

## Role of the Amino-Terminal Region of *Porphyromonas gingivalis* Fimbriae in Adherence to Epithelial Cells

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Received 21 June 1999/Returned for modification 4 August 1999/Accepted 25 August 1999

***Porphyromonas gingivalis* fimbriae elicit many responses in eukaryotic cells, including mitogenicity, cytokine production, epithelial cell invasion, and cellular immune response. Specific domains of the major fimbrial protein (FimA) have been shown to be important in triggering some of these functions. The goal of the present study was to identify the domain(s) of *P. gingivalis* FimA responsible for specific interaction with human mucosal epithelial cells. Fimbriated *P. gingivalis* strains have been shown to bind to buccal epithelial cells, whereas nonfimbriated strains bind at low levels or not at all. This and other studies provide evidence that FimA mediates the adherence of *P. gingivalis* to oral epithelial cells. To determine the specific region(s) of *P. gingivalis* FimA involved in epithelial cell binding, specific antipeptide antibodies were used to inhibit the binding of iodinated purified fimbriae as well as the binding of *P. gingivalis* cells to epithelial cells. Antibodies directed against peptides 49 to 68 (VVMANTAGAMELVGKTLAEVK) and 69 to 90 (ALTELTAENQEAAGLIMTAEP) were found to highly inhibit both the binding of fimbriae and the binding of *P. gingivalis* cells to epithelial cells. The antibody against FimA peptides 69 to 90 also reacted with *P. gingivalis* fimbriae in immunogold labeling and immunoblot analysis, thereby indicating that this peptide domain is exposed on the surface of fimbriae. Our results suggest that the amino-terminal domain corresponding to amino acid residues 49 to 90 of the fimbrillin protein is a major epithelial cell binding domain of *P. gingivalis* fimbriae.**

*Porphyromonas gingivalis* plays an important role in the initiation and progression of periodontal disease. *P. gingivalis* has been shown to attach to and invade oral epithelial cells in vitro (22, 26). Intracellular invasion of human epithelial cells is a key pathogenic property for a number of bacterial species (4–6). For these events, bacteria bind to epithelial cell membranes and induce a series of biochemical changes (27) that involve the induction of protein kinase activity (24, 25). A variety of cell surface structures have been postulated to play roles in *P. gingivalis* interaction with host cells (2), and the initial binding of *P. gingivalis* to target cells appears to be fimbriae mediated. Recent reports from several laboratories have shown that fimbriae bind to saliva-coated hydroxyapatite (13), human erythrocytes (17, 18), monocytes and macrophages (19), epithelial cells (7, 11, 16, 30), and gingival fibroblasts (8). The afimbriated mutants of *P. gingivalis* have been shown to possess diminished capacity for adherence to oral epithelial cells in vitro (7, 16, 30). In addition, antifimbrial monoclonal antibodies have been shown to block the adhesion of *P. gingivalis* to human buccal epithelial cells in an in vitro assay (11). The above studies strongly implicate the major fimbrial protein FimA in adherence to these mammalian cells. Recent studies from Ogawa et al. (21) have shown that synthetic peptides of fimbrillin corresponding to its binding domains (amino acid residues 1 to 20, 69 to 80, and 171 to 181) inhibited the binding of *P. gingivalis* fimbriae to gingival fibroblasts. Fimbriae have also been reported to induce the expression of inflammatory cytokines in human gingival fibroblasts and mouse peritoneal

macrophages, strongly suggesting that fimbriae are crucial in bacterial interactions with the host gingival tissue (9).

*P. gingivalis* fimbriae can induce protein kinase-mediated phosphorylation of a signaling protein in mouse peritoneal macrophages and in human monocytes (15, 20). Since *P. gingivalis* FimA appears to be key in the binding to and possible invasion of epithelial cells, we attempted to elucidate the important region(s) of fimbrillin that are involved in the attachment process. To this end, we have mapped the surface regions of the fimbrillin molecule by utilizing antipeptide antibodies against synthetic peptides corresponding to the FimA sequence (3). The selection of peptides was based on surface predictions incorporating three different parameters: hydrophobicity, accessibility, and mobility (23). Antipeptide antibodies that reacted with native fimbriae, thereby suggesting that the corresponding peptides are surface exposed, were used as inhibitors of *P. gingivalis* binding to epithelial cells.

The peptides synthesized are listed in Table 1. Peptide synthesis and preparation of peptide conjugates were carried out by a method described earlier (14). The composition and sequence of peptides were confirmed by amino acid analysis on a Beckman model 121MB sequencer, and the amino acid sequence was confirmed by an automated stepwise procedure on an Applied Biosystems model 477A sequencer.

