A Role for Fimbriae in *Porphyromonas gingivalis* Invasion of Oral Epithelial Cells

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Isogenic mutants of *Porphyromonas gingivalis* which differ in the expression of fimbriae were used to examine the contribution of fimbriae in invasion of a human oral epithelial cell line (KB). At a multiplicity of infection of 100, the wild-type *P. gingivalis* strains 33277, 381, and A7436 exhibited adherence efficiencies of 5.5, 0.11, and 5.0%, respectively, and invasion efficiencies of 0.15, 0.03, and 0.10%, respectively. However, adherence to and invasion of KB cells was not detected with the *P. gingivalis* *fimA* mutants, DPG3 and MPG1. Adherence of *P. gingivalis* wild-type strains to KB cells was completely inhibited by the addition of hyperimmune sera raised to the major fimbriae. Examination by electron microscopy of invasion of epithelial cells by the *P. gingivalis* wild-type strain 381 revealed microvillus-like extensions around adherent bacteria; this was not observed with *P. gingivalis* *fim* mutants. Taken together, these results indicate that the *P. gingivalis* major fimbriae are required for adherence to and invasion of oral epithelial cells.

Invasion of mammalian epithelial cells has been reported for a number of pathogenic bacteria and is an important strategy for the sequestration of bacteria from the immune system (6). The periodontal pathogen *Porphyromonas gingivalis* has been observed within gingival tissues in vivo, suggesting that as well as colonizing mucosal surfaces, it may also pass through the epithelial barrier (9, 23). *P. gingivalis* has recently been shown to bind to and invade primary gingival epithelial cells (15), multilayered pocket epithelia (23), and transformed cells in vitro (4). *P. gingivalis* has also been demonstrated to persist and multiply within epithelial cells in vitro, a mechanism postulated to contribute to its pathogenic potential (17). A variety of cell surface structures have been postulated to play a role in the interaction of *P. gingivalis* with host cells (1). Several reports have documented the role of *P. gingivalis* fimbriae in mediating adherence to salivary pellicle-coated tooth surfaces (sHAP); afimbriate variants of *P. gingivalis* have also been shown to have a diminished capacity for adherence to oral epithelial cells in an in vitro assay (8). Fimbriae have been reported to induce the expression of inflammatory cytokines in human gingival fibroblasts and mouse peritoneal macrophages, strongly suggesting that fimbriae play a crucial role in bacterial interactions with host gingival tissues (12, 20, 21). Synthetic peptides derived from the predicted amino acid sequence of the major subunit of fimbrillin (FimA) have been shown to block adherence to sHAP, and an antifimbrial monoclonal antibody (Ab) has been shown to block adhesion of *P. gingivalis* to human buccal epithelial cells in an in vitro assay (8, 13). Recent studies using *P. gingivalis* 381 with an insertional mutation in the gene encoding the fimbrillin subunit (2) have shown that this mutant (DPG3) is unable to adhere to sHAP and unable to induce bone loss in the gnotobiotic rat model (18). A separate *fimA* mutant of *P. gingivalis* 33327 (MPG1) has also been shown to exhibit a loss of adhesiveness to both gingival fibroblasts and epithelial cells in vitro (11). Although

these studies support a role for fimbriae in adherence to epithelial cells, the ability of *P. gingivalis* *fim* mutants to adhere to and invade oral epithelial cells in an invasion assay has not been examined. To define the role of fimbriae in *P. gingivalis* adherence to and invasion of epithelial cells, we have examined two *P. gingivalis* *fimA* mutants in an invasion assay.

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. *P. gingivalis* A7436 was originally isolated from a patient with refractory periodontitis and was characterized in our laboratory; strains 381 and 33277 have been described previously (11, 18). *P. gingivalis* *fimA* mutants DPG3 and MPG1 were constructed following allelic exchange in *P. gingivalis* 381 and 33277, respectively, with the insertionally inactivated *fimA* gene (11, 18). *P. gingivalis* A7436, 33277, and 381 were grown on anaerobic blood agar (ABA) plates (Remel, Lenexa, Kans.); MPG1 and DPG3 were grown on ABA containing erythromycin (5.0 and 10 µg/ml, respectively). All *P. gingivalis* strains were grown under anaerobic conditions (Coy Laboratory Products, Inc., Ann Arbor, Mich.) with 85% N₂, 10% CO₂, and 5% H₂ for 3 to 5 days, inoculated into fresh anaerobic broth, and grown to the late logarithmic phase of growth.

