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# Interaction of oral bacteria with gingival epithelial cell multilayers

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## SUMMARY

**Primary gingival epithelial cells were cultured in multilayers as a model for the study of interactions with oral bacteria associated with health and periodontal disease. Multilayers maintained at an air–liquid interface in low-calcium medium displayed differentiation and cytokeratin properties characteristic of junctional epithelium. Multilayers were infected with fluorescently labeled *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum* or *Streptococcus gordonii*, and bacterial association was determined by confocal microscopy and quantitative image analysis. *Porphyromonas gingivalis* invaded intracellularly and spread from cell to cell; *A. actinomycetemcomitans* and *F. nucleatum* remained extracellular and showed intercellular movement through the multilayer; whereas *S. gordonii* remained extracellular and predominantly associated with the superficial cell layer. None of the bacterial species disrupted barrier function as measured by transepithelial electrical resistance. *P. gingivalis* did not elicit secretion of proinflammatory cytokines. However, *A. actinomycetemcomitans* and *S. gordonii* induced interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), IL-6 and IL-8 secretion; and *F. nucleatum* stimulated production of IL-1β and TNF-α. *Aggregatibacter actinomycetemcomitans*, *F. nucleatum* and *S. gordonii*, but not *P. gingivalis*, increased levels of apoptosis after 24 h infection. The results indicate that the organisms with pathogenic potential were able to traverse the epithelium, whereas the commensal bacteria did not. In addition, distinct host responses characterized the interaction between the junctional epithelium and oral bacteria.**

## INTRODUCTION

The human oral cavity is a complex ecosystem that contains a diverse assemblage of microorganisms with differing pathogenic potential. Streptococci such as *Streptococcus gordonii* are early colonizers of the dental plaque biofilm and generally are commensals in the oral cavity, although they are capable of causing disease at systemic sites such as on defective heart valves (Nobbs *et al.*, 2009). A predominant anaerobic species in the subgingival biofilm is *Fusobacterium nucleatum*, which is prevalent in mature plaque in both health and disease (Dzink *et al.*, 1988; Tanner & Bouldin, 1989; Moore & Moore, 1994) and is considered an opportunistic pathogen. The presence of *S. gordonii* and *F. nucleatum* favors subsequent colonization by more pathogenic organisms,

such as *Porphyromonas gingivalis*, which plays a role in the initiation and progression of chronic periodontitis (Lamont & Jenkinson, 1998; Kuboniwa & Lamont, 2010). Another pathogen is *Aggregatibacter actinomycetemcomitans*, a causal agent of the clinically distinct localized aggressive periodontitis (Kachlany, 2010). Nevertheless, many recognized pathogens are frequently present in healthy individuals, and disease ensues when there is a disruption of the normally balanced host–microbe interaction (Marsh, 2003; Handfield *et al.*, 2008).

The epithelial cells that line the gingival compartment are among the first host cells encountered by subgingival bacteria. In addition to providing a mechanical barrier to microbial intrusion, gingival epithelial cells (GECs) also produce effectors of innate immunity, such as cytokines, and act as sensors of infection by signalling to immune cells in the underlying periodontal tissues (Kagnoff & Eckmann, 1997; Tribble & Lamont, 2010). The interactions between bacteria and GECs have been studied extensively using epithelial cell monolayers maintained in culture. *Porphyromonas gingivalis* is highly invasive yet demonstrates stealth-like properties in primary cultures of GECs, suppressing production of cytokines such as interleukin-8 (IL-8) and preventing epithelial cell apoptosis (Lamont *et al.*, 1995; Darveau *et al.*, 1998; Mao *et al.*, 2007; Tribble & Lamont, 2010). In contrast, *A. actinomycetemcomitans* invades poorly but stimulates expression of cytokines and induces apoptotic cell death (Huang *et al.*, 1998; Handfield *et al.*, 2005; Guentsch *et al.*, 2010; Stathopoulou *et al.*, 2010). *Fusobacterium nucleatum* can invade epithelial cells and stimulate a proinflammatory response (Darveau *et al.*, 1998; Han *et al.*, 2000; Huang *et al.*, 2004; Stathopoulou *et al.*, 2010), whereas *S. gordonii* is essentially extracellular although capable of inducing inflammatory cytokines (Lamont *et al.*, 1995; Hasegawa *et al.*, 2007; Stathopoulou *et al.*, 2010).

Although monolayers of cells are a convenient and reproducible model for the study of host–bacteria interactions, a cell multilayer more closely resembles the *in vivo* situation. GECs can be grown in organotypic culture, and express differentiation and cytokeratin markers similar to those of junctional epithelial cells (Pan *et al.*, 1995; Papaioannou *et al.*, 1999; Rouabhia & Deslauriers, 2002). It has been shown that *P. gingivalis* adheres to, invades and penetrates through multilayers of GECs (Sandros *et al.*, 1994; Andrian *et al.*, 2004). However, the responses of epithelial cell multilayers to challenge with *P. gingivalis* or with other oral organisms have not been investigated. In this study we generated multilayers of GECs and characterized their responses to *P. gingivalis*, *A. actinomycetemcomitans*, *F. nucleatum* and *S. gordonii*. The results show that distinct profiles of multilayer interactions are exhibited by the bacteria, which may contribute to the pathogenic personalities that characterize each organism.

