelium is characterized by the presence of human beta defensin-2 and a gradient of interleukin-8 that guides the transmigration of neutrophils through the junctional epithelium (8,9). Inflamed gingiva are heavily infiltrated with neutrophils and other leukocytes around the pocket and junctional epithelia, accompanied by the elevated expression of interleukin-8, interleukin-1, tumor necrosis factor-α and intercellular adhesion molecule (10). According to current hypotheses, commensal bacteria are relatively well tolerated by the host immune system and may even benefit the overall readiness of oral epithelia to respond to challenge by inducing human beta defensin-2 and interleukin-8 (1,11). Then, what causes the conversion from the established immune tolerance to chronic inflammation is not fully understood.

The ability of several bacterial species, including *Streptococcus gordonii*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* to induce human beta defensins from oral epithelial cells has been studied (12–15). Cytokine production by oral epithelial cells in response to bacteria such as *F. nucleatum*, *P. gingivalis*, *A. actinomycetemcomitans*, *Eikenella corrodens* and *Prevotella intermedia*, has been extensively studied (13,16–22). However, the induction of antimicrobial peptides and cytokine production by nonperiodontopathic vs. periodontopathic bacteria has not been evaluated in parallel. To address this, we examined the expression of human beta defensin-1, -2, -3 and of LL-37, and the secretion of interleukin-8 and interleukin-1α, by human oral epithelial cells exposed to three nonperiodontopathic and five periodontopathic bacterial species.

Material and methods

Bacterial strains and culture

Streptococcus sanguinis NCTC 10904, *S. gordonii* ATCC 10558, *Veillonella atypica* ATCC 17744, *F. nucleatum* ATCC 10953, *P. intermedia* ATCC 25611, *P. gingivalis* ATCC 49417, *Tannerella forsythia* ATCC 43407 and *Treponema denticola* ATCC 33521, obtained from the ATCC (Bethesda, MD, USA), were cultured as described previously (5). Bacteria in the log phase were harvested and washed twice with phosphate-buffered saline. Part of the bacterial suspension was stained with 5 μM SYTOX-Green nucleic acid stain (Molecular Probes, Eugene, OR, USA), and the concentration and viability of bacteria were determined by flow cytometry (23). A fraction of the bacterial suspension with determined concentration was sonicated (Sonic Dismembrator 300; Fisher Scientific, Fair Lawn, NJ, USA) for 15 min on ice-water to prepare lysates. The breakage of bacterial cells was confirmed by examination under a light microscope. To analyze invasion into HOK-16B cells, we used bacteria stained with 5 μM 5 (and 6-)-carboxyfluorescein diacetate succinimidyl ester (Molecular Probes).

Epithelial cell culture

HOK-16B was obtained from Dr N.-H. Park (University of California Los Angeles, Los Angeles, USA) and maintained in keratinocyte growth medium (Clonetics, San Diego, CA, USA) containing 0.15 mM calcium and a supplementary keratinocyte growth medium bullet kit that includes antibiotics GA-1000 (gentamicin and amphotericin B) and growth factors.

Infection of epithelial cells with oral bacteria

HOK-16B cells were plated at 6×10^4 cells/500 μL/well in triplicate into 24-well plates 1 d before infection. At 80% confluence, cells were infected with eight different species of live or lysed bacteria in keratinocyte growth medium containing 2% heat-inactivated human sera (Sigma, St Louis, MO, USA), and then cultured at 37°C in a water-saturated atmosphere of 95% air and 5% CO_2 for 24 h. The multiplicities of infection of 200 and 1000 were based on the total number of bacteria. The culture supernatant was collected separately from triplicate wells for cytokine enzyme-linked immunosorbent assay (ELISA), and total RNA was extracted from combined cells of triplicate wells using TRIzol (Invitrogen, Carlsbad, CA, USA). Experiments were repeated two or three times. In the separate preliminary experiments, the concentration and viability of bacteria were re-analyzed after coculture. In addition, the effect of bacteria on HOK-16B cell viability was determined by flow cytometry after staining with Trypan blue, as previously described (24). None of the bacteria studied were cytotoxic to HOK-16B cells, up to a multiplicity of infection of 2000.

