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ORAL MICROBIOLOGY AND IMMUNOLOGY

The immune response of oral epithelial cells induced by single-species and complex naturally formed biofilms

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Introduction: In the oral cavity, the surfaces are constantly exposed to a complex variety of microorganisms organized in biofilms. As part of a sophisticated local immune response, gingival epithelial cells (GECs) express antimicrobial peptides, such as human β-defensin-2 (hBD-2), ribonuclease 7 (RNAase-7), and psoriasin (PSO), and pro-inflammatory mediators, such as interleukin-8 (IL-8) and 5-lipoxygenase (5-LO). The aim of the present study was to test whether GECs show a differential immune response to single-species biofilms compared with multi-species biofilms.
Methods: GECs were cultured from biopsies derived from three different healthy donors ($n = 3$). To obtain naturally formed biofilm (NFB), polymer disks were attached to prostheses and carried intraorally for 12, 24, 36, and 48 h. In addition, single-species biofilms (SSB; *Streptococcus mutans* and *Streptococcus mitis*) were cultured on polymer disks *in vitro* (12, 24, 36, and 48 h). The messenger RNA (mRNA) expression of hBD-2, RNAase-7, PSO, IL-8, 5-LO, and glycerylaldehyde-3-phosphate dehydrogenase was analysed using semi-quantitative reverse transcription-polymerase chain reaction.
Results: In GECs, the hBD-2 mRNA expression was significantly upregulated in response to *S. mitis*-biofilm stimulation compared with *S. mutans*-biofilm stimulation ($P < 0.0001$). In contrast, the RNAase-7 mRNA expression was significantly higher in GECs when responding to both *S. mutans* biofilms and naturally formed biofilms compared with *S. mitis* biofilms ($P < 0.0001$ and $P < 0.001$, respectively). The IL-8 and 5-LO mRNA was significantly upregulated in response to *S. mutans* biofilms ($P < 0.0001$ and $P = 0.0002$, respectively).
Conclusion: This *in vitro* study found biofilm-dependent expression of antimicrobial peptides and inflammatory mediators in GECs.

Key words: biofilm; cytokine; defensin; immune response; oral epithelium

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The surfaces of the oral cavity are constantly exposed to a wide variety of microorganisms, which are capable of forming biofilms on not only teeth and oral mucosa, but also prostheses (29). If not disturbed, biofilm formation starts a few hours after ingestion and may proceed for days. However, the oral cavity maintains healthy homeostasis during the early phase of biofilm formation. For this maintenance, the mucosal epithelium exhibits a sophisticated defense mechanism by providing a physical and a chemical barrier (6, 13). While the physical barrier is represented by rigid intercellular connections, the chemical barrier is composed of a number of antimicrobial peptides, such as human β-defensins (hBDs) (6) and the recently described psoriasin (PSO) and ribonuclease 7 (RNAase-7) (11, 14, 18). Antimicrobial peptides are mainly responsible for maintaining oral health by fighting pathogenic bacteria (4). In addition to the mucosal epithelium, antimicrobial peptides are also synthesized by oral salivary glands and secreted into the saliva (2, 25, 28), which covers the oral epithelium by

forming a thin pellicle-like layer. Certain epithelia, such as the oral epithelium (gingival epithelium), are populated by distinct commensal bacterial species, and recent studies suggest that host epithelial tissues promote associations with these distinct bacteria, which may have beneficial effects on healthy oral status (30, 32, 37).