## METHODS

### Primary cell culture

Primary cultures of GECs were generated as described previously (Oda & Watson, 1990). Briefly, healthy gingival tissue was collected from patients undergoing surgery for removal of impacted third molars and following Institutional Review Board Guidelines. Basal epithelial cells were separated and cultured in flasks in keratinocyte growth medium (KGM; Cambrex, Rockland, ME) containing 0.06 mM calcium and in the absence of antibiotics at 37°C in 5% $CO_2$. At 80% confluence cells were removed by trypsination for multilayer culture. Polyester (PET) Membrane Transwell Clear Inserts (12-mm diameter, 0.4-μm pore size; Tissue Culture Treated, Corning 3450; Corning Inc., Corning, NY) were placed in 12-well culture dishes (Corning Inc.), and GECs were seeded onto the membranes at $2 \times 10^5$ cells per well. KGM was added to the membrane and in the well under the membrane insert, and the cells were cultured until a confluent monolayer was attained (approximately 1 week). Medium was then removed from the insert to produce an air–liquid interface and the cells were cultured to a three-layer multilayer (approximately 3 weeks) that contained approximately $9 \times 10^5$ cells (see supplementary material, Fig. S1).

### Bacteria and culture conditions

Bacteria were maintained as frozen stock cultures. Both *P. gingivalis* ATCC 33277 and *F. nucleatum* ATCC 25586 were cultured anaerobically at 37°C in trypticase soy broth (TSB) supplemented with yeast extract (1 mg $ml^{-1}$), hemin (5 μg $ml^{-1}$), and menadione (1 μg $ml^{-1}$). *Aggregatibacter actinomycetemcomitans* VT1169 was grown in TSB with yeast extract

(0.6 mg $ml^{-1}$) in 10% $CO_2$ at 37°C. *Streptococcus gordonii* DL-1 was cultured anaerobically at 37°C in TSB with yeast extract (5 mg $ml^{-1}$) and glucose (5 mg $ml^{-1}$).

### Bacterial challenge

Bacteria were stained with BacLight Green (Invitrogen, Carlsbad, CA) anaerobically at 37°C for 30 min, suspended in KGM and added to the top of the multilayers at a multiplicity of infection of 100. After incubation at 37°C in 5% $CO_2$, the multilayers were fixed with 4% paraformaldehyde for 20 min, washed with phosphate-buffered saline (PBS), and blocked overnight at 4°C in 10% normal goat serum in PBS. The cells were permeabilized for 20 min at room temperature with 0.2% saponin in PBS with 10% normal goat serum. Actin microfilaments were stained with Alexa 635 Phalloidin (Invitrogen) 1 : 200 for 30 min at room temperature. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) (1 μg $ml^{-1}$; Sigma-Aldrich, St Louis, MO). Membranes were excised from the insert and mounted on a glass slide with Vectashield (Vector Laboratories, Burlingame, CA). Images were acquired using a Leica DM IRM confocal microscope with MICRO-MANAGER software (Applied Scientific Instrumentation, Eugene, OR). Three optical slices at 10-μm intervals were collected and IMARIS software (V6; Bitplane, Zurich, Switzerland) was used to compile the optical slices into a three-dimensional volume view. The Ortho-Slicer function was used to 'slice' the volume compilation of images into three sections (representing the three cell layers). The Spot-Counter function was then used to detect and count the number of fluorescent signals in a specified channel (corresponding to individual bacterial cells) and also within a segmented area of the volume. Counting was performed in 20 random fields.

### Fluorescent antibody and annexin-V staining

The cells were fixed and permeabilized as above and allowed to react with primary antibodies for 1 h at room temperature. Antibodies used were: mouse anti-human cytokeratins 1/10, 5/6, 13 and 19, or mouse anti-human Toll-like receptors (TLR) 2 and 4 (Invitrogen), 1 : 1000. Antigen–antibody binding was detected with goat anti-mouse Alexa 555 secondary antibody (Invitrogen). For quantification of apoptosis, cells were stained with fluorescein isothiocyanate-Annexin-V and propidium iodide (PI) using the Apo*Target* kit (Invitrogen). Membranes were washed with PBS, excised from the insert, mounted on glass slides and images were acquired on a Leica DM IRM confocal microscope with MICRO-MANAGER software as described above.

### Transepithelial electrical resistance

Transepithelial electrical resistance was measured using the Millicell-ERS (Millipore, Billerica, MA) system. Membranes were submerged in PBS, electrodes were inserted above and below the membrane, and electrical resistance was measured. Background resistance from cell-free membranes was subtracted from initial resistance values.

### Cytokine measurements

Supernatants from the upper chambers of cell membranes were collected, centrifuged to remove any bacteria, and stored at −20°C until use. The supernatants were analysed in the Millipore MILLIPLEX Map kit Human Cytokine/Chemokine custom 7-Plex Multi-Cytokine Detection System for IL-1β, IL-10, IL-12(p40), IL-6, IL-8, monocyte chemoattractant protein 1 (MCP-1) and tumor necrosis factor-α (TNF-α). The Luminex 100 System was used to acquire the results and MILLIPLEX Analyst Software (VigeneTech, Carlisle, MA) was used to analyse the results.