Examination of bacterial invasion into HOK-16B cells

HOK-16B cells were plated at a density of 6×10^4 cells/cm^2 onto 12-mm diameter acid-washed glass coverslips (Fisher Scientific). Cells infected, as described above, were fixed with 2% paraformaldehyde in phosphate-buffered

saline, neutralized with 0.1 M glycine in phosphate-buffered saline and then permeabilized for 1 min with 0.5% Triton-X-100 in phosphate-buffered saline. Cells were then stained with Rhodamine-phalloidin (Sigma) for 15 min, washed with phosphate-buffered saline and stained with Hoechst 33342 (Molecular Probes). Finally, cells were washed with phosphate-buffered saline and mounted with a mounting solution (Citifluor, London, UK) before analysis using a Zeiss LSM 510 confocal microscope (Carl Zeiss, Jena, Germany).

Real-time semiquantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA (2 μg) was subjected to reverse transcription with $(dT)_{18}$ and Superscript II enzyme (Invitrogen) in a 25 μL reaction mix at 42°C for 1 h. Real time-PCR was performed in a 20-μL reaction mix containing 1 μL of template cDNA, SYBR Premix Ex *Taq*, ROX Reference Dye (Takara Bio, Otsu, Japan) and each primer (0.2 μM). Oligonucleotide primers were designed to amplify at least two exons in order to prevent the amplification of contaminating genomic DNA. Primer sequences are listed in Table 1. Amplification was performed in a fluorescence thermocycler (Applied Biosystems 7500 Real-time PCR; Applied Biosystems, Foster City, CA, USA) under the following conditions: initial denaturation at 94°C for 1 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 15 s and elongation at 72°C for 33 s. The specificity of the PCR product was verified by melting curve analysis and examination on a 2% agarose gel. The housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase, was amplified in parallel with the gene of interest. Relative copy numbers compared with glyceraldehyde-3-phosphate dehydrogenase were calculated using $2^{-\Delta Ct}$. Real-time PCR was performed in triplicate for each RNA sample. The mean ± standard error of the mean of the relative copy numbers from six to nine assays were expressed as induction fold compared with that of the control culture without bacteria.

ELISA

The amounts of interleukin-8 and interleukin-1α secreted into the medium during coculture with bacteria were measured using ELISA kits (R & D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. The mean ± standard error of the mean of six to nine assays were expressed as induction percentages compared with that of the control culture without bacteria.

Statistics

Statistical analysis was performed using the relative copy numbers of antimicrobial peptides and the concentrations of cytokines to determine the difference between the control and cells infected with each bacterial species. At the same time, induction rates were used to determine the difference in the responses induced by live vs. lysed bacteria. The differences between the two groups were analyzed with the two-tailed nonpaired Student's *t*-test. Data were considered statistically significant at a *p*-value of < 0.05.

Table 1. Primer sequences employed for real-time reverse transcription-polymerase chain reaction

	Primer sequences	
GAPDH	Forward	CAGCCTCAAGATCATCAGCA
	Reverse	CCATCCACAGTCTTCTGGGT
HBD-1	Forward	GTCGCCATGAGAACTTCCTA
	Reverse	GTTCATTTCACTTCTGCGTCATTTCTTCT
HBD-2	Forward	ATCAGCCATGAGGGTCTTGT
	Reverse	GGATCGCCTATACCACCAAA
HBD-3	Forward	TGTTTGCTTTGCTCTTCCTG
	Reverse	CTTTCTTCGGCAGCATTTC
LL-37	Forward	GCTAACCTCTACCGCCTCCT
	Reverse	TCTGGTGACTGCTGTGTCGT

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HBD, human beta defensin; LL-37, a cathelicidin.