Antimicrobial peptides (AMPs) as part of the chemical epithelial barrier are defined as proteins that are <100 amino acids in length and have a molecular weight typically ranging between 3.5 and 6.5 kDa (6, 21). The hBDs are cationic antimicrobial peptides made primarily by epithelial cells and expressed in all human epithelial tissues tested. In general, defensins are secreted in biological fluids, including urine, bronchial fluid, nasal secretions, saliva, and gingival crevicular fluid, where these peptides show subtype-specific expression patterns (1, 5, 8, 28, 35). While hBD-1 represents a constitutively expressed peptide (17, 20, 25), the expression of hBD-2 and hBD-3 is inducible by bacteria, interleukin-1 (IL-1) or tumor necrosis factor-α (TNF-α) (17, 19, 27). Although hBD-2 is induced and expressed only during inflammation of epithelial tissues, in the oral epithelium it is also expressed in healthy, clinically uninflamed gingival tissue (7, 9). The hBD-2 was first isolated from psoriatic skin scales and exhibits strong bactericidal effects on gram-negative bacteria as well as a high antimycotic potency against *Candida albicans*, but it possesses only weak bacteriostatic effects on gram-positive bacteria (16). PSO and RNAase-7 are identified as members of the S100 protein super family, and have recently been described as novel epithelial antimicrobial peptides (14, 18). The expression of both Rnase-7 and PSO is inducible by pro-inflammatory mediators such as IL-1β, interferon-γ (IFN-γ), and TNF-α, or by *Escherichia coli* culture supernatants, respectively (14, 18). In our laboratory, gene expression of PSO and RNAase-7 has been discovered in cultured gingival epithelial cells. In addition, it was demonstrated that the messenger RNA (mRNA) expression of PSO and RNAase-7 was inducible by naturally formed biofilms, but with interindividual variations (11).

IL-8 represents a chemokine that specifically activates neutrophils. IL-8 is known as an inducible chemotactic factor that is synthesized in a number of cells, such as macrophages, monocytes, fibroblasts, and epithelial cells (33). Unlike IL-8, 5-lipoxygenase (5-LO) is an enzyme that is mainly involved in eicosanoid biosynthesis and therefore influences local immune responses. 5-LO is highly expressed by human oral keratinocytes after bacterial challenge (11).

The exact role of gingival epithelial cells regarding the expression profiles of AMPs in response to naturally biofilms is not yet apparent. A number of studies showed expression profiles of AMPs in gingival epithelial cells exposed to bacteria that have been cultured separately (3, 4, 10, 19, 20). These stimulation experiments using bacteria of single species may not reflect the environmental complexity of biofilms. Biofilms consist of multiple microorganisms of different species that live in a well-structured ecosystem, which partly protects bacteria from microbicidal host defense responses (34). Therefore, the aim of the present study was to specifically test the hypothesis that epithelial cells show a differential gene expression pattern of antimicrobial peptides and inflammatory mediators in response to different stages of naturally formed biofilms in contrast to two biofilm models consisting of single oral pathogens (*Streptococcus mutans* and *Streptococcus mitis*). Furthermore, it was of interest to determine the influence of biofilm growth phases, representing early intraoral plaque formation, on the expression of genes involved in host immunity.

Materials and methods

Cell culture

Gingival biopsy specimens were surgically obtained from young healthy patients who underwent third molar extraction at the Department of Operative Dentistry and Periodontology, University Hospital Kiel. The local Ethics Committee of the University of Kiel had approved the study and the subjects had signed a letter of informed consent.

Donors did not show any clinical signs of acute or chronic gingival inflammation in the molar or retro-molar region before surgical third molar extraction (no redness, no swelling, no bleeding on probing). Primary human gingival epithelial cells were isolated from the biopsy specimens and grown in a serum-free keratinocyte growth medium (Keratinocyten SFM; Invitrogen, Karlsruhe, Germany). The growth medium was supplemented with 1000 μg/ml penicillin and 500 μg/ml amphotericin (Biochrom, Berlin, Germany). For experimental studies, second to fifth passage cultures were grown to 80% confluence. The cultures were routinely screened for the mRNA expression of keratin 13 to confirm the epithelial character of the cells. For stimulation experiments, gingival epithelial cells derived from a total of three different donors.

Biofilm formation

Subjects that underwent third molar extraction also volunteered for biofilm generation after wound healing was completed. Therefore, only young healthy patients (with inconspicuous general health, no medication, no periodontal disease, no active caries, no prosthodontic restorations or insufficient fillings) were asked to carry polymer disks as described below.

