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ORAL MICROBIOLOGY
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The stage of native biofilm formation determines the gene expression of human β-defensin-2, psoriasin, ribonuclease 7 and inflammatory mediators: a novel approach for stimulation of keratinocytes with *in situ* formed biofilms

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Background/aims: Antimicrobial peptides such as human β-defensin-2 (hBD-2), psoriasin (PSO), and ribonuclease 7 (RNase 7) play an important role in innate immunity. The aim of the present study was to test the hypothesis that epithelial cells show a differential gene expression pattern of antimicrobial peptides (hBD-2, PSO, RNase 7) and inflammatory mediators such as interleukin-8 (IL-8) and 5-lipoxygenase (5-LO) in response to different stages of naturally formed biofilms.
Methods: Epithelial cells were cultured from biopsies obtained from five healthy individuals. Native bacterial biofilms were taken from the same subjects that donated the gingival biopsies. To obtain different stages of biofilm formation, polymer disks were attached to prostheses and carried intraorally for 1, 3, 5, and 9 days. The expression of genes for hBD-2, PSO, RNase 7, 5-LO, and IL-8 was examined using semi-quantitative reverse transcription–polymerase chain reaction. The bacterial composition of the individual biofilms was defined using a microarray system (Parocheck®), which showed the presence of 20 different bacterial species that are associated with plaque formation.
Results: The expression of the messenger RNAs of hBD-2, RNase 7, and 5-LO was upregulated as a result of the exposure to early biofilm stages, whereas the gene expression of IL-8 was increased in response to matured biofilms. Inter-individual differences in the innate immune response were observed.
Conclusion: The results of the present study showed a time-dependent messenger RNA expression of antimicrobial peptides (hBD-2, RNase 7), 5-LO, and IL-8 in oral epithelial cells responding to different stages of biofilm formation.

Key words: defensins; inflammatory mediator; innate immunity; keratinocytes; oral biofilm

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The oral cavity is in constant contact with bacteria but generally maintains a homeostasis that is associated with oral health. This phenomenon is partly promoted by the physical barrier of the oral epithelium as well as by saliva. However, no clinical signs of inflammation are noticeable in spite of the fact that some oral pathogenic bacteria are capable of dividing in the space of 20 minutes under optimal conditions. The low number of inflammatory cells, e.g. polymorphonuclear cells, in the oral tissues is probably not sufficient to maintain healthy conditions. Thus, it has been hypothesized that human epithelial cells express a chemical barrier provided by antimicrobial peptides/proteins, which is commonly present in plants and insects. This chemical barrier represented by antimicrobial peptides may vary between individuals based on genetically determined differences in DNA copy numbers (23). These interindividual differences led to the consideration that defensins might play a crucial role in the development of severe diseases such as colonic Crohn's disease (9) and potentially also in chronic inflammatory diseases of the oral cavity.

In mammals, this chemical defense shield is composed of antimicrobial peptides including the α-defensins of the intestinal epithelium and the β-defensins of the skin and mucosal epithelia. In humans, β-defensins, such as hBD-1 and hBD-2, have been identified in various epithelial tissues (3, 11, 22, 33). Ribonuclease 7 (RNase 7) and psoriasin (PSO), a member of the S100 protein super family, have been recently discovered as new epithelial antimicrobial peptides (12). While hBD-1 shows constitutive expression in epithelial cells, hBD-2 expression is induced in oral keratinocytes *in vitro* by specific microorganisms as well by pro-inflammatory cytokines (interleukin-1, tumor necrosis factor-α, and interferon-γ). The hBD-2 is also upregulated in inflamed epithelial tissues (24). Expression of RNase-7 is inducible by pro-inflammatory cytokines such as interleukin-1β and interferon-γ, as well as by tumor necrosis factor-α, and PSO is induced by *Escherichia coli* culture supernatants (12).

The exact role and physiological function of each antimicrobial peptide is not yet apparent. Previous models utilizing bacterial extracts and/or heat-killed organisms to study defensin induction may not adequately depict the interactions between keratinocytes and viable bacteria during the initiation of oral and other common infections. On various epithelial surfaces, as well as in the oral cavity, structured bacterial biofilms can be pathogenic and can induce the chemical defense mechanisms of epithelial cells. Biofilms consist of multiple different microorganisms living in a structured ecosystem that protects the bacteria from the microbicidal host defense system. The release of pathogenic factors by such structured ecosystems is dependent on time- and population-controlled mechanisms. These complex biofilms have not yet been modeled to study the interactions between films and epithelial cells *in vitro*.

The aim of the present study was to test the specific 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. Therefore, 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. Early phases of plaque formation were used to examine the induction of inflammatory processes rather than subgingival biofilms that are associated with established inflammatory reactions. Furthermore, we presume that there are genetically determined interindividual epithelial immune responses as well as microbiological flora inhabiting each oral cavity.

To address this question, we developed a new model of *in vivo*-formed biofilm and applied it *in vitro* to investigate early local immune responses of gingival epithelial cells.