Results

Induction of antimicrobial peptides and inflammatory responses from HOK-16B cells by live bacteria

Nonperiodontopathic bacteria were selected to include both gram-positive facultative and gram-negative absolute anaerobes that are thought to be compatible with periodontal health (1). Five species chosen from the list of bacteria often referred to as periodontopathogens included two from the orange-complex bacteria and three from the red-complex bacteria, according to Socransky's classification (25). HOK-16B is an immortalized cell line established by the transfection of human papillomavirus type 16 DNA into primary human oral keratinocytes cultured from excised gingival tissue (26). HOK-16B cells were cocultured with various oral bacteria at multiplicities of infection of 200 and 1000 in a 95% air and 5% CO_2 atmosphere for 24 h to simulate chronic stimulation *in vivo*. To assess the effects of oral bacteria on the barrier function of epithelia, the expression levels of four antimicrobial peptides, including human beta defensin-1, -2 and -3, and LL-37, were evaluated by real-time semiquantitative RT-PCR. The ability of various oral bacteria to induce inflammatory responses from epithelial cells was assessed by measuring the amounts of interleukin-8 and interleukin-1α secreted into the medium during coculture.

Unstimulated HOK-16B cells expressed all four antimicrobial peptides, with human beta defensin-1 expression the strongest and human beta defensin-2 expression the weakest (Fig. 1A). In addition, the cells secreted substantial basal levels of interleukin-8 and interleukin-1α (Fig. 1B). Coculture with oral bacteria had

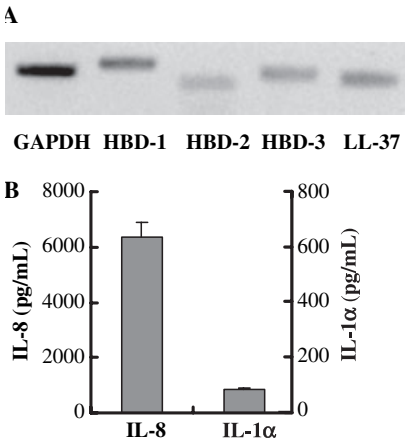

Fig. 1. Basal levels of antimicrobial peptides (human beta defensin-1, -2, -3 and LL-37), interleukin-8, and interleukin-1α expressed by immortalized gingival epithelial cells, HOK-16B. The basal levels of antimicrobial peptides, interleukin-8, and interleukin-1α expressed by HOK-16B cells were analyzed using RNA samples and culture medium from control culture without bacteria. (A) The expression of human beta defensin-1, -2 and -3, and of the cathelicidin, LL-37, was examined by reverse transcription-polymerase chain reaction (RT-PCR), and the PCR products were visualized on a 2% agarose gel. The number of PCR cycles was adjusted to 32 in order to present differential expression. (B) The amounts of interleukin-8 and interleukin-1α secreted into the culture medium were measured by enzyme-linked immunosorbent assay. The graph presents the mean ± standard error of the mean of all control samples shown in Figs 2 and 3. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HBD, human beta defensin; IL, interleukin.

various effects on the levels of antimicrobial peptides, interleukin-8 and interleukin-1α. Among the three nonperiodontopathic bacterial species, only *S. gordonii* was a relatively efficient inducer of antimicrobial peptides, up-regulating two out of four peptides significantly, but did not affect the levels of interleukin-8 and interleukin-1α (Fig. 2A,B). *V. atypica* induced only human beta defensin-3 significantly. Interestingly, *S. sanguinis* inhibited human beta defensin-1 expression, although it up-regulated the levels of human beta defensin-3 and interleukin-1α. The orange-complex and the red-complex bacteria in the periodontopathic group demonstrated quite different effects. The orange-complex bacteria *F. nucleatum* and *P. intermedia* induced both interleukin-8 and antimicrobial peptides, up-regulating three and all four peptides, respectively (Fig. 2A,B). In contrast, the red-complex bacteria often demonstrated suppressive, rather than inductive, effects on the levels of antimicrobial peptides and interleukin-8 (Fig. 2A,B). In particular, *T. denticola* significantly suppressed human beta defensin-1 transcription and interleukin-8 accumulation in a dose-dependent manner. *P. gingivalis* down-regulated interleukin-8 and human beta defensin-3 at a multiplicity of infection of 200, but not at a multiplicity of infection of 1000. The expression of human beta defensin-3 was also suppressed by *T. forsythia*. None of the periodontopathic bacteria affected the level of interleukin-1α. Vankeerberghen *et al.* reported the induction of antimicrobial peptides by periodontopathic bacteria at time-points earlier than 24 h (15). However, we did not observe the induction of antimicrobial peptides by *P. gingivalis* or *T. denticola* at 4, 8, or 16 h.