The application of polymer disks for intraoral biofilm growth has been described in our previous report (11). The prostheses carrying the polymer disks were placed intraorally for 12, 24, 36, and 48 h for the formation of biofilms by three different volunteers. The polymer disks were gently removed from the support prosthesis without affecting the surface of the native biofilm.

Single-species biofilms were grown in a stationary biofilm forming system modified from Guggenheim et al. (15). Briefly, polymer disks were incubated with saliva for 4 h to allow for the formation of a salivary pellicle. Afterwards, the polymer disks were placed in polystyrene cell culture plates and were covered with a layer of bacterial growth medium. For the cultivation of *S. mutans* and *S. mitis*, tryptic soy broth and brain–heart infusion broth, respectively, were used. The plates were incubated at 37°C (5% CO_2) for 12, 24, 36, or 48 h. Medium was renewed after 24 h by aspiration of the medium and addition of fresh medium. A total of three different polymer disks per species were incubated.

Epithelial biofilm interactions

The disks containing the biofilm were washed gently with 500 μl phosphate-buffered saline (PBS; Biochrom) to remove any loosely adherent bacteria and saliva and placed at a distance of 500 μm above the primary epithelial cell cultures for 2 h in antibiotic-free medium. To achieve reproducible distances, a sterile spacer (orthodontic wire) was used. For control purposes, the PBS used for washing the biofilms was collected and used for stimulation of epithelial cell cultures. Afterwards, the polymer disks were removed from the epithelial cells and frozen at −80°C until further processing. The

epithelial cells were washed three times with PBS before cell lysis and mRNA extraction. For control experiments, disks without the biofilms were used for stimulation. Each stimulation and control experiment was performed in triplicate.

RNA extraction and analysis

After stimulation, the cells were harvested using 500 μl lysis buffer (RNeasy Protect mini kit; Qiagen, Hilden, Germany). Total RNA was extracted according to the manufacturer's protocols. Reverse transcription-polymerase chain reaction (RT-PCR) was conducted to semi-quantitatively analyse mRNA expression of genes for hBD-2, RNAase 7, PSO, IL-8, 5-LO and glycerylaldehye-3-phosphate dehydrogenase (GAPDH, housekeeping gene) using previously described protocols (24). RNA was reverse transcribed using oligo-(dT) primers (Invitrogen). The complementary DNA (cDNA) was amplified in a total volume of 25 μl PCR mixture containing 1 μl cDNA, 10× PCR buffer, 1.5 mM $MgCl_2$, 10 mM dNTP Mix, 250 nM of both forward and reverse primer, and 2.5 U *Taq* DNA polymerase (Invitrogen). The specific sequences and annealing temperatures of the oligo-nucleotide primers have been published elsewhere (11). Reactions were carried out with the Personal Cycler (Bio-Rad Laboratories, Munich, Germany). The general PCR conditions were initial denaturation at 95°C for 5 min, continued by denaturation step at 95°C for 30 s, annealing at primer-specific temperatures for 30 s, elongation at 72°C for 1 min for 20–25 cycles, and a subsequent extension step at 72°C for 5 min. The PCR conditions for semi-quantitative determination were optimized for each gene investigated. Conditions for each dataset were chosen so that the PCR product did not reach its plateau at the end of the amplification steps, but represented the exponential amplification phase. The beginning of the plateau phase was determined in control experiments (data not shown). Overall, the plateau phase started around cycle 30, and was characterized by a constant PCR band intensity up to cycle 45. Each set of amplification always included a no-sample negative control (water) and a positive control with cloned target sequences.

The PCR products were loaded onto ethidium-bromide-stained, 1.5% agarose gels. A 1 kbp DNA ladder molecular weight marker (Gibco Life Technologies, Karlsruhe, Germany) was used to confirm the expected length of the amplification product. Images of the RT-PCR ethidium-bromide-stained agarose gels were acquired and quantification of the bands was performed using the IMAGE J software (http://rsb.info.nih.gov/ij/index.html). The ratio between the sample RNA to be determined and the internal control GAPDH was calculated to normalize for initial variations in sample concentration. Band intensity was expressed as relative absorbance units normalized to the unstimulated control. Median values and ranges for all experiments were calculated and differences between biofilms were tested for statistical significance by the Kruskal–Wallis test followed by the Mann–Whitney test for signed-differences. Differences with $P \leq 0.05$ were considered significant.