Materials and methods

Cell culture

Gingival biopsy specimens were surgically removed from five young healthy patients who underwent third molar extraction at the Department of Operative Dentistry and Periodontology, University Hospital Kiel. The subjects showed no clinical signs of gingival or periodontal inflammation and no radiographic signs of bone loss. The local Ethics Committee of the University of Kiel had approved the study and the subjects had signed a letter of informed consent.

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). Second-passage to fifth-passage cultures were used for experimental studies after they had reached approximately 80% confluence. The cultures were routinely screened for the messenger RNA (mRNA) expression of keratin 13 to confirm the epithelial character of the cells.

Biofilm formation

Bacterial biofilms were grown in the same subjects that donated the epithelial biopsies. For this purpose, individual intraoral support prostheses carrying two polymer disks were manufactured. For every subject, the disks were placed at the same location within the oral cavity at the palate. Polycarbonate polymer disks were used because preliminary experiments have shown sufficient biofilm formation on these surfaces. The polymer disks were ground (4000 grit) and sterilized before the beginning of the experiment. The prostheses with the polymer disks were placed intraorally for 1, 3, 5, and 9 days for the formation of biofilms approximately 4 weeks after surgery to be certain that the extraction wounds were completely healed. The polymer disks were gently removed from the support prosthesis without affecting the surface of the native biofilm. The disks containing the biofilm were washed gently with 500 μl phosphate-buffered saline (PBS) to remove any loosely adherent bacteria and saliva and were placed at a distance of 500 μm above the primary epithelial cell cultures for 2 h in antibiotic-free medium. For control purposes the PBS used for washing the biofilms was collected and 300 μl was used to stimulate epithelial cell cultures in a total volume of 3 ml. In addition, the washing liquid was tested by polymerase chain reaction (PCR) for the mRNA of the investigated defensin and inflammatory mediators. 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 (Biochrom, Berlin, Germany) 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 duplicate.

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 (RT) PCR was conducted to

Table 1. Primer sequences and annealing temperatures

Primer	Sequence								Annealing temp (°C)
GAPDH (5′)	ATG	AGC	CCC	AGC	CTT	CTC	CAT		60
GAPDH (3′)	CCA	GCC	GAG	CCA	CAT	CGC	TC		
hBD-2 (5′)	ATC	AGC	CAT	GAG	GGT	CTT	GT		62
hBD-2 (3′)	GAG	ACC	ACA	GGT	GCC	AAT	TT		
IL-8 (5′)	CTT	TCA	GAG	ACA	GCA	GAG	CAC		60
IL-8 (3′)	ACT	GTG	AGG	TAA	GAT	GGT	GGC		
PSO (5′)	TTC	TTC	TAC	TCG	TGA	CGC	TTC		57
PSO (3′)	CTC	TGC	TTG	TTG	TAG	TCT	GTG		
RNase 7 (5′)	GGA	GTC	ACA	GCA	CGA	AGA	CCA		60
RNase 7 (3′)	CAT	GGC	TGA	GTT	GCA	TGC	TTG	A	
5-LO (5′)	ACC	TGC	ACC	TGA	ATG	ACT	ACT	G	62
5-LO (3′)	CTA	CCT	CCT	TCA	GTT	CTG	CTC	T	

analyze mRNA expression semi-quantitatively for hBD-2, RNase 7, PSO, interleukin-8 (IL-8), 5-lipoxygenase (5-LO) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; a housekeeping gene) using previously described protocols (25). RNA was reverse transcribed using oligo-(dT)-primers (Invitrogen). The complementary DNA was amplified in a total volume of 25 µl PCR mixture containing 1 µl complementary DNA, 10× PCR buffer, 1.5 mM $MgCl_2$, 10 mM dNTP mix, 250 nM of both forward and reverse primer, and 2.5 U of *Taq*DNA polymerase (Invitrogen). The primers represented specific sequences for hBD-2, RNase 7, PSO, IL-8, 5-LO and GAPDH. The specific sequences and annealing temperatures of the oligo-nucleotide primers are summarized in Table 1. 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, followed by a second 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. PCR conditions for semi-quantitative determination were optimized for each gene investigated. The conditions for each data set 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. Each set of amplifications always included a no-sample negative control (water) and a positive control with cloned target sequences.

The PCR products were loaded on to ethidium-bromide-stained, 1.5% agarose gels. A 1 kb DNA ladder molecular weight marker (Gibco Life Technologies, Karlsruhe, Germany) was run on every gel 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 with the program IMAGE J (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 significances using the Kruskal–Wallis test followed by the Wilcoxon test for signed differences. Differences with $P \leq 0.05$ were considered significant.