Adherence to and invasion of KB cells by *P. gingivalis*. Adherence and invasion of *P. gingivalis* was assessed in an oral epithelial cell invasion model as previously described (4, 5). The oral epithelial KB cell line ATCC CCL17 (American Type Culture Collection, Rockville, Md.) was maintained in Dulbecco's modified Eagle medium (DMEM) (GIBCO, United Kingdom) supplemented with 10% fetal calf serum (GIBCO) and gentamicin. Subcultivation was performed on confluent cultures, plated 24 h earlier. Cells were plated at a concentration of 10⁶ cells per ml, determined by cell counting on a Coulter counter (4, 5). Bacterial cultures grown to late log phase were centrifuged, washed in phosphate-buffered saline (PBS), and resuspended in DMEM containing 1 mM MgCl₂ and 0.2 mM CaCl₂ at a final concentration of 10⁸ cells per ml. Bacterial suspensions (1.0 ml) were added to confluent KB monolayers and incubated at 37°C in 5% CO₂ for 2 h. After incubation, unattached bacteria were removed following washing of the monolayers twice in PBS. KB cells were lysed in 1 ml of sterile

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TABLE 1. Bacterial strains used in this study

<i>P. gingivalis</i> strain	Genotype or relevant properties	Source
A7436	Wild type	C. A. Genco
381	Wild type; produces intact major fimbriae	R. J. Genco
DPG3	381 <i>fimA</i> mutant; does not produce major fimbriae	R. J. Genco
33277	Wild type	American Type Culture Collection
MPG1	33277 <i>fimA</i> mutant; does not produce major fimbriae	N. Hamada

distilled water (dH₂O) per well and incubated for 10 min. Lysates were serially diluted, plated on ABA plates, and incubated anaerobically at 37°C for 7 days. All assays were performed in triplicate.

The optimal multiplicity of infection for *P. gingivalis* invasion of KB cells has previously been determined to be 100 (4). Thus, for the studies described here, we typically infected 10⁶ KB cells with 10⁸ bacterial cells. Under these conditions, we found that the level of adherence observed with the *P. gingivalis* wild-type strains A7436 and 381 was similar to that previously reported for other *P. gingivalis* strains (5.0 and 5.5% adherence, respectively) (Table 2) (4). In contrast to the results obtained with the *P. gingivalis* wild-type strain 381, we did not detect adherence of the *P. gingivalis* *fimA* mutants DPG3 and MPG1 to KB cells. These results indicate that production of the major *P. gingivalis* fimbriae is required for adherence of *P. gingivalis* to epithelial cells.

Invasion of KB cells by *P. gingivalis* strains was quantified by determining the CFU recovered following antibiotic treatment (Table 2). Confluent KB cells were infected with 1.0 ml of a bacterial suspension (final concentration, 10⁸ cells), and tissue culture plates were centrifuged at 2,000 × g for 10 min at 4°C to enhance contact between KB cells and bacteria. Infected monolayers were incubated at 37°C for 4 h in a 5% CO₂ incubator. After incubation, unattached bacteria were removed following washing of the monolayers twice in PBS. External adherent cells were killed by incubating the infected monolayers with DMEM containing 100 µg of metronidazole/ml. Monolayers were washed twice with PBS, and KB cells were lysed in 1 ml of sterile dH₂O per well and incubated for 10 min. Lysates were diluted and plated on ABA plates, and cultures were incubated anaerobically at 37°C for 7 days. All assays were performed in triplicate.

TABLE 2. Invasion of KB cells by *P. gingivalis*

Strain	% Adherence ^a	Invasion efficiency (%) ^b
A7436	5 ± 0.1	0.01
381	5.5 ± 0.23	0.1
DPG3	<0.0001	<0.0001
33277	0.112 ^c	0.0255
MPG1	ND ^d	0.004

^a Defined as the percentage of CFU input that bound to KB cells. Values represent the means of triplicate independent determinations from a typical experiment ± standard deviations.

^b Percentage of the inoculum (*P. gingivalis*) protected from metronidazole killing after the infection period. Values represent the means of triplicate independent determinations from a typical experiment. For all values, the standard deviation was <0.001.