Microbiological analysis

The bacterial composition of the intraorally formed biofilms was evaluated as follows: cultivation of *S. mutans* and *S. mitis* was performed on non-selective culture media under capnoaerophilic conditions with 5% CO_2. Streptococci were isolated by colony morphology, and *S. mitis* and *S. mutans* were identified by biochemical characteristics using the Api 20 Strept test system (Biomérieux, Nürtingen, Germany). The total number of colony-forming units (CFU) was determined on the basis of serial dilution from 10^{-1} to 10^{-3} after characterization on non-selective media. One viable bacterium per 10 μl of sample volume was detectable by the bacterial cultivation method used.

Results

Gene expression of hBD-2, RNAase-7, and PSO in gingival epithelial cells

To examine the expression of hBD-2, PSO, and RNAase-7 mRNA *in vitro*, human epithelial cells were isolated from gingival biopsies and challenged with intraorally formed biofilms from three different subjects or with single-species biofilms of *S. mutans* or *S. mitis*. The epithelial character of the investigated epithelial cells was confirmed by the detection of keratin 13 mRNAs using RT-PCR. For the experiments, biofilms were grown for 12, 24, 36, and 48 h within the oral cavity or *in vitro*. Total RNA was extracted and analysed by semi-quantitative RT-PCR according to established protocols. The viability of epithelial cells and the yield of total RNA were checked during each experiment, and no differences were found between untreated and treated cells. For standardization of the semi-quantitative approach, the gene expression of GAPDH was analysed to show equivalent loading of samples under each condition tested. The size of the amplified products was as predicted. Although different time-points for either biofilm growth were tested, no statistical analysis for individual time-points was performed because of inter-individual variations. Unstimulated gingival epithelial cells showed low basal expression of hBD-2 mRNA; however, no statistically significant differences regarding the expression of antimicrobial peptides or inflammatory mediators were observed. Control experiments using PBS from the washing procedure of the biofilms did not affect the mRNA expression of all genes tested.

The expression of hBD-2 was induced by a factor of 2.0-fold to 4.4-fold after stimulation with *S. mitis* biofilms for 12–48 h. In comparison to stimulation with *S. mutans* biofilms and naturally formed biofilm, the gene expression of hBD-2 was significantly higher in gingival epithelial cells when exposed to *S. mitis* biofilms ($P = 0.0001$ and $P = 0.0001$, respectively). Although the overall gene expression level of hBD-2 was significantly higher after stimulation of epithelial cells with naturally formed biofilm compared with stimulation with *S. mutans* biofilms ($P = 0.031$), no substantial change was observed in relation to unstimulated gingival epithelial cells (Fig. 1).

The mRNA expression of RNAase-7 showed a mean expression level of 1.3–1.6 in epithelial cells exposed to naturally formed biofilms from 12 to 48 h. The mRNA expression of RNAase-7 was reduced following stimulation with *S. mitis* biofilms compared with the unstimulated epithelial cells, and there were no obvious differences for distinct time periods of biofilm formation. Stimulation with *S. mutans* biofilms showed a mean RNAase-7 gene expression level of 1.4 in cells exposed to biofilm after 12–24 h of incubation, but a decreased RNAase-7 gene expression (0.3) was detected in cells stimulated with biofilm after 48 h of incubation. In comparison to *S. mitis* biofilms, the gene expression of RNAase-7 was significantly higher in gingival epithelial cell stimulated with *S. mutans* biofilms ($P = 0.0001$). No statistical differences were observed when comparing the RNAase-7 mRNA level in epithelial cells after stimulation with *S. mutans* biofilms or naturally formed biofilms. The expression level of RNAase-7 was significantly higher in epithelial cells after stimulation with natural biofilms compared