Microbiological analysis

The detection of the biofilm composition was performed for every subject and every time point in duplicate using a commercially available microarray system (ParoCheck®; Greiner Bio-One GmbH, Frickenhausen, Germany), which allows the simultaneous detection of up to 20 different oral bacterial species based on species-specific highly conserved regions from the 16S rRNA gene. The ParoCheck® chip is a coated glass slide with a total of 86 DNA measuring points, which can be evaluated by all commercially available microarray scanners.

In this study, samples were assayed in a 20-µl reaction mixture containing 1 µl template, 0.2 µl *Taq* DNA polymerase and 18.8 µl Master Mix supplied with the Parocheck® kit (containing buffers, $MgCl_2$, dNTPs, DNase-free water and fluorophore-labeled primers). PCR was performed to amplify the target sequences and the cycling conditions used were as follows: 94°C for 1 min followed by 45 cycles at 95°C for 20 s, annealing at 60°C for 20 s, 72°C for 30 s and final elongation at 72°C for 1 min. Next, the labeled amplified products were hybridized to pathogen-specific oligomers according to the manufacturer's instructions. This step was first performed at 60°C in a steam-saturated atmosphere for 5 min. Next, 30 µl hybridization buffer was mixed with 5 µl PCR product at room temperature and incubated for 2 min at 95°C using a heating block. Then, 25 µl hybridization mix was transferred into each well of the chip and incubated for 10 min at 60°C. After washing and then drying using an air spray, the chip was read using a scanner (Axon 4100 A; Axon Instruments Inc., Union City, CA) and the software PARO-REPORT 20. The bacterial counts were semi-quantitatively analyzed on a graduated scale ranging from 0 to 4 according to the dot intensity measured and were calibrated to serial dilutions of the relevant microorganisms. Median counts were calculated for all five subjects for each bacterium for each period of biofilm formation. As an additional control experiment, polymer disks containing the freshly formed biofilms were placed in culture medium for 2 h. Afterwards the medium was analyzed according to the described protocol for the detection of bacteria. This procedure was performed to determine whether bacteria might detach from the biofilm and so directly stimulate the epithelial cells.

Results

Expression of hBD-2 in oral epithelial cells co-incubated with intraorally formed biofilms

To examine the expression of hBD-2 mRNA *in vitro*, human epithelial cells were isolated from gingival biopsies and challenged with intraorally formed biofilms derived from the same subjects. The epithelial character of the investigated epithelial cells was confirmed by the detection of keratin 13 mRNA using RT-PCR. The biofilms were grown for 1, 3, 5, and 9 days within the oral cavity and attention was taken that these time periods were accurately maintained. Total mRNA was extracted and analyzed 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. The GAPDH gene is a housekeeping gene control included to show equivalent loading of samples under all conditions and to standardize for the semi-quantitative approach. The size of the amplified products was as predicted. For

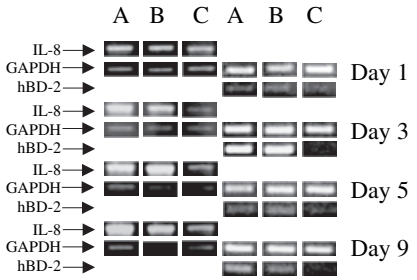

Fig. 1. Comparison of hBD-2 and IL-8 induction by native oral biofilms of an individual subject. In this example IL-8 is upregulated in oral keratinocytes by biofilms that were grown for 5 and 9 days intraorally. The human defensin hBD-2 is upregulated by biofilms that were grown for 3 days in the oral cavity. The results shown are representative of five different subjects. Lanes A and B, stimulations in duplicate; lane C, control stimulation.

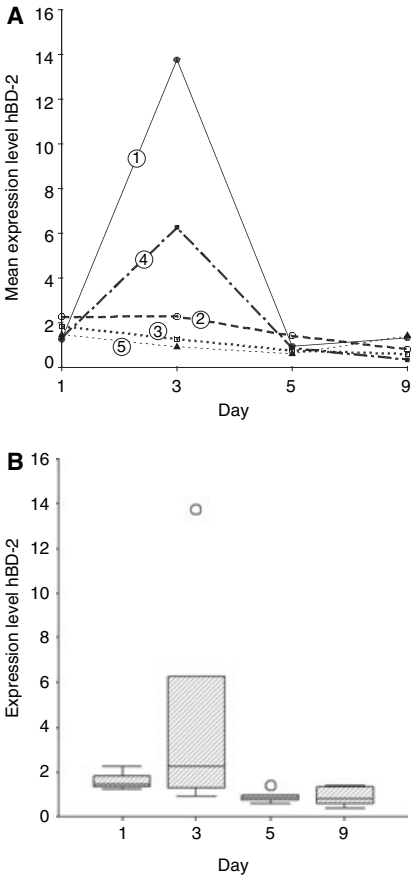

Fig. 2. (A) Individual hBD-2 expression in subjects 1 to 5 upregulated by biofilms that were grown for 1, 3, 5, and 9 days intraorally. Subjects 1 and 4 showed a significant induction of mRNA expression by 3-day-old biofilms. The numbers in circles indicate individuals 1, 2, 3, 4, and 5. (B) Box-plot of median values and range for hBD-2 expression in oral keratinocytes; 'o' indicates outliers.

