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Epithelial Interleukin-8 Responses to Oral Bacterial Biofilms[▽]

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An *in vitro* model of bacterial biofilms on rigid gas-permeable contact lenses (RGPLs) was developed to challenge oral epithelial cells. This novel model provided seminal data on oral biofilm-host cell interactions, and with selected bacteria, the biofilms were more effective than their planktonic counterparts at stimulating host cell responses.

The organization of multispecies biofilms found in the supra- and subgingival regions in the oral cavity demonstrates structural features reflecting the pattern of accretion of individual species (12). These patterns are likely related to interrelationships of species in the multicellular structures that presumably reflect both specific cognate interactions leading to coaggregation (19) and symbiotic nutritional partnerships (5). Responses of oral host cells, e.g., epithelial cells, fibroblasts, lymphocytes, macrophages, and osteoclasts, following challenge with oral bacterial species have been routinely studied with planktonic bacteria and carried out under aerobic conditions (10). However, it is clear that the apical aspect of the subgingival sulcus at sites of periodontal lesions is characterized by an anaerobic microenvironment (3). Still remaining ill defined are the patterns of host cell responses that occur when biofilms interface with these host cells in this type of altered local environment. This report describes a model system using rigid gas-permeable contact lenses (RGPLs) to create bacterial biofilms and the use of this model in examining the production of a biomarker of host inflammatory responses, interleukin 8 (IL-8), by epithelial cells.

Porphyromonas gingivalis FDC381, *Fusobacterium nucleatum* ATCC 25586, *Actinomyces naeslundii* ATCC 49840, *Streptococcus sanguinis* ATCC 10556, *Streptococcus oralis* ATCC 10557, and *Streptococcus gordonii* ATCC 10558, representing common members of the subgingival plaque (17), were cultured in broth under anaerobic conditions. Biofilms were grown on 10-mm-diameter RGPLs (Advanced Vision Technologies, Golden, CO) precoated with 1% fetal bovine serum (FBS) (Invitrogen) (13) by adding 5 ml of planktonic culture at an optical density (OD) A_{600} of 0.3 in a single well of a 6-well polystyrene tissue culture plate (BD Falcon, Franklin Lakes, NJ). Biofilms were grown for 3 days in an anaerobic chamber, where spent medium was replaced by fresh medium each day. For the first time, this RGPL/adherent biofilm model was used in host cell challenge studies, allowing us to evaluate the response of epithelial cells challenged with either biofilm or planktonic cells

under anaerobic conditions (1, 6). IL-8 was initially targeted for assessment in this model due to its significant elevations in inflamed gingival tissues and gingival crevicular fluid (4) in periodontitis and its critical role as a chemoattractant for polymorphonuclear leukocytes, a hallmark of periodontal inflammation (16).

An immortalized epithelial cell line, OKF4 (14), was cultured in keratinocyte serum-free medium (KSM) (Invitrogen, Carlsbad, CA) and seeded into 48-well tissue culture plates (Costar, Cambridge, MA) at a density of 10^5 cells per well in a 1-ml volume and allowed to adhere overnight in a 5% CO_2 chamber at 37°C (aerobic conditions) to form a confluent monolayer. The RGPL was overlaid on the adherent OKF4 cells and maintained under anaerobic and aerobic conditions for a further 24 h. The metabolic activity assessed using the cell proliferation reagent WST-1 (Roche Diagnostics Corp., Indianapolis, IN) demonstrated a negligible effect of anaerobic conditions and cultivation under RGPLs on the metabolism of this substrate (Fig. 1). RNA was extracted, using a PicoPure RNA isolation kit (Arcturus Bioscience, California), from OKF4 cells that were lysed (Purelink RNA minikit; Invitrogen, California) for 15 min and stored at −80°C. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH), human β-actin, RPL19F, and 18S (Integrated DNA Technologies, Indiana, and Operon, Alabama) housekeeping genes (Table 1) were analyzed using real-time PCR (11). The mean crossing point value of duplicate determinations for GAPDH (mean ± standard deviation [SD], 17.42 ± 1.12 and 16.77 ± 0.74 under aerobic and anaerobic conditions, respectively), for β-actin (17.07 ± 1.73 and 17.32 ± 1.04), for RPL19F (18.65 ± 1.53 and 18.16 ± 0.44), and for 18S (17.37 ± 1.92 and 16.70 ± 0.58) were determined. Thus, all 4 housekeeping genes were expressed similarly under both aerobic and anaerobic growth conditions.