### Statistical analyses

One-way analysis of variance multiple pairwise comparisons test with the Tukey or Bonferroni post tests were used to determine statistical significance.

## RESULTS

### Keratin expression by GEC multilayers

The GEC multilayers were probed with keratin antibodies and fluorescently labeled secondary antibodies. Keratin expression was examined by confocal microscopy. Cells in multilayers exhibited uniform strong expression of cytokeratins 13 and 19 (see Supplementary material, Figs S2 and S3), markers of junctional epithelium (Papaioannou *et al.*, 1999). In

addition, there was moderate expression of cytokeratin 5/6, a marker of basal epidermal cells. Expression of cytokeratins 1/10, markers of terminal differentiation, was not detected. These results indicate that the GEC multilayers provide a model that reflects important aspects of the basal layer of the junctional epithelium.

### Bacterial interactions with GEC multilayers

Multilayers were challenged with fluorescently labeled bacteria and the physical association examined by confocal microscopy. *Porphyromonas gingivalis* penetrated the multilayers both intercellularly and intracellularly (Fig. 1). After 2 h, approximately 80% of the total bacteria associated with the layers were present in the top layer. Over time, the organisms moved between the layers and after 24 h approximately 40% of the bacteria were located in the middle layer (Fig. 1 B). The majority of the *P. gingivalis* associated with the multilayers were intracellular as determined by co-localization with actin (Fig. 1C). In contrast, *A. actinomycetemcomitans*, *F. nucleatum* and *S. gordonii* penetrated to the lower epithelial layers but were not co-localized with actin, indicating that they were not found intracellularly. *Aggregatibacter actinomycetemcomitans* penetrated the multilayers rapidly (Fig. 2A), and after 2 h almost 40% of the bacteria had reached the middle layer (Fig. 2B). At 24 h, almost 30% of the bacteria had reached the bottom layer and the organisms were more evenly dispersed through the three layers (Fig. 2B). This contrasts with *P. gingivalis*, which did not penetrate to the basal layer during the experiment. *Fusobacterium nucleatum* spread through the three layers by 24 h (Fig. 3A), although at the 2 h time-point only 20% of the bacteria had reached the middle layer (Fig. 3B). *Streptococcus gordonii*, which also traversed the epithelial layers through an extracellular route, penetrated them slowly, and after 24 h 70% of the organisms remained in the superficial layer (Fig. 4).

### Transepithelial resistance

The GEC multilayers exhibited a stable transepithelial electrical resistance of 150 $\Omega \times cm^2$. There was no significant change in transepithelial resistance following challenge with any of the test species of bacteria over 24 h (Fig. 5). Several of the bacteria penetrated

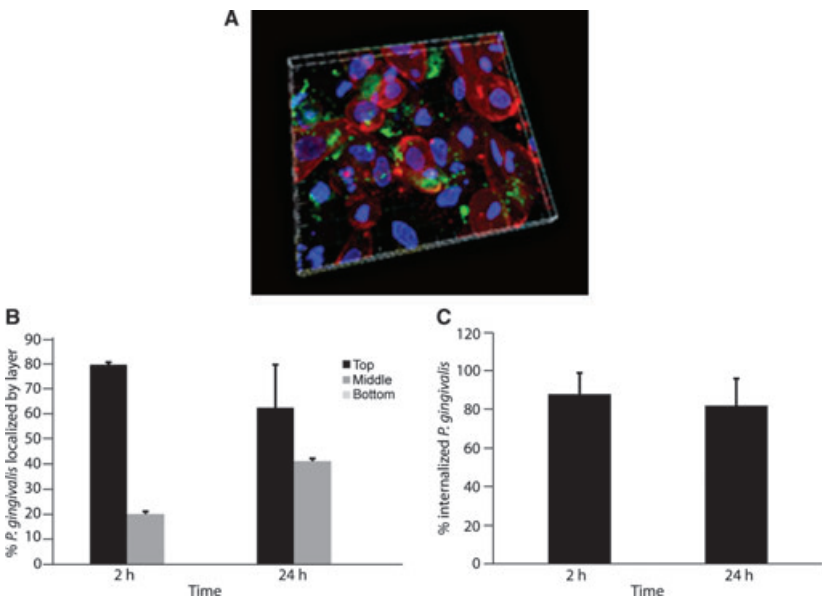


**Figure 1** Physical association between *Porphyromonas gingivalis* and gingival epithelial cell (GEC) multilayers. (A) Confocal microscopy of GECs stained with Alexa 635-Phalloidin (red) after infection for 24 h with *P. gingivalis* stained with Baclight Green. Nuclei are stained with DAPI (blue). A series of fluorescent optical 0.2-μm *x–y* sections were collected to create digitally reconstructed images with IMARIS software. (B) Percentage of *P. gingivalis* cells in the upper, middle or lower layers of the GEC multilayer. Layers were digitally separated using the Ortho-Slicer function of IMARIS and the Spot-Counter function was used to detect and count the number of bacterial fluorescent signals. (C) Percentage of total *P. gingivalis* that were internalized within all layers of the GECs at 2 and 24 h. Images are representative of three independent experiments. Data are means of three independent experiments in duplicate with standard deviation.