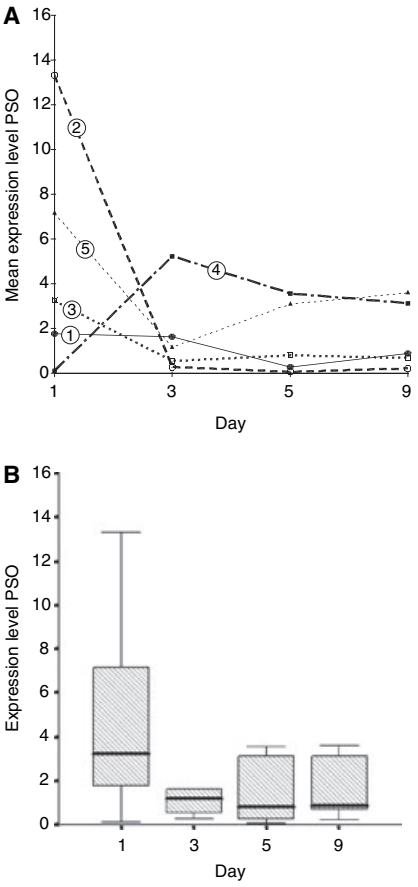

Fig. 3. (A) The antimicrobial peptide PSO is upregulated in subjects 1, 2, 3, and 4 after stimulation with biofilms that were grown for 1 day intraorally. The numbers in circles indicate individuals 1, 2, 3, 4, and 5. (B) The box-plots of PSO expressions show an overlap of the 75% quartile for days 1 to 9.

each subject the unstimulated controls did not show any statistically significant differences regarding the expression of antimicrobial peptides or inflammatory mediators. Control unstimulated gingival epithelial cells did express low basal amounts of hBD-2 mRNA (Fig. 1). Control experiments using PBS from the washing procedure of the biofilms did not show any effect on the mRNA expression of the genes tested. In addition, the solution was not capable of inducing any mRNA expression in epithelial cell cultures above the levels of control experiments.

Individual responses of hBD-2 expression after stimulation are presented in Fig. 2A. The expression of hBD-2 was induced by a factor of 2.3–13.8 after stimulation with 3-day-old plaque for subjects 1 and 4. For subjects 2, 3, and 5 no hBD-2 mRNA induction was observed. For all five subjects a median factor of induction of 1.5 and a range of 1.02 was observed for 1-day plaque growth, which was statistically different from the expression level of hBD-2 after stimulation with biofilms that had aged for 5 days (0.87, range 0.08, $P = 0.032$) (Fig. 2B).

Expression of the newly characterized antimicrobial peptides RNase 7 and PSO in oral epithelial cells

To determine the expression of RNase 7 and PSO mRNA *in vitro* the same experiments were conducted as reported above with biofilms grown for 1, 3, 5, and 9 days within the oral cavity.

For the antimicrobial peptide PSO, individual responses are shown in Fig. 3A. Epithelial cells from subjects 2, 3, and 5 showed significant induction of PSO after stimulation with plaque formed after 1 day intraorally. Subject 4 showed an induction after stimulation with plaque formed after 3 days intraorally. The factor of PSO mRNA induction ranged from 3.2 to 13.3. Plaque matured for 1 day increased the expression of PSO in epithelial cells by a median factor of 3.2 with a range of 13.2. The box-plots illustrate the overlap between the four experimental groups (Fig. 3B).

Individual responses of RNase 7 expression after stimulation are presented in Fig 4A. Individuals 1, 2, and 3 showed a significant increase of RNase 7 expression after stimulation with plaque grown for 1 day ranging from 2.9 to 13.0 compared to the untreated controls. Subjects 4 and 5 did not significantly change the expression of RNase 7 for any tested biofilms. A median value (range) of induction compared to the untreated controls of 2.9 (11.9) was calculated for 1-day biofilms, which was statistically significant compared to the induction of mRNA expression with 3-day (0.85, range 1.16; $P = 0.032$), 5-day (0.90, range 0.69; $P = 0.008$) and 9-day (0.85, range 1.42; $P = 0.016$) biofilms (Fig. 4B).