^c Standard deviation was <0.001.

^d ND, not detected.

TABLE 3. Adherence of *P. gingivalis* to KB cells following addition of Ab to the major fimbriae

Strain	CFU recovered ^a from culture		% Inhibition ^b
	Control	With Ab	
A7436	6.27 × 10 ⁶	1.15 × 10 ⁵	98% ± 2.0
381	2.46 × 10 ⁶	4.33 × 10 ²	100% ± 7.0
DPG3	1.60 × 10 ⁶	1.6 × 10 ⁶	0

^a Bacterial cells (10⁹) were preincubated with PBS (control) or antifimbrial Ab for 60 min at 37°C and used to infect 10⁶ KB cells. Cultures were incubated for 2 h, and the number of CFU recovered was determined as described in the text.

^b Reduction in adherence following pretreatment of *P. gingivalis* with antifimbrial Ab (1:500) compared with adherence of a PBS-treated control. % Adherence was calculated as CFU recovered following Ab addition/CFU recovered in control samples × 100. % Inhibition = 100% - % adherence. Values represent the means of triplicate independent determinations from a typical experiment ± standard deviations.

Although we observed similar levels in the adherence of the *P. gingivalis* wild-type strains A7436 and 381 to KB cells, these strains were observed to invade epithelial cells at different levels. We found that *P. gingivalis* 381 exhibited an invasion efficiency 10-fold higher than that observed with A7436 (Table 2). In agreement with the results obtained in the adherence assay, we did not detect invasion of KB cells by *P. gingivalis* DPG3. The invasion efficiency of the independently isolated 33277 *fimA* mutant MPG1 was also reduced significantly compared to that of the wild-type strain 33277 (Table 2). Although we observed the internalization of *P. gingivalis* 33277 into KB cells, there was a 1-log-unit difference between the observed invasive capability of this strain in our studies and that in previous reports (4); this may be due to differences in laboratory strains of 33277.

Inhibition of adherence of *P. gingivalis* to KB cells by anti-fimbrial Ab. The role of fimbriae in adherence of *P. gingivalis* to epithelial cells was also examined by preincubating *P. gingivalis* with rabbit antisera raised to the major fimbriae prior to the addition of KB cells. Fresh *P. gingivalis* cultures were resuspended in 0.05 M PBS (pH 7.2) at a concentration of 10⁹ cells/ml and incubated anaerobically at 37°C for 60 min with a 1:500 dilution of hyperimmune sera raised to *P. gingivalis* 381 major fimbriae. Since this involved multiple washes following incubation with Ab, we used a multiplicity of infection of 1,000 (10⁹) to ensure that sufficient bacteria could be detected. *P. gingivalis* cultures incubated with normal rabbit sera were used as a negative control. After 100 µl of each *P. gingivalis* culture was plated on ABA plates to confirm viability, cultures were used to infect KB monolayers as described above.

Preincubation of *P. gingivalis* strains with normal rabbit sera did not inhibit the ability of *P. gingivalis* to adhere to epithelial cells (data not shown). As shown in Table 3, preincubation of the *P. gingivalis* wild-type strains A7436 and 381 with antisera to the major fimbriae resulted in the complete inhibition of adherence of *P. gingivalis* to KB cells. These results are in agreement with the findings of previous studies in which antisera to fimbriae were shown to block adherence of *P. gingivalis* to epithelial cells in an vitro assay (13), and together these indicate that the *P. gingivalis* major fimbriae are required for adherence to epithelial cells.

Electron microscopic analysis of *P. gingivalis* strains. Previous studies have established that *P. gingivalis* DPG3 and MPG1 do not produce fimbriae as detected by electron microscopy or Western blot analysis (11, 18). We also examined *P. gingivalis* strains by electron microscopy to confirm surface characteristics. Preparation of *P. gingivalis* cultures for visualization of