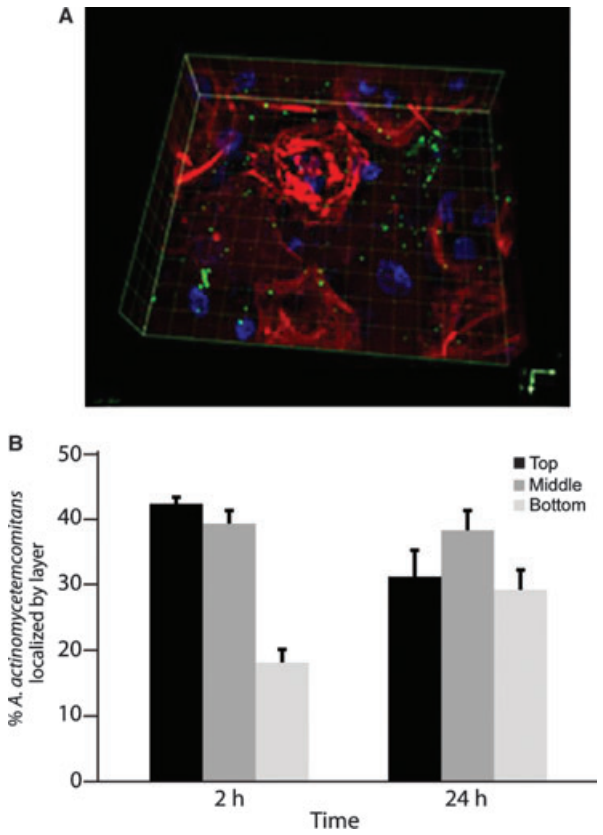


**Figure 2** Physical association between *Aggregatibacter actinomycetemcomitans* and gingival epithelial cell (GEC) multilayers. (A) Confocal microscopy of GECs stained with Alexa 635-Phalloidin (red) after infection for 24 h with *A. actinomycetemcomitans* stained with Baclight Green. Nuclei are stained with DAPI (blue). A series of fluorescent optical 0.2-μm *x–y* sections were collected to create digitally reconstructed images with IMARIS software. (B) Percentage of *A. actinomycetemcomitans* cells in the upper, middle or lower layers of the GEC multilayer. Bacteria were counted as described in the legend to Fig. 1. Images are representative of three independent experiments. Data are means of three independent experiments in duplicate with standard deviation.

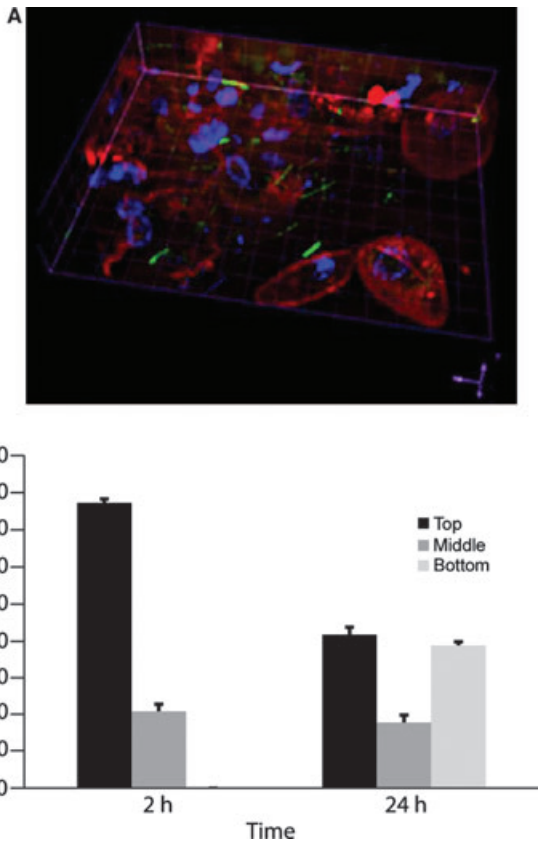


**Figure 3** Physical association between *Fusobacterium nucleatum* and gingival epithelial cell (GEC) multilayers. (A) Confocal microscopy of GECs stained with Alexa 635-Phalloidin (red) after infection for 24 h with *F. nucleatum* stained with Baclight Green. Nuclei are stained with DAPI (blue). A series of fluorescent optical 0.2-μm *x–y* sections were collected to create digitally reconstructed images with IMARIS software. (B) Percentage of *F. nucleatum* cells in the upper, middle or lower layers of the GEC multilayer. Images are representative of three independent experiments. Data are means of three independent experiments in duplicate with standard deviation.

the epithelial layers through an extracellular route but did this without disrupting the integrity of the epithelial barrier.