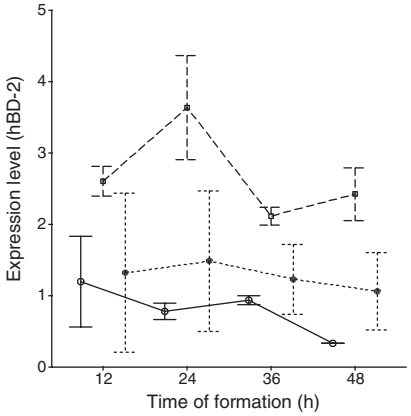

Fig. 1. The expression of human β-defensin-2 (hBD-2) after stimulations with single-species biofilms of *Streptococcus mitis* (open square) or *Streptococcus mutans* (circle) and complex intraorally (filled circle) formed biofilms. The biofilms were grown for 12, 24, 36, and 48 h. The statistical analyses demonstrated statistically significant differences between the three different biofilms ($P = 0.0001$). Graphs represent mean ± standard deviation for each time-point.

with *S. mitis* biofilms ($P = 0.0001$) (Fig. 2).

In gingival epithelial cells, the mRNA expression of the antimicrobial peptide PSO showed no changes after stimulation with natural biofilms compared with the control groups. The stimulation with *S. mitis* biofilms (after from 12 to 48 h of incubation) led to an increased mRNA expression level for PSO ranging from 0.6 to 1.7. In contrast, the mRNA expression level for PSO decreased in gingival epithelial cells from 1.1 to 0.4 after stimulation with *S. mutans* biofilms. Although no statistically significant differences were observed between the three treatment modalities ($P = 0.1$), stimulation with *S. mitis* and naturally formed biofilm (after 48 h of incubation) resulted in similar trends regarding a slightly increased gene expression of PSO (Fig. 3).

Gene expression of IL-8 and 5-LO in gingival epithelial cells

To determine the expression of IL-8 mRNA *in vitro*, epithelial cells were challenged with intraorally formed biofilms and single species biofilms grown for 12–48 h. After stimulation with *S. mutans* biofilms, the mRNA expression level of IL-8 ranged from 7.5 to 5.7, and this level was statistically higher compared with the induction levels after stimulation with *S. mitis* biofilms (0.1–0.4) and naturally formed biofilms (1.8–2.8) ($P = 0.0001$ and $P = 0.0001$, respectively). The statistical analysis did not reveal significant differences regarding the gene expression of IL-8 in cells either exposed to *S. mitis* biofilms or naturally formed biofilms (Fig. 4).

The induction levels for the 5-LO enzyme ranged from 1.9 to 2.8 after stimulation with *S. mutans* biofilms, from 1.2 to 1.6 after stimulation with *S. mitis* biofilms, and from 1.5 to 1.8 after stimulation with naturally formed biofilms. The comparison between stimulations by *S. mitis* biofilms and the intraorally formed biofilms showed no significant differences. Stimulation of gingival epithelial cells with *S. mutans* biofilms, however, resulted in a significantly increased gene expression of 5-LO mRNA compared with cells exposed to *S. mitis* and naturally formed biofilms ($P = 0.002$ and $P = 0.016$, respectively) (Fig. 5).

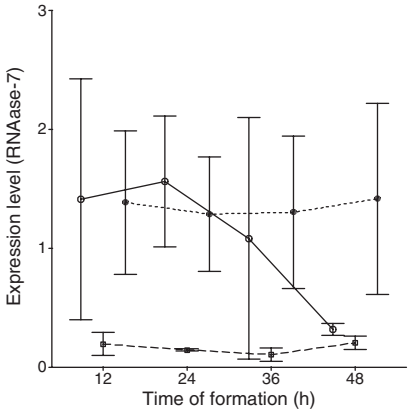

Fig. 2. The expression of RNAase-7 after stimulations with single-species biofilms of *Streptococcus mitis* (open square) or *Streptococcus mutans* (circle) and complex intraorally (filled circle) formed biofilms. The statistical analyses demonstrated statistically significant differences