Expression of IL-8 in oral epithelial cells treated with native biofilms

To determine the expression of IL-8 mRNA *in vitro*, the epithelial cells were challenged with intraorally formed biofilms of the same subjects that were donors for the epithelial cell cultures. The biofilms were grown for 1, 3, 5, and 9 days within the oral cavity. Total RNA was harvested and analyzed. Individual responses of IL-8 induction after stimulation are presented in

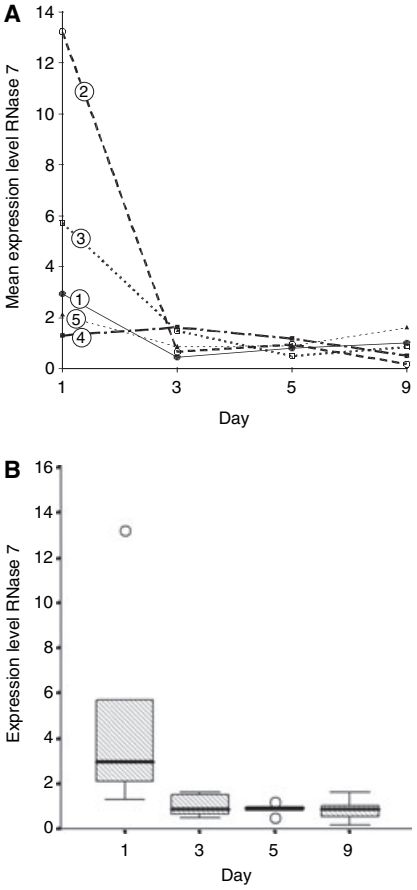

Fig. 4. (A) The newly recognized antimicrobial peptide RNase 7 was expressed in keratinocyte cultures of five subjects after challenge with native biofilms after 1 day of aging. The numbers in circles indicate individuals 1, 2, 3, 4, and 5. (B) The box-plot showed an elevated expression of RNase 7 at day 1 in comparison to days 3, 5, and 9; 'o' indicates outliers.

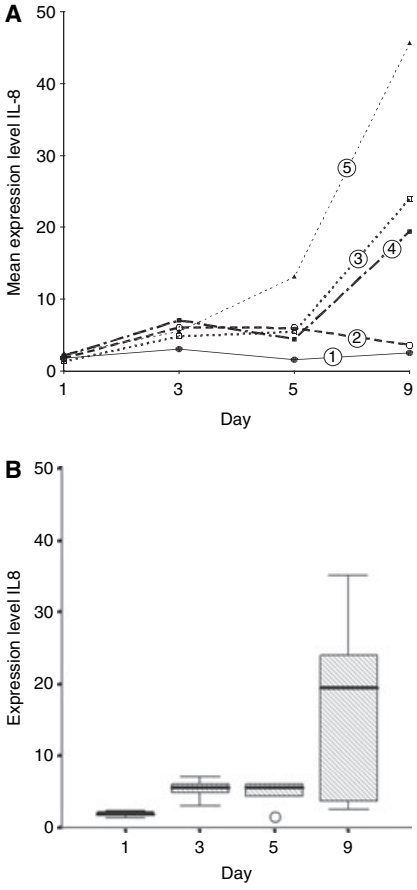

Fig. 5. (A) Individual expression of the pro-inflammatory mediator IL-8 after stimulation with native biofilms. Subjects 3, 4, and 5 showed a marked increase at day 9. The numbers in circles indicate individuals 1, 2, 3, 4, and 5. (B) The box-plot of the median IL-8 expression showed a significant increase of IL-8 mRNA at day 9 in comparison to day 1 ($P = 0.008$); 'o' indicates outliers.

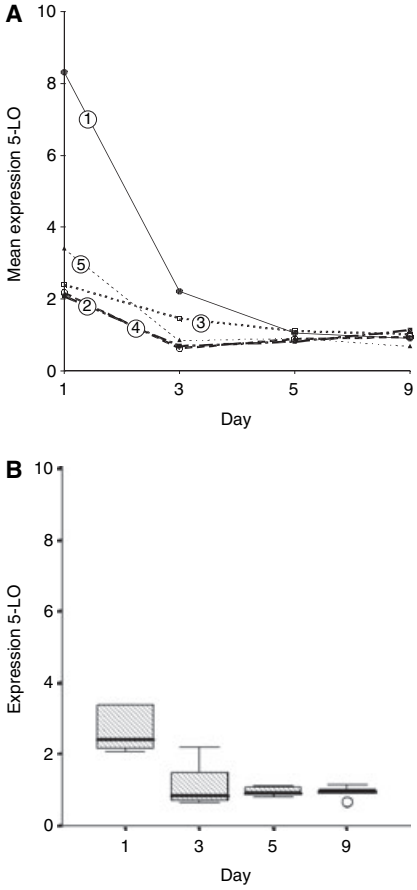

Fig. 6. (A) The individual expression of the 5-LO enzyme in oral keratinocytes. The numbers in circles indicate individuals 1, 2, 3, 4, and 5. (B) Median values and ranges are depicted in the box-plot. The expression of the enzyme was statistically different at day 1 compared to days 3, 5, and 9; 'o' indicates outliers.