Induction of antimicrobial peptides and inflammatory responses from HOK-16B cells by lysed bacteria

Live bacteria and bacterial debris present in subgingival plaque may induce different responses from epithelial cells, because some oral bacteria can invade epithelial cells and may inject bacterial substances directly into the cytoplasm of a host cell (27–29). In our coculture system, the invasion of five periodontopathic bacterial species into HOK-16B cells was confirmed (data not shown). Interestingly, HOK-16B cells also internalized bacterial lysates nonspecifically, as evidenced by the internalization of their own lysates (Fig. S1). Upon coculture with HOK-16B cells, bacterial lysates sometimes induced responses different from those induced by live bacteria (Fig. 3A,B, indicated by a dagger). Out of eight different occasions, four were a result of the reduced ability of lysates to induce antimicrobial peptides, and the other four were a result of the abrogation of the inhibitory effects on either antimicrobial peptide expression or interleukin-8 accumulation observed by live bacteria (Fig. 3A,B).

Discussion

In this study, we investigated the ability of various oral bacteria to induce innate immune responses from gingival epithelial cells and showed that nonperiodontopathic and orange-complex bacteria induce weak and strong responses, respectively, but that red-complex bacteria suppress immune responses. In addition, live and lysed bacteria showed a difference in their ability to induce immune responses from epithelial cells.

Aerobic conditions were chosen for the coculture of bacteria with HOK-16B cells, because culture under anaerobic conditions was too stressful for HOK-16B cells. To prevent the outgrowth of facultative anaerobic bacteria, antibiotics were included in the keratinocyte growth medium. Under these culture conditions, 35–60% of bacteria survived but showed no bacterial growth (Fig. S2).

HOK-16B cells secreted a substantial amount of interleukin-8 as basal levels (Fig. 1B), representing 100-fold more interleukin-8 than that secreted by A549 epithelial cells derived from lung carcinoma (data not shown). Primary normal gingival epithelial cells secreted equivalent levels of interleukin-8 with HOK-16B cells without stimulation (data not shown). Therefore, gingival epithelia seem to be equipped with interleukin-8 to chemoattract neutrophils as well as antimicrobial peptides in the absence of bacteria.

In general, nonperiodontopathic bacteria up-regulated some antimicrobial peptides without affecting the interleukin-8 level (Fig. 2A,B). Among the three nonperiodontopathic bacterial species, only *S. gordonii* was a relatively efficient inducer of antimicrobial peptides, up-regulating human beta defensin-3 and LL-37. In contrast, *S. sanguinis* may not change the total amount of antimicrobial peptides significantly, as it up-regulates