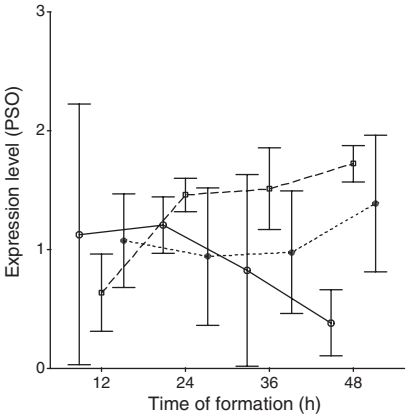

Fig. 3. The expression of the antimicrobial peptide psoriasin (PSO) after stimulations with single-species biofilms of *Streptococcus mitis* (square) or *Streptococcus mutans* (circle) and complex intraorally (filled circle) formed biofilms. The biofilms were grown for 12, 24, 36, and 48 h. The statistical analyses demonstrated no statistically significant differences between the three different biofilms ($P = 0.0001$) after 12, 24, 36, and 48 h biofilm growth. Graphs represent mean ± standard deviation for each time-point.

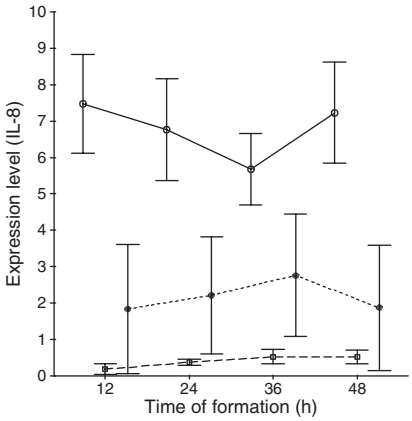

Fig. 4. The expression of the proinflammatory mediator interleukin-8 (IL-8) after stimulation with single-species biofilms of *Streptococcus mitis* (open square) or *Streptococcus mutans* (circle) and complex intraorally (filled circle) formed biofilms. The biofilms were grown for 12, 24, 36, and 48 h. The statistical analyses demonstrated statistically significant differences between the three different biofilms ($P = 0.0001$). Graphs represent mean ± standard deviation for each time-point.

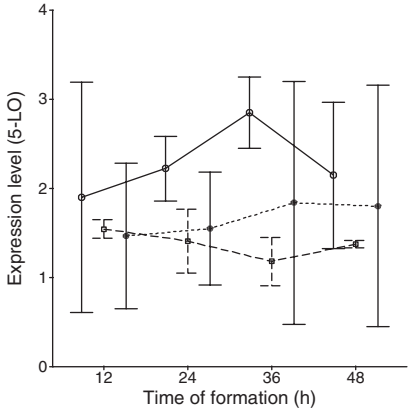

Fig. 5. The expression of the enzyme 5-lipoxygenase (5-LO) after stimulation with single-species biofilms of *Streptococcus mitis* (open square) or *Streptococcus mutans* (circle) and complex intraorally (filled circle) formed biofilms. No statistically significant differences were observed. Graphs represent mean ± standard deviation for each time-point.

Microbiological composition of biofilms

S. mitis was detected in all intraorally formed biofilms of the three subjects during the time period 12–48 h. In contrast, *S. mutans* was not detected as a component of the biofilms. For all three natural biofilms the mean number of *S. mitis* was 1.3×10^5, 1.4×10^5, 0.6×10^5, and 0.6×10^5 for the time periods 12, 24, 36, and 48 h, respectively. The quantitative analysis of the *in-vitro*-formed biofilms showed 1.8×10^5 CFU *S. mitis* and *S. mutans* for the different time periods.

Discussion

The present study aimed to investigate differences between naturally formed oral biofilm-induced and single-species biofilm-induced immune responses in gingival epithelial cells. Based on previous studies the mRNA expression of the antimicrobial peptides hBD-2, RNAase-7, and PSO, and of IL-8 and 5-LO was analysed (11).