Fig. 5A. Every individual epithelial cell culture expressed IL-8 mRNA after stimulation with biofilms ranging from 1.5 to 45.6 times compared to the unstimulated control. Individuals 3, 4, and 5 showed significantly elevated expression of IL-8 after stimulation with 9-day-old plaque in contrast to epithelial cells from subjects 1 and 2, which did not respond equally. A median and range of IL-8 mRNA expression level after stimulation of 1.8 (1.1) was observed for 1-day-old biofilms, of 5.5 (4.0) for 3-day-old biofilms, and of 5.5 (11.5) for 5-day-old biofilms. Biofilms that had matured for 9 days intraorally upregulated the expression of IL-8 mRNA in epithelial cells by a factor of 19.4 (43.1) compared to the unstimulated controls. The median expression of IL-8 was significantly different to stimulation with plaque grown for 1 day ($P = 0.008$) (Fig. 5B).

Expression of 5-LO in oral epithelial cells is upregulated by newly formed biofilms

It has been reported that components of the arachidonic acid cascade, the leukotrienes, synthesized in epithelial cells, are potent inducers of inflammation after bacterial challenge (8). The results of the present study confirm this and show the induction of a key enzyme for the leukotriene B_4 synthesis – 5-LO. Individual levels of induction are presented in Fig. 6A. All subjects showed increased induction of the enzyme after stimulation with 1-day-old plaque, superior to the stimulation caused by 3-, 5-, and 9-day-old plaque, ranging from factors of 2.1 to 8.3. No significant induction was observed after stimulation of epithelial cells with native plaque aged 3, 5 and 9 days. A median value and range of induction of 2.4 (6.2) was calculated for 5-LO expression in epithelial cells after stimulation with 1-day-old matured native plaque, which was statistically different from the median values calculated for stimulations with 3-day (0.84, range 1.59; $P = 0.032$), 5-day (0.91, range 0.30; $P = 0.008$) and 9-day (0.95, range 0.45, $P = 0.008$) plaque (Fig. 6B).

Stimulation of epithelial cells by native biofilms showed interindividual differences

We observed individual immune responses in epithelial cells from different donors. From Figs 2–6 it is obvious that subjects 3, 4, and 5 showed a very strong IL-8 mRNA response after stimulation with plaque biofilms that had been grown for 9 days. The same subjects showed a strong induction of hBD-2 (subject 4), RNase 7 (subject 3), and PSO (subject 5) after stimulation with plaque grown for 1 or 3 days. Subject 5 also showed an

increased 5-LO expression after stimulation with 1-day-old biofilms. In contrast, subjects 1 and 2 did not show increased expression of IL-8 at any time point, but responded with elevated expression levels for hBD-2 and 5-LO (subject 1) or PSO and RNase 7 (subject 2). From these data two response patterns can be deduced: One group of subjects showed defensin expression followed by the induction of the inflammatory mediator IL-8 induced by 9-day-old biofilms while the second group of subjects exclusively expressed defensins without extensive expression of IL-8.

Microbiological composition of native biofilms

For the detection of the biofilm composition the bacteria were harvested from the polymer disks and RNA was extracted for each experiment. Determination of microorganisms was conducted using a commercially available DNA test. On the basis of this test it was possible to discriminate 20 different species associated with early plaque formation from the disks. *Actinobacillus actinomycetemcomitans*, *Prevotella nigrescens*, *Prevotella intermedia* and *Porphyromonas gingivalis* were not found within the biofilms after 1 to 9 days of maturation. *Actinomyces viscosus*, *Tanerella forsythus*, *Campylobacter rectus*, *Peptostreptococcus micros*, *Campylobacter concius*, and *Eubacterium nodatum* were found infrequently on the disks and were not included in further analyses. It has to be considered that healthy subjects were included in the present study that did not show any signs of periodontitis and in consequence periodontitis-related bacteria were not expected on a regular basis as components of the biofilms. The composition of the biofilms is presented in Fig. 7. *Actinomyces odontolyticus*, *Veillonella parvula*, *Streptococcus gordonii*, *Streptococcus mitis*, *Capnocytophaga* sp., *Eikonella corrodens*, *Fusobacterium nucleatum*, and *Treponema denticola* were found on a regular basis as components of the bacterial biofilms. The bacterial composition of individual biofilms showed considerable differences that did not allow any correlation with the expression of defensins or inflammatory mediators. For example in subject 1 *A. odontolyticus* only became apparent in day-9 biofilms while this bacterium was highly abundant in subject 4 biofilms. In contrast, *Capnocytophaga* sp. was not detectable in subject 4, but was a prominent component of subject 1 biofilms. In addition, subjects 1 and 4 showed a significant increase of the total bacterial counts within the biofilms from days 1 to 9, in contrast to subjects 2, 3, and 5, which showed very high levels of bacterial counts even within day-1 biofilms. These results documented the variable growth conditions for biofilms within individuals. *Treponema denticola* was detectable in oral biofilms from day 1 to day 9 with no changes of quantity. A noticeable increase in counts was observed for *V. parvula*, *Capnocytophaga* sp., *E. corrodens*, and *F. nucleatum*. The total bacterial counts increased between the days 1 and 9 biofilms. However, it was not possible to find any correlation with the different expression level of antimicrobial peptides or inflammatory components expressed by the cultured human epithelial cells.