## Cytokine secretion

After 2 and 24 h of bacterial challenge, culture supernatants were analysed for cytokine levels using Luminex technology. There was an increase in the secretion of IL-1β and TNF-α following 24 h of challenge with *S. gordonii*, *F. nucleatum* and *A. actinomycetemcomitans* (Figs 6 and 7). Levels of IL-6 and IL-8 increased following challenge with *S. gordonii*, and to a lesser degree *A. actinomycetemcomitans* (Figs 8 and 9). In contrast, *P. gingivalis* did not increase the extracellular levels of IL-1β, TNF-α, IL-6 or IL-8 (not shown). There were no statistically significant increases in the amounts of IL-10, IL-12 or MCP-1 following challenge with any of the bacterial species (not shown).

## Apoptosis

The ability of the test bacteria to induce apoptosis in the GEC multilayers was tested with Annexin-V staining (Fig. 10). *Streptococcus gordonii*, *F. nucleatum* and *A. actinomycetemcomitans* stimulated the induc-

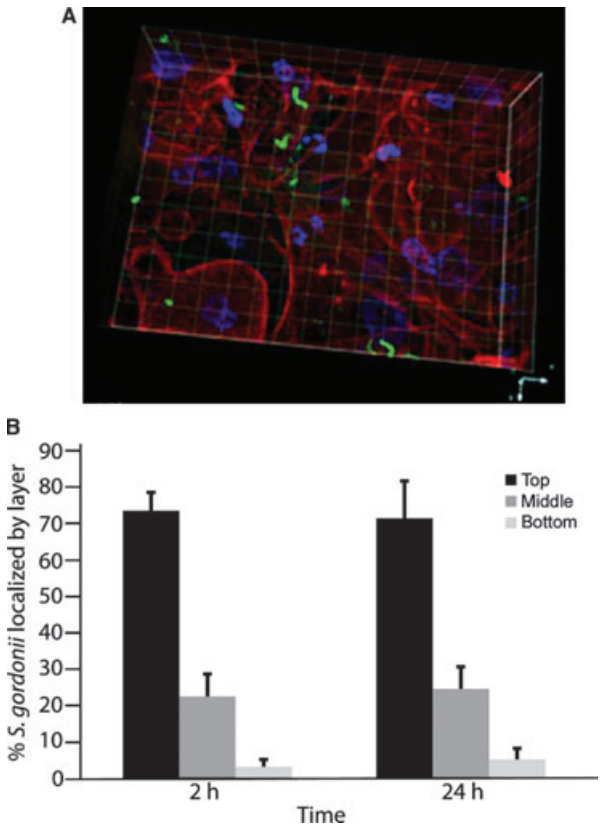


**Figure 4** Physical association between *Streptococcus gordonii* and gingival epithelial cell (GEC) multilayers. (A) Confocal microscopy of GECs stained with Alexa 635-Phalloidin (red) after infection for 24 h with *S. gordonii* stained with Baclight Green. Nuclei are stained with DAPI (blue). A series of fluorescent optical 0.2-μm *x–y* sections were collected to create digitally reconstructed images with IMARIS software. (B) Percentage of *S. gordonii* cells in the upper, middle or lower layers of the GEC multilayer. Bacteria were counted as described in the legend to Fig. 1. Images are representative of three independent experiments. Data are means of three independent experiments in duplicate with standard deviation.

tion of apoptosis after 2 h, and apoptosis was sustained through 24 h. In contrast, *P. gingivalis* caused a transient increase in Annexin-V staining after 2 h but at 24 h apoptosis levels had returned to the control value.

## DISCUSSION

The junctional epithelium is characterized by a relatively permeable structure, lack of keratinization and limited differentiation. Primary cultures of GECs maintained in monolayers exhibit these properties (Oda & Watson, 1990), but they lack the stratified cell layers of tissue. A number of investigators have established multilayers of oral epithelial cells, although often the cells terminally differentiate when in an air–liquid interface or when cultured with serum or high calcium concentrations (Pan *et al.*, 1995; Papaioannou *et al.*, 1999; Rouabhia & Deslauriers, 2002). We have established multilayers of primary GECs in an air–liquid interface that retain limited differentiation status (K1/10-negative) by culture in medium lacking serum and with a low calcium concentration. This model provides an organotypic culture of phenotypically relevant cells that facilitates study of bacterial interactions with the junctional epithelium.