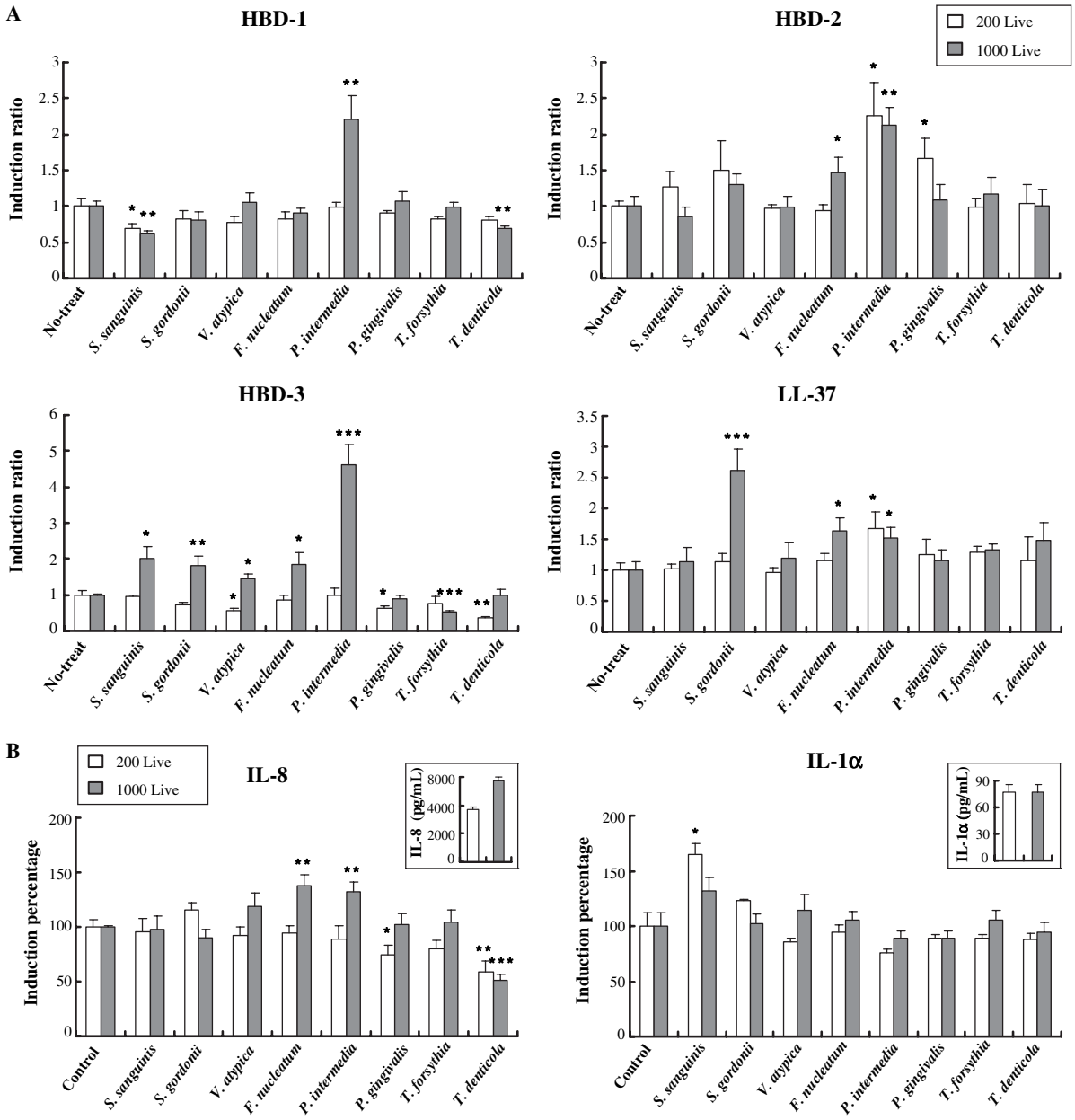

Fig. 2. Induction of antimicrobial peptides and cytokines from HOK-16B cells by live bacteria. HOK-16B cells were cocultured with live bacteria, at multiplicities of infection of 200 or 1000, for 24 h. (A) Induction of human beta defensin-1, -2, -3, and of the cathelicidin, LL-37, by various oral bacteria was evaluated by real-time reverse transcription-polymerase chain reaction. The mean ± standard error of the mean of the relative copy numbers were expressed as induction fold, compared with those of the control culture without bacteria. (B) The amounts of interleukin-8 and interleukin-1α secreted into the culture medium were measured by enzyme-linked immunosorbent assay. The mean ± standard error of the mean of the measured amounts were expressed as induction percentages, compared to control culture without bacteria. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, vs. control. HBD, human beta defensin; IL, interleukin; No-treat, no treatment. *F. nucleatum*, *Fusobacterium nucleatum*; *P. gingivalis*, *Porphyromonas gingivalis*; *P. intermedia*, *Prevotella intermedia*; *S. gordonii*, *Streptococcus gordonii*; *S. sanguinis*, *Streptococcus sanguinis*; *T. denticola*, *Treponema denticola*; *T. forsythia*, *Tannerella forsythia*; and *V. atypica*, *Veillonella atypica*.