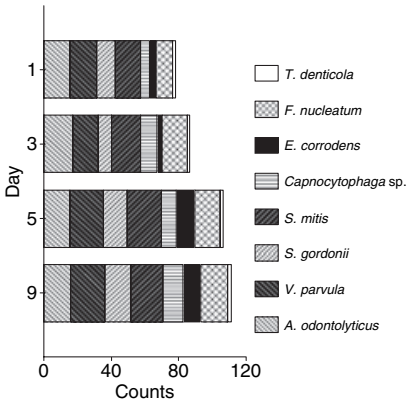

Fig. 7. The microbial composition of the biofilms from all five subjects is presented. Although, the bacterial test could discriminate 20 different species only eight were found on a regular basis on the polymer disks and were analyzed. The bacterial counts were semi-quantitatively analyzed on a graduation ranging from 0 to 4 according to the dot intensity. Median counts were calculated for each bacterium for each period of biofilm formation. The total bacterial counts of the biofilms increased from day 1 to day 9. A noticeable increase in counts from day 1 to day 9 was observed for *Veillonella parvula*, *Capnocytophaga* sp., *Eikonella corrodens*, and *Fusobacterium nucleatum*.

Discussion

The oral epithelium functions as a protective mechanical and chemical barrier against pathogenic microorganisms present in oral biofilms. In the present study, a new approach has been tested to study the physiological role of the innate immune system provided by oral epithelial cells, which are constantly exposed to commensal and pathogenic microorganisms. The oral epithelial cells and the growing biofilms are easy attainable and could be used for the characterization of individual inflammatory responses *in vitro*.

In the present study, the expression of antimicrobial peptides and inflammatory mediators showed several new characteristics of the innate immune response. First, 1-day-old and 3-day-old biofilms significantly induced the expression of the antimicrobial peptides hBD-2 and RNase 7 as well as the expression of the enzyme 5-LO. Second, 9-day-old matured biofilms significantly upregulated the expression of the inflammatory mediator IL-8 in epithelial cell cultures. These results verified the initial hypothesis that the innate immune response of epithelial cells depends on different growth stages of native biofilms. Third, the interindividual differences of epithelial cells in response to the challenge with oral biofilms were observed. While some individual epithelial cell cultures showed an increase in IL-8 mRNA synthesis after stimulation with 9-day-old biofilms, the remaining cell cultures did not respond with increased IL-8 mRNA to any of the biofilms used. Fourth, the microbiological characterization of different biofilms did not reveal specific microorganisms that could be related to the observed mRNA expression patterns suggesting that a general cellular immune response to oral biofilm components maintains the biological balance.

Previous models utilizing bacterial extracts and/or heat-killed organisms to study defensins or mediator induction may not adequately depict the interactions between keratinocytes and viable bacteria during the initiation of oral infections (10, 13, 21, 24, 35). The present study used naturally formed biofilms for the stimulation of oral epithelial cells and the consequent characterization of defensin and inflammatory mediator expression. The biofilms were separated from the epithelial cell layer during stimulation by a distance of approximately 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 (27). Other experimental approaches for imitating *in vivo* conditions used organotypic epidermal models (1, 20, 24). It is of particular interest that in the present study biofilms were used for the stimulation of epithelial cells that correspond to supragingival plaque from 1 to 9 days old. That is relevant for studies of early immune response in contrast to the use of subgingival plaque. As a result of the ongoing inflammatory process in the gingival tissues, consequent changes of the conditions within the gingival sulcus

facilitated the formation of subgingival plaque that was different from supragingival plaque. The localized expression of hBD-2 in gingival epithelial cells and not in the junctional epithelium (2) supports the hypothesis that antimicrobial peptides are secreted for defense against supragingival plaque and not subgingival biofilms.

This experimental approach enabled the first demonstration of the mRNA expression of the antimicrobial peptides PSO and RNase 7 in oral epithelial cells. PSO shows antibacterial activity against *E. coli* and is a major component of defensins derived from psoriatic scales (12). RNase 7 also demonstrates antibacterial activity against *E. coli*, but in contrast to PSO it is highly abundant in healthy skin (15). For PSO no differences were found between different biofilms, but RNase 7 showed high median expression levels after stimulation with day 1 biofilms compared with 3-, 5-, and 9-day-old biofilms. This indicates that RNase 7 may be especially relevant for the defense of early plaque biofilms in the oral cavity. RNase 7 exhibits broad-spectrum antimicrobial activity against gram-negative bacteria (*Pseudomonas aeruginosa* and *E. coli*), gram-positive bacteria (*Staphylococcus aureus* and *Propionibacterium acnes*), and the yeast *Candida albicans*. RNase 7 represents on a per-molar basis the most potent human antimicrobial protein known (14). The biological role of both PSO and RNase 7 antimicrobial peptides in the oral cavity remains to be determined; however, only a concerted induction of all the antimicrobial peptides observed in this study may be capable of a cytoprotective function in the oral cavity.