fimbriae was performed as previously described (18). For negative staining, bacteria were applied to Formvar- and carbon-coated grids and then air dried. The cells were then negatively stained with 2% uranyl acetate in dH_2O . Sections were examined by transmission electron microscopy (TEM) with a Zeiss CEM 902 electron microscope. TEM was also performed on designated samples to confirm adherence to epithelial cells and internalization within epithelial cells. KB cells were cultured in 24-well plates and treated as described for the invasion experiments, with the following modifications. Following a 4-h incubation with bacteria, monolayers were washed four times in PBS and detached from the plastic surface with dH_2O . The cell slurry was then immediately centrifuged for 4 min at $15,000 \times g$. Fixation of the pellet was performed by addition of 2.5% glutaraldehyde in 0.05 M sodium cacodylate to the cell suspension for 2 h. Cells were pelleted and postfixed in 1% OsO_4 in 0.1 M sodium cacodylate for 3 min. After postfixation the ultrathin sections were contrasted with lead citrate and uranyl acetate before examination by TEM. For scanning electron microscopy, human gingival epithelial layers (Ca9-22) (11) cultured on round glass slides were incubated for 60 min with *P. gingivalis* 33277 or MPG1. Bacterial cells attached to human gingival epithelial cells were observed with a scanning electron microscope (Nippon Denshi Co.).

As seen previously (27), *P. gingivalis* 381 cultures were electron dense and produced long, slender fimbriae (ca. 15 nm in diameter) easily seen in negatively stained preparations (Fig. 1A). By contrast, we did not detect these long fimbriae on the surface of the corresponding *P. gingivalis* *fimA* mutant, DPG3 (Fig. 1B), indicating that the insertion mutation in DPG3 blocked fimbrial production. Expression of long fimbrial structures from the surface of the *P. gingivalis* wild-type strain 33277 has been demonstrated previously (11). These structures were not observed in the corresponding *fimA* mutant, MPG1 (14). Previous electron microscopic analysis has also demonstrated that the cell envelopes of DPG3 and MPG1 appeared typical for gram-negative bacteria and were not significantly different from the ultrastructures of wild-type strains (11, 18).

We also examined the nature of the interaction of *P. gingivalis* with epithelial cells by electron microscopy. In epithelial cells infected with the *P. gingivalis* wild-type strain 381, we observed microvilli surrounding bacteria which appeared intimately associated with the epithelial cell surface (Fig. 2A). Close examination of micrographs of KB cells infected with the wild-type strain 381 revealed the engulfment of bacteria by microvilli (Fig. 2B); this process may represent the initial formation of a cytoplasmic vacuole. In contrast to our observations with the *P. gingivalis* wild-type strain 381, we did not detect attachment of the corresponding *fimA* mutant, DPG3, to the epithelial cell surface (Fig. 2C). Similar to the results obtained with DPG3 in transmission electron micrographs, we found that the 33277 *fimA* mutant MPG1 was not associated with the oral epithelial cell surface (data not shown). We also examined the interactions of *P. gingivalis* 33277 and MPG1 with human gingival epithelial cells by scanning electron microscopy. As seen in Fig. 3A, numerous bacteria were observed to be attached to epithelial cells following infection with the wild-type strain 33277. In contrast, few bacteria were observed following infection with the 33277 *fimA* mutant MPG1 (Fig. 3B).

Concluding remarks. In this study we have confirmed a role for the major fimbriae in the adherence to and invasion of oral epithelial cells by *P. gingivalis*. Our results are in agreement with those of studies recently reported by Weinberg et al. (30) on the involvement of the *P. gingivalis* major fimbriae in the invasion of normal human gingival epithelial cells. In our study

A

B

FIG. 1. Transmission electron micrographs of *P. gingivalis* 381 and DPG3. Samples were prepared by negative staining with uranyl acetate. (A) *P. gingivalis* 381 had numerous thin, long fimbriae on its surface. Magnification, $\times 45,500$. (B) DPG3 did not possess these thin, long fimbriae. Magnification, $\times 27,300$.

we found that incubation of *P. gingivalis* with antisera raised to the major fimbriae prior to infection of epithelial cells resulted in the complete inhibition of adherence to these cells. In addition, adherence to and invasion of oral epithelial cells was not detected with the *fimA* knockout strains DPG3 and MPG1. Electron microscopic analysis confirmed the intimate interaction of the *P. gingivalis* wild-type strains with the epithelial cell surface. The interactions of *P. gingivalis* with epithelial cells appears to involve at least a two-stage process of initial and intimate attachment to the epithelial cell. The initial attachment appears to be mediated by the major fimbriae; this is followed by intimate attachment to the surface of the epithelial cell and the engulfment of bacteria. Our recent results indicate that cytoskeletal rearrangements as well as calcium signals are induced following invasion of epithelial cells by *P. gingivalis* (7). If invasion of *P. gingivalis* follows a similar pathway as that observed for enteropathogenic *Escherichia coli* (3, 14, 19, 26), the *P. gingivalis* major fimbriae may be required for the initial