*Porphyromonas gingivalis* rapidly invades monolayers of primary GECs in high numbers (Lamont *et al.*, 1995; Belton *et al.*, 1999), and can also adhere to and invade GECs in multilayers (Sandros *et al.*, 1994; Papaioannou *et al.*, 2003; Andrian *et al.*, 2004). In our organotypic model, the majority of *P. gingivalis* organisms traverse the multilayers of GECs intracellularly, corroborating the importance of the intracellular lifestyle for *P. gingivalis*. Whereas *A. actinomycetemcomitans* and *F. nucleatum* can invade monolayers of transformed epithelial cells (Meyer *et al.*, 1997; Han *et al.*, 2000; Edwards *et al.*, 2006), and *S. gordonii* can invade HeLa cells and endothelial cells (Stinson *et al.*, 2003; Nobbs *et al.*, 2007), in GEC multilayers any intracellular invasion was below the limit of detection. However, *A. actinomycetemcomitans* and *F. nucleatum* did spread through the multilayers intercellularly whereas *S. gordonii* was more confined to the upper cell layer. Although *P. gingivalis* can effectively penetrate tissue by the paracellular route (Hintermann *et al.*, 2002; Katz *et al.*, 2002; Balkovetz & Katz, 2003), the slower spread of the organism through the multilayers in this model may be a reflection of the predominantly intracellular location and the need for specialized cell-to-cell transmission systems (Yilmaz *et al.*, 2006). Indeed, *P. gingivalis* did not disrupt the barrier function of the multilayers as measured by transepithelial electrical resistance, which may be related to the downregulation of protease production that occurs when *P. gingivalis* is in contact with GECs (Xia *et al.*, 2007). With monolayers of MDK cells, a critical threshold concentration of *P. gingivalis* is required to disrupt the barrier function (Katz *et al.*, 2000) so higher numbers of *P. gingivalis* may be capable of inducing disruption of the integrity of the multilayers. Neither *A. actinomycetemcomitans* nor *F. nucleatum*

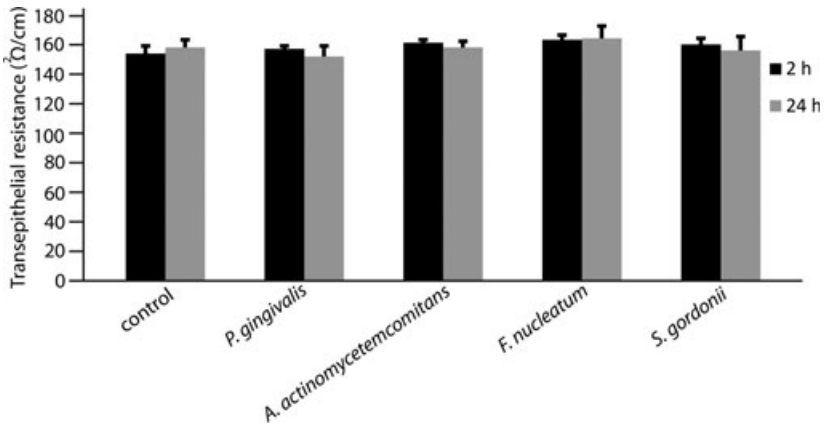


**Figure 5** Transepithelial electrical resistance of gingival epithelial cell (GEC) multilayers infected with *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, or *Streptococcus gordonii* for 2 and 24 h. Control is uninfected cells. Data are means of three independent experiments in duplicate with standard deviation.

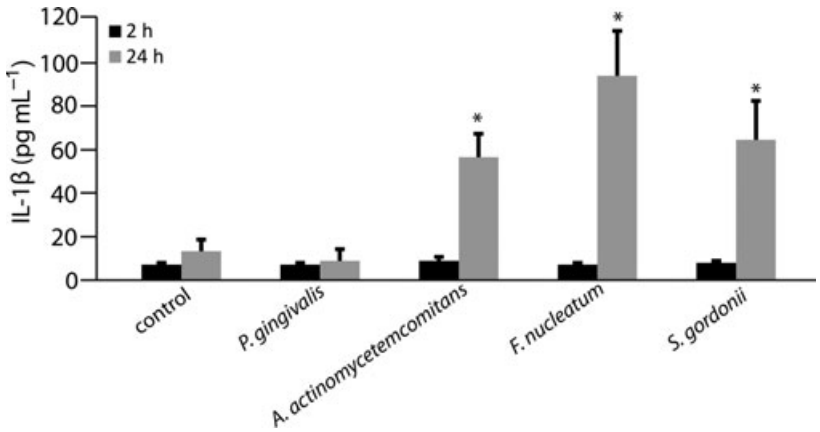


**Figure 6** Interleukin-1β (IL-1β) accumulation in supernatants of gingival epithelial cell (GEC) multilayers following infection with *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, or *Streptococcus gordonii* for 2 or 24 h as determined by a Luminex cytokine array. Control is uninfected cells. The error bars indicate standard deviations ($n = 3$). Asterisk denotes $P < 0.05$ compared with control.

disrupted the barrier function, indicating that the permeable structure of the multilayers is sufficient to allow intercellular bacterial penetration. The basis for the lack of *S. gordonii* spread through the multilayers is unknown; however, *S. gordonii* possess multiple adhesins for epithelial cells, including Hsa and AgI/II proteins (Nobbs *et al.*, 2009), so may remain attached to the first cells that are encountered.