The observed lack of defensin induction by aging biofilms could be a pathogenic mechanism aimed at overwhelming the chemical defense shield by the inhibition of defensin synthesis by molecules released from the growing biofilms. Dinulos et al. (4) investigated the hBD-2 expression in keratinocytes challenged by the skin pathogen *Streptococcus pyogenes* and concluded that this may be important for its ability to evade innate defenses and cause disease, as it is highly sensitive to killing by hBD-2. It was also demonstrated that *S. mitis*, a major endogenous bacterium in the oral microflora, might possess a certain resistance mechanism against hBD-2 (28). On the other hand, strong antimicrobial activity of hBD-2 has been shown against early aerobic colonizers such as *Streptococcus sanguis*, *Actinomyces naeslundii*, and *Actinomyces israelii* (17). The hypothesis of a downregulation of the chemical defense shield by pathogenic biofilms is partially contradicted by *in vivo* studies showing that levels of hBD-2 expression were similar in healthy and inflamed oral tissues (5, 7, 21); however, the cellular source of hBD-2 was not specified in these studies.

Remarkably, the epithelial cells derived from three healthy donors showed high levels of IL-8 mRNA in response to their own individual flora in the matured biofilm (9 days old) contrasted with two individuals that did not increase IL-8 mRNA synthesis. These observations agree with those of other reports on interindividual variations of defensin and inflammatory mediator expression profiles in epithelial cells (1, 18). Genetic variabilities may account for these differences (16, 19). It may be possible that the IL-8 mRNA was increased in response to invasive bacteria released from the biofilms. However, washing the biofilms with PBS before stimulation removed any loosely attached bacteria and controls revealed that no bacteria were found in the stimulation medium after 2 h. The experiments suggested that epithelial cells provide specific genetic profiles of innate immune response elements. From a biological point of view it could be suggested that mucosal and gingival health is best maintained in individuals presenting high basal expression levels of the defensins but low levels of inflammatory mediators. Genetically determined synthesis of IL-8 enhanced by matured biofilm may lead to pronounced inflammatory reactions in these individuals and to chronic diseases like gingivitis or periodontitis. This may put individuals at higher risk for certain inflammatory diseases in that there is a variable ability to respond to an infection or foreign insult. Increased susceptibility to recurrent infections because of genetic defects is known for human disorders and may also be relevant for interindividual differences in the presence of epithelial antimicrobial peptides and inflammatory mediators in oral diseases.

The bacterial composition of the biofilms observed in the present study correlated with *de novo* plaque formation on natural teeth (31, 32), indicating the validity of the experimental approach and the use of polymer disks. Furthermore, the bacteria detected in this study are associated with the red to green complex as described by Socransky et al. (30). Several studies have shown that bacterial adhesion is related to surface roughness and, to a lesser extent, to material characteristics like the surface free energy (30, 34). Significantly different biofilms with various polymer materials were not expected. We used a semi-quantitative 16S rRNA approach to analyze the composition of bacterial biofilms, detecting viable and dead bacteria. In contrast to bacterial culture analysis of biofilm components, this method detects all relevant changes within the biofilms, irrespective of the viability of the microorganisms. To facilitate equal biofilm formation for individual experiments the polymer disks were placed intraorally at the dorsum of the palate in every patient. In agreement with multiple abortive attempts to correlate specific microorganisms with oral infection, the microbiological composition of the biofilms showed no correlation to the observed expression profiles for defensins or IL-8. With respect to the results of the present study, in the future it will be more important to characterize the time-dependent release of pathogen-associated molecules in biofilms rather than the bacterial composition.

Various links have been revealed referring to interactions between the innate and the acquired immune systems, including antimicrobial peptides and regulatory enzymes of the arachidonic acid cascade (8, 26). Epithelial cells upon induction secrete both IL-8 and leukotriene B_4 in response to *A. actinomycetemcomitans* (8). In contrast to IL-8, the enzyme 5-LO of the arachidonic acid cascade was upregulated by short-time growth of biofilms. Interactions between metabolites of the arachidonic acid cascade and of defensin synthesis have been reported (6, 29); however, the exact nature of the regulatory connections between these metabolites and defensin synthesis remains to be determined.

In summary, the results of the present study confirmed our hypothesis that mRNA expression of antimicrobial peptides (hBD-2, RNase 7), 5-LO, and IL-8 depends on different stages of native biofilm formation. In addition, this is the first report of inducible expression of RNase 7 mRNA in oral epithelial cells and their potential cytoprotective function in the oral cavity. The reluctance of matured biofilms to induce the release of defensins and the consequent activation of the synthesis of IL-8 mRNA might be a mechanism by which mature biofilms alter the epithelial chemical defense shield provided by antimicrobial peptides. Both the chemical defense and the inflammatory reaction showed interindividual differences that may reflect the susceptibility of individuals to develop chronic inflammatory diseases.

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