human beta defensin-3 but down-regulates human beta defensin-1. Nonperiodontopathic bacteria were susceptible to antimicrobial peptides and/or phagocytosis, except for this particular *S. gordonii* strain that is highly resistant to human beta defensin-3, LL-37 and phagocytosis (5). Colombo *et al.* recently reported the detection of a wide range of oral species on or in sulcular epithelial cells, and *S. gordonii* was one of the species more prevalent in healthy sites than in diseased sites (30). Therefore, *S. gordonii* may be a true beneficial commensal that interacts with oral epithelia. Collectively, our results indicate that nonperiodontopathic oral

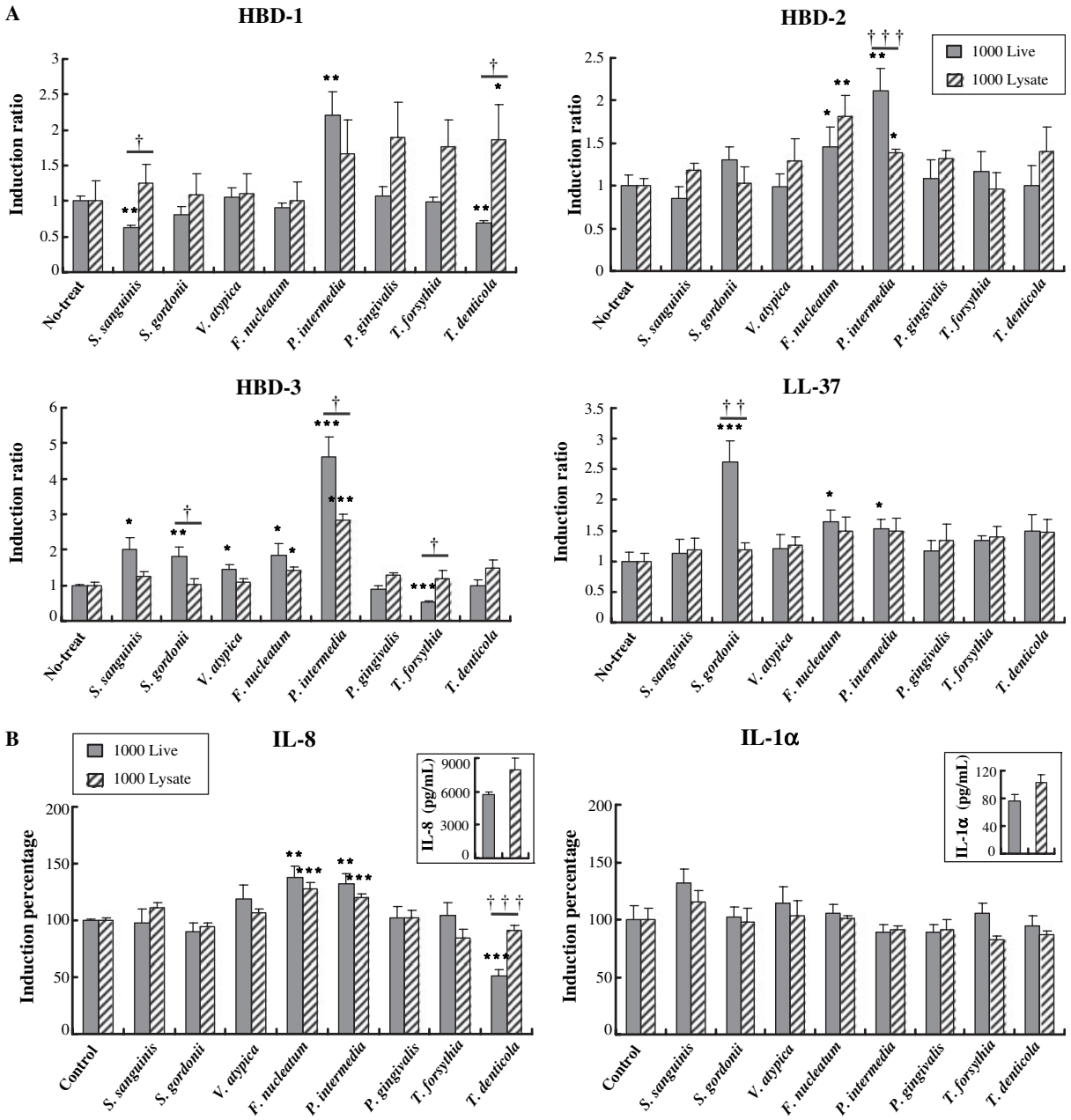

Fig. 3. Induction of antimicrobial peptides and cytokines from HOK-16B cells by bacterial lysates. HOK-16B cells were cocultured with bacterial lysates, of a multiplicity of infection 1000, for 24 h. The results were compared with those obtained by live bacteria at a multiplicity of infection of 1000, shown in Fig. 2. (A) Induction of human beta defensin-1, -2 and 3, and of the cathelicidin, LL-37, by various oral bacteria was evaluated by reverse transcription-polymerase chain reaction. (B) The amounts of interleukin-8 and interleukin-1α secreted into the culture medium were measured by enzyme-linked immunosorbent assay. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, vs. control. †$p < 0.05$, ††$p < 0.01$, †††$p < 0.001$, vs. live bacteria. *F. nucleatum*, *Fusobacterium nucleatum*; *P. gingivalis*, *Porphyromonas gingivalis*; *P. intermedia*, *Prevotella intermedia*; *S. gordonii*, *Streptococcus gordonii*; *S. sanguinis*, *Streptococcus sanguinis*; *T. denticola*, *Treponema denticola*; *T. forsythia*, *Tannerella forsythia*; and *V. atypica*, *Veillonella atypica*.