FIG. 2. Transmission electron micrographs of KB monolayers infected with *P. gingivalis* 381 and DPG3. (A) Intimate interaction of *P. gingivalis* 381 with the epithelial cell surface. Magnification, $\times 45,000$. (B) Engulfment of *P. gingivalis* 381 by the epithelial cell. Magnification, $\times 60,000$. (C) Absence of intimate interaction of *P. gingivalis* DPG3 with the epithelial cell surface. Magnification, $\times 45,000$.

association of *P. gingivalis* with epithelial cells. The lack of initial association with the epithelial cells, as observed with *fimA* mutants, would preclude the next stage of intimate attachment.

Other molecules in addition to fimbriae may be required for the adherence of *P. gingivalis* to epithelial cells. For example, it is well documented that the production of a polysaccharide capsule may interfere with the initial attachment of bacteria to epithelial cells. In *Neisseria meningitidis* acapsulate bacteria are more adhesive than capsulate bacteria, and the capsule appears to modulate the function not only of outer membrane

FIG. 3. Adherence of *P. gingivalis* to human gingival epithelial cells. Human gingival epithelial cells were incubated with *P. gingivalis* 33277 or MPG1 and examined by scanning electron microscopy. (A) *P. gingivalis* 33277 adhering to epithelial cell with numerous microvilli present. (B) *P. gingivalis* MPG1. Few bacteria or microvilli are present. (C) Uninfected epithelial cell. Bars, 1.0 μm .

opacity proteins but also of pilus-dependent interactions (29). Whether the partial inhibitory effect of the capsule on pilus-dependent adhesion is due to partial masking of adhesive ligands that may be present along the length of the pili or whether it is a generalized charge-mediated phenomenon, or both, remains to be determined. It has also been demonstrated that polysaccharide capsule-deficient mutants of *Haemophilus influenzae* type b exhibit enhanced adherence to and invasion of human cells (28). High-molecular-weight proteins appear to mediate the attachment of *H. influenzae* to human epithelial cells, and thus the production of a polysaccharide capsule may mask the surface exposure of these proteins. The differences observed in our studies in the adherence and invasion efficien-

cies for different wild-type *P. gingivalis* strains may reflect the differences in other cell surface proteins and/or the production of a capsule. The polysaccharide capsule may serve to mask the major fimbriae, resulting in a lack of intimate attachment to the epithelial cell surface. It is clear, however, that fimbriae mediate adherence of *P. gingivalis* to epithelial cells, and this adherence may be modified by other cell surface molecules.

Short structures representing a second type of fimbria have recently been observed on the surface of the *P. gingivalis* 33277 *fimA* mutant MPG1 (10). Subsequent purification of the minor fimbriae has confirmed these structures to be composed of a 67-kDa protein (10). The specific role of these minor fimbriae in the interactions of *P. gingivalis* with host cells has not been defined. Although the *fimA* mutant MPG1 produces these minor fimbriae, our results indicate that *P. gingivalis* MPG1 is a poor invader of KB cells. In addition, the lack of microvillus involvement on the epithelial cell surface following infection with MPG1 suggests that the expression of the minor fimbriae, in the absence of the expression of the major fimbriae, does not enable *P. gingivalis* to interact with and invade epithelial cells.

Recent reports by Madianos et al. (17) and by Lamont et al. (15) have confirmed the ability of *P. gingivalis* to replicate within primary gingival epithelial cells. In addition, *P. gingivalis* has been shown to advance into deeper epithelial layers (22, 23). These observations indicate that *P. gingivalis* has evolved mechanisms for survival within epithelial cells, resulting in the subversion of the host immune response. The results presented here indicate that the *P. gingivalis* major fimbriae are required for the initial attachment of *P. gingivalis* to oral epithelial cells and subsequent invasion. Ongoing studies on the cytoskeletal rearrangements induced following *P. gingivalis* invasion should enable us to further define the role of the major fimbriae as well as the roles of other surface molecules in the host signaling process.

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