Planktonic bacteria, biofilms, and control challenges were each carried out in 6 wells of a 48-well plate containing fresh cell culture medium and incubated for 6 h under anaerobic conditions. Planktonic bacterial challenge (10^8, 10^7, and 10^6 cells/well) corresponding to a multiplicity of infection (MOI) at 1,000:1, 100:1, and 10:1 and 3-day-old biofilms grown on and adherent to the RGPLs were used to stimulate the OKF4 cells for 6 h. We estimated that an MOI of 10:1 to 50:1 bacterial cells in the biofilms were in contact with the epithelial cells (13). Both planktonic cells and biofilms were washed twice to remove metabolic by-products and incubated in KSM medium for challenge of the epithelial cells. OKF4 cells with or without

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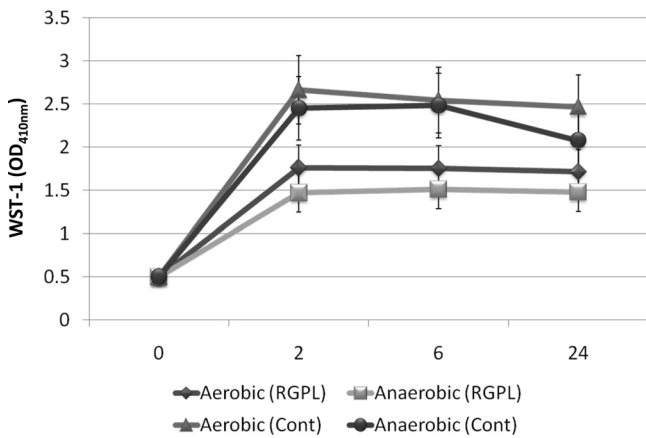
FIG. 1. Metabolic activity of OKF4 epithelial cells under aerobic (5% CO_2 and air) and anaerobic (85% N_2, 5% CO_2, and 10% H_2) conditions. At each time point, the mean and standard deviation of triplicate determinations of WST-1 colorimetric change in OKF4 cells alone (control) or OKF4 cells cultivated under RGPLs is shown.

overlaid RGPLs (minus biofilms) and phorbol 12-myristate 13-acetate (PMA) (10 nm)-treated OKF4 cells were used as controls. OKF4 cell supernatants from each of two wells were pooled and stored at −80°C for IL-8 determination.

Universal Probe Library (UPL) probes (Roche, Indiana) were used to enhance the specificity of gene expression for IL-8 determination (Table 1). Relative quantification analysis was performed with LightCycler software, version 4.0 (Roche, Indiana). The concentration ratios of the target genes were calculated by normalizing to the GAPDH housekeeping gene. Assessment of mRNA for IL-8 gene expression showed that with the exception of *P. gingivalis*, generally the biofilms of each species elicited significantly greater IL-8 mRNA levels than the planktonic bacteria of the same species (Table 2). *P. gingivalis* as a planktonic challenge did appear to elicit a message for this protein within the initial 6 h of challenge, while the biofilms appeared to downregulate (4-fold) the basal level of IL-8 message produced by the OKF4 cells. Additionally, among the 3 streptococcal species, only *S. gordonii* and *S. oralis* biofilms elicited IL-8 mRNA. Both *A. naeslundii* and *F. nu-*

cleatum appeared most active for IL-8 induction, with the greatest levels derived from *F. nucleatum* biofilm challenge.