bacteria may co-exist with the host as tolerant or beneficial bacteria, at least in the gingival sulcus.

F. nucleatum is a well-known stimulatory bacterium that induces both antimicrobial peptides and interleukin-8 efficiently (13,15,16,18). The stimulatory effect of a glycoprotein fraction from *P. intermedia* on the secretion of interleukin-8, granulocyte colony-stimulating factor and granulocyte–macrophage colony-stimulating factor has also been reported (21). Our results were in good agreement with earlier studies, because *F. nucleatum* and *P. intermedia* were quite efficient inducers of both antimicrobial peptides and interleukin-8 (Fig. 2A,B). In par-

ticular, *P. intermedia* was the strongest inducer of antimicrobial peptides, up-regulating all four peptides, including human beta defensin-1 that has been regarded as noninducible (Figs 2 and 3). It is interesting that both of the two strong inducers of antimicrobial peptides and interleukin-8 are highly susceptible to antimicrobial peptides and phagocytosis by neutrophils, raising the issue of whether they are true periodontopathogens (5). In fact, *F. nucleatum* is often regarded as a commensal (12,15); however, it also has pathogenic characteristics. *F. nucleatum* facilitates the colonization of many periodontopathic bacteria (3), invades oral epithelia (28), produces a serine protease (31,32) and induces inflammatory cytokines from host cells (13,33). Similarly, *P. intermedia* invades oral epithelia and has proteolytic activity (1,34). Furthermore, a weak (but significant) association between the prevalence of these two bacteria and the clinical index of periodontitis has been reported (25). Our results indicate that *F. nucleatum* and *P. intermedia* induce active immune responses from the host that eliminate them efficiently and thus may have self-limiting characteristics.