Epithelial cell monolayers display a limited innate immune response following challenge with *P. gingivalis* (Darveau *et al.*, 1998; Hajishengallis, 2009). *Porphyromonas gingivalis* exhibited even greater 'stealth-like' characteristics with the GEC multilayers, and none of the tested cytokines were stimulated by the organism. As *P. gingivalis* can induce the secretion of IL-1β, TNF-α and IL-6 from monolayers of GECs (La *et al.*, 2010; Stathopoulou *et al.*, 2010), this prompted us to question whether multilayers of GECs have diminished expression of TLRs. However, staining with fluorescent antibodies to TLR2 and TLR4 and quantitative image analysis revealed no significant differences in expression of TLR2 and TLR4 between monolayers and multilayers (not shown). Both *F. nucleatum* and *S. gordonii* tended to be more proinflammatory, and induced secretion of IL-1β and TNF-α. Moreover, IL-6 and IL-8 levels were also increased in response to *S. gordonii*. These data support the contention that a degree of inflammation,

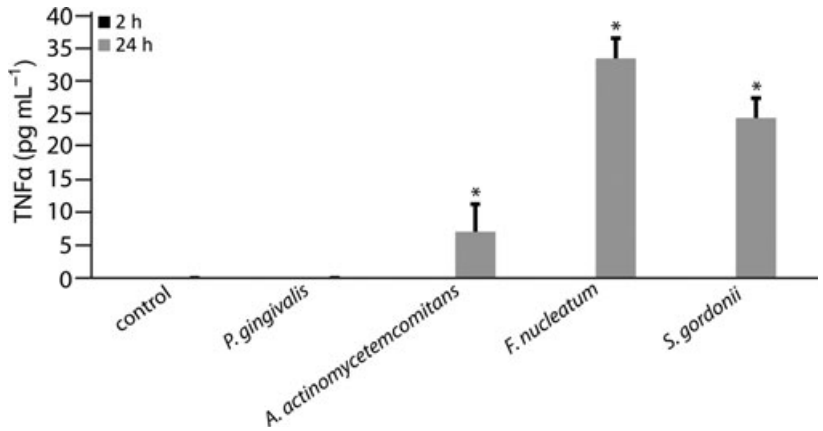


**Figure 7** Tumour necrosis factor-α (TNF-α) accumulation in supernatants of gingival epithelial cell (GEC) multilayers following infection with *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, or *Streptococcus gordonii* for 2 or 24 h as determined by a Luminex cytokine array. Control is uninfected cells. The error bars indicate standard deviations ($n = 3$). *$P < 0.05$ compared with control.

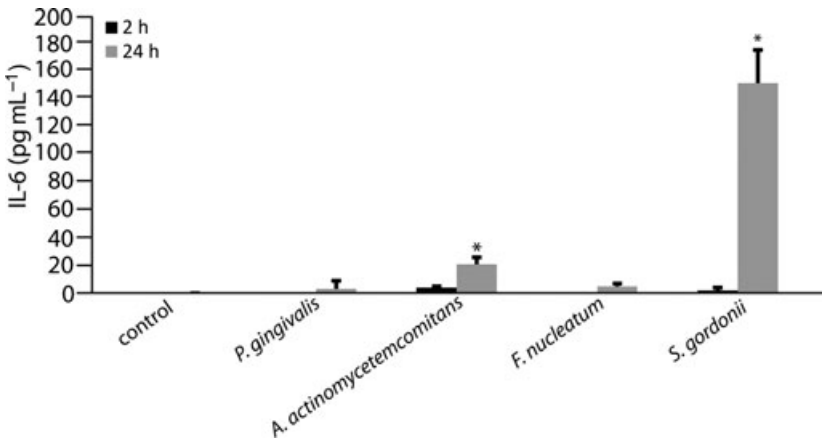


**Figure 8** Interleukin-6 (IL-6) accumulation in supernatants of gingival epithelial cell (GEC) multilayers following infection with *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, or *Streptococcus gordonii* for 2 or 24 h as determined by a Luminex cytokine array. Control is uninfected cells. The error bars indicate standard deviations ($n = 3$). *$P < 0.05$ compared with control.

induced by commensal organisms, contributes to the control of potential pathogens, and to the maintenance of gingival health (Dixon *et al.*, 2004). *Aggregatibacter actinomycetemcomitans* stimulated the secretion of IL-1β, TNF-α, IL-6 and IL-8 although levels of the last three cytokines were significantly lower than those induced by *F. nucleatum* or *S. gordonii*. In combination with the *P. gingivalis* data, these findings suggest that there may be threshold concentrations of cytokine production that are associated with health and with the type and severity of disease.

Epithelial cell responses to bacterial challenge often include modulation of apoptotic pathways. Apoptosis in the GEC multilayers was increased following challenge with *A. actinomycetemcomitans*, *F. nucleatum* or *S. gordonii*, indicating that the gingival epithelial response to infection with different types of bacteria ultimately includes increased cell death, which may facilitate removal of excess bacteria. Interestingly, these three organisms also induced cytokine expression, which may contribute to the apoptotic process. In contrast, *P. gingivalis* induced a transient increase in early apoptotic markers, which returned to control levels after 24 h. This mimics the situation found in monolayers of GECs and is related to the ability of *P. gingivalis* to upregulate anti-apoptotic