The amount of IL-8 secreted into the supernatants of OKF4 cells was determined using an IL-8 enzyme-linked immunosorbent assay (ELISA) kit (R & D Systems, Minneapolis, MN). The levels were compared with those of unchallenged epithelial cells and RGPL-only-overlaid OKF4 cells, respectively, using the nonparametric Mann-Whitney U test (SigmaStat 3.5; Systat Corp., San Jose, CA). IL-8 was present in supernatants of the OKF4 cells following challenge with biofilms or planktonic forms of the 6 oral bacterial species (Fig. 2). Of the streptococcal species, only *S. gordonii* biofilm or planktonic cells induced measurable levels of secreted IL-8 above those for control or RGPL-treated cells at 6 h, consistent with the literature (7, 18). *P. gingivalis*, *Treponema denticola*, and *Tannerella forsythia* have been reported to suppress the production of this chemokine (7). Killing or protease treatments enhanced the capacity of *P. gingivalis* to elicit IL-8 (8, 9), although *P. gingivalis* did appear to induce a strong expression of IL-8 mRNA that was related to the capacity of the strain to adhere to and invade the epithelial cells (15). In this study, neither *P. gingivalis* biofilms nor planktonic challenge resulted in detectable IL-8 in the supernatants, as has been associated with gingipain degradation of the molecule (2). Both *A. naeslundii* and *F. nucleatum* biofilms elicited significant elevations in IL-8 compared to challenge with comparable levels of planktonic bacteria (10^6 to 10^7). However, the highest MOI challenge (1,000:1) with planktonic *A. naeslundii* induced significant elevations in IL-8 secretion compared to all other conditions. *F. nucleatum* routinely upregulates IL-8 mRNA and induced production of IL-8 by epithelial cells (7). We observed that the *F. nucleatum* biofilms elicited levels of IL-8 that were significantly greater than any level for planktonic challenge.

Various investigations of host responses to oral bacteria have targeted IL-8 product or message levels as a comparative biomarker of response capacity of epithelial cells to individual species or their components. Extensive literature has reported a range of responses from host cell types (e.g., epithelial cells, fibroblasts, and immune cells) when challenged *in vitro* with individual planktonic bacteria or components isolated from individual species (10). However, *in vivo*, it would be expected

TABLE 1. Primer sequences, melting temperatures, and amplicon sizes for the housekeeping genes and IL-8 gene

Probe	Gene or product	Primers[a]	T_m (°C)	Amplicon (bp)
SYBR green	GAPDH	F: 5′-AAGGTCATCCCTGAGCTGAA-3′	59.80	310
		R: 5′-TGCTGTAGCCAAATTCGTTG-3′	59.87	
	β-Actin	F: 5′-AGAGCTACGAGCTGCCTGAC-3′	59.96	300
		R: 5′-AGTACTTGCGCTCAGGAGGA-3′	60.16	
	RPL19	F: 5′-AGATCCGGAAGCTCATCAAA-3′	59.77	301
		R: 5′-TCCATGAGAATCCGCTTGTT-3′	60.60	
	18S	F: 5′-ATAGCCTTTGCCATCACTGC-3′	60.24	304
		R: 5′-AGAAGTGACGCAGCCCTCTA-3′	60.16	
UPL probe 9	GAPDH	F: 5′-GGAAGCTTGTCATCAATGGAA-3′	60	102
		R: 5′-TGGACTCCACGACGTACTCA-3′	60	
UPL probe 72	IL-8	F: 5′-AGACAGCAGAGCACACAAGC-3′	59	62
		R: 5′-ATGGTTCCTTCCGGTGGT-3′	60	

[a] F, forward; R, reverse.