It was of interest to show the influence of naturally formed biofilms on the gene expression of antimicrobial peptides (hBD-2, RNAase-7, and PSO) and proinflammatory mediators (IL-8 and 5-LO) in comparison with biofilm represented by *S. mitis* that is involved in early dental plaque formation or *S. mutans*, which is not present in the early plaque (31). *S. mutans* is a major etiological factor during caries development (23) and has therefore been chosen for stimulation experiments in this study. It was not intended to analyse the complex microbiological composition of the biofilms, but to show differences between host immune responses to single-species vs. those to naturally formed biofilms. The composition of the biofilms used in this study was reported in previous publications and was similar to the biofilm formation on tooth surfaces, indicating the validity of the experimental approach including the use of polymer disks. To facilitate equal biofilm formation for individual experiments the polymer disks were placed intraorally at the dorsum of the palate in each patient. The microbiological analysis demonstrated that *S. mitis* was indeed present in the naturally formed biofilms (from 12 to 48 h), while *S. mutans* could not be detected. These results reflect previous reports on oral biofilm composition (31). The biofilms were separated during stimulation from the epithelial cell layer by a distance of 500 μm, because recent studies have shown that molecules that are released from bacteria are most potent for the induction of epithelial cell immune reactions (26). The control experiments confirm that before stimulation any bacteria loosely attached to the biofilms were removed and that no bacteria were found in the stimulation medium after 2 h.

In the present study, the expression of antimicrobial peptides and inflammatory mediators by oral epithelial cells showed new aspects of the innate immune response towards different conditions of biofilm formation.

In stimulation experiments using *S. mutans* biofilms, the mRNA expression of the proinflammatory mediators IL-8 and 5-LO was induced, whereas the gene expression of the antimicrobial peptides hBD-2, RNAase-7, and PSO was poorly affected in gingival epithelial cells. In contrast, *S. mitis* biofilms showed clear stimulatory effects on the gene expression of hBD-2 and PSO, but no influence on the gene expression of both inflammatory mediators (IL-8 and 5-LO). These results may point to a mechanism of innate immunity that bacteria involved in early plaque formation are capable of inducing the synthesis of antimicrobial peptides, in contrast to bacteria that are not involved in early biofilm formation and therefore are not recognized as pathogenic by epithelial cells and in consequence did not stimulate the synthesis of antibacterial peptides. The results for stimulation experiments using naturally formed biofilms demonstrated mRNA expression levels for the antimicrobial peptides as well as the proinflammatory mediators ranging between levels gained from stimulation experiments using *S. mutans* and *S. mitis* biofilms. These results led us to the suggestion that a complex bacterial environment, such as the dental plaque, produces factors that enhance and/or reduce local host immune responses.

The findings in the present study also demonstrated that models using bacterial extracts or heat-killed organisms to study defensins or mediator induction may not adequately depict the interactions between keratinocytes and biofilms during the initiation of oral infections (12, 17, 19, 22, 36). For future research of bacteria and epithelial interactions, it might be critical to verify results from single-species biofilms with experiments from mixed-species biofilms. In the present study, however, only *in vitro* experiments were performed, and conclusions drawn from the results must be interpreted with caution. Although this study showed distinct differences in the immune responses of oral keratinocytes when stimulated with either single-species biofilm or naturally formed biofilm, it is very much expected that there may be differences between immune responses in tissue as opposed to cells in a monolayer culture.

In summary, the results of the present study showed highly variable immune responses to single-species biofilms and complex naturally formed biofilms in gingival epithelial cells. Both *S. mutans* and *S. mitis* had effects on the gene expression of antimicrobial peptides (hBD-2, RNAase-7, and PSO) and proinflammatory mediators (IL-8 and 5-LO), but in opposite ways. In gingival epithelial cells, stimulation experiments using single-species biofilms did not reflect immune responses demonstrated in experiments using naturally formed biofilms. Consequently, stimulation experiments using single-species biofilms or bacteria may not help to uncover complex host immune processes that occur in response to biofilms composed of multiple organisms, such as the dental plaque. The authors therefore propose to include naturally formed biofilms for studies on bacteria and epithelial cell interactions.

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