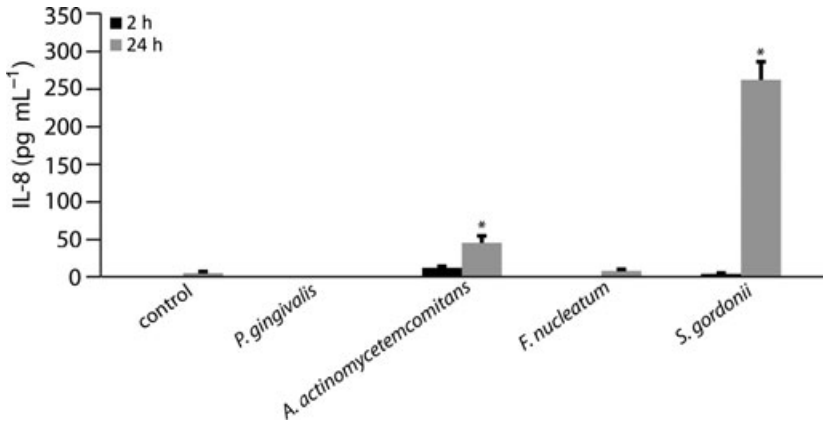


**Figure 9** Interleukin-8 (IL-8) accumulation in supernatants of gingival epithelial cell (GEC) multilayers following infection with *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, or *Streptococcus gordonii* for 2 or 24 h as determined by a Luminex cytokine array. Control is uninfected cells. The error bars indicate standard deviations ($n = 3$). *$P < 0.05$ compared with control.

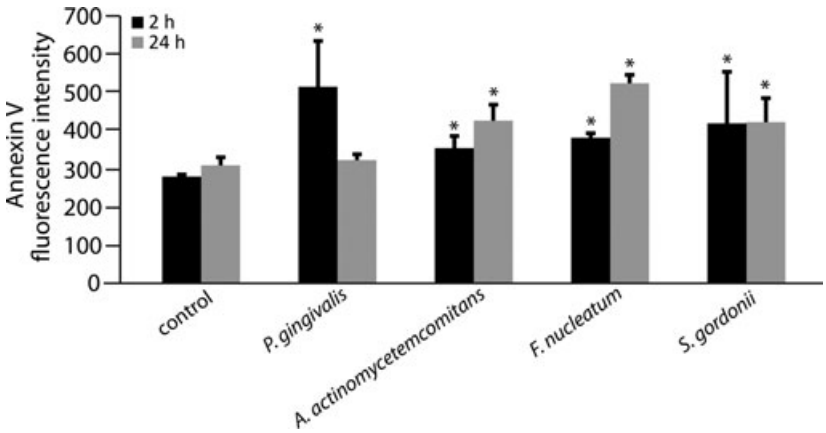


**Figure 10** Annexin V staining in gingival epithelial cell (GEC) multilayers infected with *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, or *Streptococcus gordonii* for 2 or 24 h. Control is uninfected cells. Fluorescein isothiocyanate-Annexin V signals were collected by confocal microscopy. Mean intensity was calculated using SLIDEBOOK software (Olympus, Center Valley, PA). Error bars indicate standard deviations ($n = 3$). *$P < 0.05$ compared with control.

pathways in GECs in a time-dependent manner (Nakhjiri *et al.*, 2001; Mao *et al.*, 2007). As *P. gingivalis* is predominantly located intracellularly, this is thought to represent a strategy of the bacteria to prolong the life of its eukaryotic host cell.

The bacterial inhabitants of the gingival compartment are diverse and exhibit a range of context-dependent pathogenic potentials. These pathogenic personalities may be established to a degree by the nature of the interaction with the junctional epithelium. *Porphyromonas gingivalis*, a pathogen found in severe and chronic manifestations of periodontitis, resides intracellularly and in GECs does not stimulate the production of proinflammatory cytokines. Persistence of the organism will be facilitated by the inhibition of apoptotic cell death. *Aggregatibacter actinomycetemcomitans*, a pathogen found in more acute, aggressive forms of periodontitis, can penetrate multilayers and induce a degree of inflammation, but this may be insufficient to control the organism. *Fusobacterium nucleatum*, which also has pathogenic potential, penetrates the multilayers; however, the induction of proinflammatory cytokines may be sufficient to control the organism in the absence of other pathogenic bacteria or host factors that predispose to disease. *Streptococcus gordonii* resides predomi-

nantly on the surface of the multilayers and over time induces the secretion of proinflammatory cytokines, which may help to control overgrowth of other bacterial species. An additional host strategy to control bacterial overgrowth may be to initiate apoptotic cell death that will contribute to the removal of cell-associated bacteria. Interestingly, the three organisms with pathogenic potential all had the capacity to traverse the epithelium, whereas the commensal organism did not. These studies provide insight into a mechanism that has been underappreciated, and indicate that strategies of traversing the epithelium represent an important component of bacterial pathogenicity.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Confocal microscopy of gingival epithelial cell multilayers stained with Alexa 635-Phalloidin (red). Nuclei are stained with DAPI (blue). A series of fluorescent optical 0.2-μm *x–y* sections were collected to create digitally reconstructed images with IMARIS software.

**Figure S2.** Confocal microscopy of gingival epithelial cell multilayers stained with cytokeratin 13 antibodies and detected with Alexa 555 secondary antibody.

**Figure S3.** Confocal microscopy of gingival epithelial cell multilayers stained with cytokeratin 19 antibodies and detected with Alexa 555 secondary antibody.

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