The down-regulation of interleukin-8 by *P. gingivalis* in both mRNA and protein levels, so-called 'chemokine paralysis' has been well characterized (16,18). In our study, *P. gingivalis* down-regulated not only interleukin-8 but also human beta defensin-3 at a multiplicity of infection of 200 (Fig. 2A,B). The reason why the same bacterium at the increased multiplicity of infection did not demonstrate similar inhibitory effects is not clear. The expression of human beta defensin-3 was also suppressed by *T. forsythia* (Fig. 2A). The suppression of epithelial cell responses by *T. denticola* was most evident, down-regulating human beta defensin-1 transcription and interleukin-8 accumulation in a dose-dependent manner (Fig. 2A,B). The protease of *T. denticola* can suppress interleukin-8 accumulation without affecting transcription (35,36). However, the level of interleukin-1α was not affected by coculture with *T. denticola*, and the lysate form of *T. denticola* did not suppress interleukin-8 accumulation (Fig. 3B), suggesting that the protease may not be sufficient for the suppressive phenomenon. Although the underlying mechanisms are not fully understood, the red-complex bacteria tend to silence the innate immune responses of oral epithelial cells. Furthermore, the red-complex bacteria are resistant to both LL-37 and phagocytosis by neutrophils (5). Therefore, the red-complex bacteria have various mechanisms to evade the host immune system, which may lead to persistent infection.

Live and lysed bacteria had different abilities to induce innate immune responses from HOK-16B cells. Different pattern recognition receptors that sense microbes are distributed at the surface, cytosol and endosomal compartments of cells (37). Five periodontopathic bacteria that invade epithelial cells would activate the intracellular microbial sensors as well as those on the cell surface. Although lysates were also internalized, the process was less efficient and may have a different subcellular destination from the invaded bacteria (Fig. S1). A type III secretion system (one of the protein secretion pathways employed by gram-negative bacteria) mediates the injection of bacterial effector proteins directly into host cells, and is best characterized in several pathogenic enteric bacteria (27). Cytolysin-mediated translocation by *S. pyogenes* has been described as a functional equivalent of type III secretion in gram-positive bacteria (29). Oral bacteria may have similar mechanisms that inject bacterial substances directly into the cytoplasm of a host cell. In that case, live bacteria, but not bacterial lysates, would stimulate the pattern recognition receptors, such as NOD-1 and -2, present in cytosol (37). Which particular pattern recognition receptor is involved in the regulation of antimicrobial peptides and interleukin-8 by live vs. lysed bacteria is under investigation in our laboratory.

In summary, we studied the innate immune responses of gingival epithelial cells to the nonperiodontopathic, the orange-complex and the red-complex bacteria, and have provided evidence supporting that the outcome of their host–microbial interaction in gingival sulcus may be characterized as a tolerant/beneficial co-existence, efficient clearance by active innate immune responses and persistent infection as a result of immune evasion, respectively.

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Supplementary material

Fig. S1. Internalization of live and lysed bacteria by HOK-16B cells. HOK-16 cells were cocultured with live or lysed *Fusobacterium nucleatum*, or with the lysates of their own cells that were labelled with carboxyfluorescein diacetate succinimidyl ester. After fixation and permeabilization, cells were stained with Rhodamine-phalodin and Hoecht 33342, and examined under a confocal microscope. *F. nucleatum*, *Fusobacterium nucleatum*.

Fig. S2. The effect of coculture on the viability of bacteria. Various oral bacteria were cocultured with HOK-16B cells, aerobically for 24 h, in keratinocyte growth medium containing 2% human sera and antibiotics. After coculture, the viability of bacteria was re-analyzed by flow cytometry after staining with SYTOX green. *F. nucleatum*, *Fusobacterium nucleatum*; *P. gingivalis*, *Porphyromonas gingivalis*; *P. intermedia*, *Prevotella intermedia*; *S. gordonii*, *Streptococcus gordonii*; *S. sanguinis*, *Streptococcus sanguinis*; *T. denticola*, *Treponema denticola*; *T. forsythia*, *Tannerella forsythia*; and *V. atypica*, *Veillonella atypica*.