TABLE 2. Expression of IL-8 mRNA following challenge of OKF4 cells for 6 h[a]

Condition	Fold change in expression with:						
	No challenge	*F. nucleatum*	*P. gingivalis*	*A. naeslundii*	*S. gordonii*	*S. oralis*	*S. sanguinis*
RGPL	1.47 ± 0.72						
Biofilm		11.97 ± 5.84	0.35 ± 0.11	5.88 ± 1.33	4.81 ± 2.43	4.91 ± 2.98	1.56 ± 0.64
Planktonic (cells/well)							
10^8		1.94 ± 0.88	2.56 ± 1.03	1.00 ± 0.29	0.98 ± 0.14	0.65 ± 0.30	0.39 ± 0.17
10^7		1.75 ± 0.80	1.82 ± 0.67	0.46 ± 0.06	1.39 ± 0.45	0.45 ± 0.24	0.48 ± 0.08
10^6		0.84 ± 0.32	1.01 ± 0.42	0.41 ± 0.03	2.56 ± 0.87	1.59 ± 0.74	0.56 ± 0.17

[a] The values represent mean fold change ± 1 SEM compared to results for cultures of untreated epithelial cells in triplicate determinations from 2 to 6 independent experiments.

that numerous host cells, particularly epithelial cells, interact with the oral bacteria growing as complex biofilms. Our findings clearly demonstrate that certain oral microbial species, as biofilms, significantly increase the induction of both IL-8 product and mRNA from oral epithelial cells. These findings provide a basis for utilization of this RGPL biofilm model system to explore multispecies biofilms (13) and their capacity to trigger a portfolio of host response molecules from epithelial cells. This model system can also be used to examine other host cell responses to mono- or multispecies biofilms targeting gene and product expression levels related to inflammation, innate immunity, and even adaptive immunity. The system is also amenable to evaluating the response of the bacteria to these interactions, either affected by direct host cell contact or in response to a milieu of host-derived factors that could alter the microbial physiology and/or cell communications, e.g., quorum sensing, between the various bacterial species. Moreover, the system will enable the assessment of how modifications within the biofilm-host cell environment could alter the host response profiles.
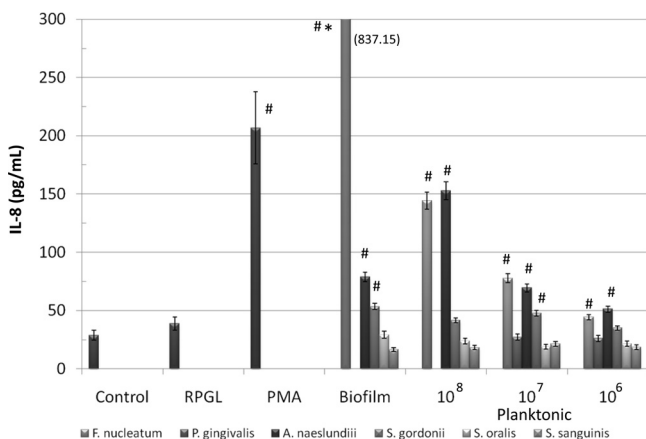
FIG. 2. IL-8 protein accumulation in OKF4 supernatants, determined by ELISA. Confluent OKF4 monolayers were stimulated continuously with monospecies biofilm or planktonic (10^8, 10^7, or 10^6, corresponding to an MOI of 100:1, 10:1, or 1:1, respectively) cultures of *S. gordonii*, *S. oralis*, *S. sanguinis*, *A. naeslundii*, *F. nucleatum*, and *P. gingivalis* under anaerobic (85% N_2, 5% CO_2, and 10% H_2) conditions for 6 h. OKF4 cells stimulated with or without RGPLs and PMA were used as controls. Data represent means ± standard errors of triplicate determinations. Statistically significant ($P < 0.05$) induction of IL-8 is indicated by "#," compared to RPGL treatment only (biofilms) or compared to the control (planktonic). The asterisk denotes significantly increased IL-8 levels compared to results for all planktonic challenge conditions ($P < 0.001$).

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