**Immunization.** For the production of polyclonal antibodies to synthetic peptides, each peptide was conjugated to thyroglobulin using *m*-maleinimido-benzoyl-*N*-hydroxy-succinimide ester (MBS) as a cross-linker by a method described by Schmidt et al. (28). Briefly, 10 mg of thyroglobulin, dissolved in 3 ml of phosphate buffered saline (PBS), pH 7.4, was activated by mixing with 5 mg of MBS in 1 ml of *N,N*-dimethylformamide. The solution was stirred for 2 h at room temperature, and unreacted MBS was removed by gel filtration on Sephadex G-25 in 0.1 M PBS, pH 6.0. Synthetic peptides (5 mg each)

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TABLE 1. Sequence of synthetic peptides corresponding to the segments of *P. gingivalis* fimbriin<sup>a</sup>

Peptide position	Amino acid sequence
42-61.....	SAGQRTLVMANTGAMELVG
49-68.....	VVMANTGAMELVGKTLAEVK
69-90.....	ALTTELTAEENQEAAGLIMTAEP
81-98.....	AAGLIMTAEPKTTIVLKAG
99-110.....	KNYIGYSGTGEG
226-245.....	IHPITLCVYGKLGKNGADLA
287-306.....	NKIGRNHKYDIKLTITGPGT

<sup>a</sup> Numbering amino acids did not take into account that the first amino acids appeared as the leader sequence in the fimbriin sequence by Dickinson et al. (3).

were reduced with sodium borohydride, and excess borohydride was destroyed with acetic acid. The neutralized and reduced peptide was combined with MBS-activated thyroglobulin and was stirred overnight at room temperature. The resulting peptide-carrying conjugate was subsequently isolated by gel filtration on Sephadex G-25 in 0.1 M ammonium bicarbonate buffer, pH 8.0.

New Zealand White rabbits (2 to 3 kg) were injected subcutaneously at multiple sites with 500 µg (total dosage) of the appropriate conjugated peptide carrier in complete Freund's adjuvant. Beginning 2 weeks after the first injection, the rabbits were boosted weekly with immunogens in incomplete adjuvant. Six weeks after the first injection, each rabbit was bled, and the antibodies were tested against the corresponding antigen by enzyme-linked immunoabsorbent assay and immunoblot analysis. After appropriate antibody titer was obtained, rabbits were bled by heart puncture, and the sera were collected and stored at -70°C.

**Bacterial culture conditions.** *P. gingivalis* 2561 was grown in one-half-strength (18 mg/ml) brain heart infusion broth, supplemented with 5 mg of yeast extract per ml and buffered at a pH of 7.4. Cells were then incubated for 2 days in an anaerobic chamber (85% N<sub>2</sub>, 10% H<sub>2</sub>, 5% CO<sub>2</sub>).

**Fimbrial preparation and iodination.** *P. gingivalis* fimbriae were purified by the procedure of Sojar et al. (29), and purity was confirmed by a sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (12) (Fig. 1A). Purified fimbriae were iodinated using the chloramine T method (10) with the following modification. Ten micrograms of purified fimbriae was labeled with 0.5 mCi of sodium iodine-125 (Amersham Pharmacia Biotech Inc., N.J.) in 0.5 M PBS, pH 7.2, in the presence of 10 µl of chloramine T (1 mg/ml) for 60 s. Adding 20 µl of sodium metabisulfite (2 mg/ml) terminated iodination. After termination, 100 µl of PBS containing 10% sucrose and 10% potassium iodide was added, and the mixture was loaded on a Sephadex G-75 column (1 by 30 cm) saturated with 1% bovine serum albumin (BSA) to prevent nonspecific binding. The column was extensively washed after saturation with BSA. The iodinated fimbrial peak was collected without a carrier protein, and the integrity of labeled fimbriae was confirmed by autoradiography (Fig. 1B).

**Dot blot assay.** Five micrograms of purified fimbriae was spotted on nitrocellulose membranes. Unoccupied sites were blocked with 1% BSA, and membranes were incubated with respective antipeptide antibodies. Following washing with Tris-buffered saline (20 mM Tris-Cl, 0.5M NaCl [pH 7.5]), membranes were incubated with goat anti-rabbit immunoglobulin G horseradish peroxidase conjugate for 1 h at room temperature. The blots were washed as above, and bound antibodies were visualized by adding 4-chloro-1-naphthol color-developing re-

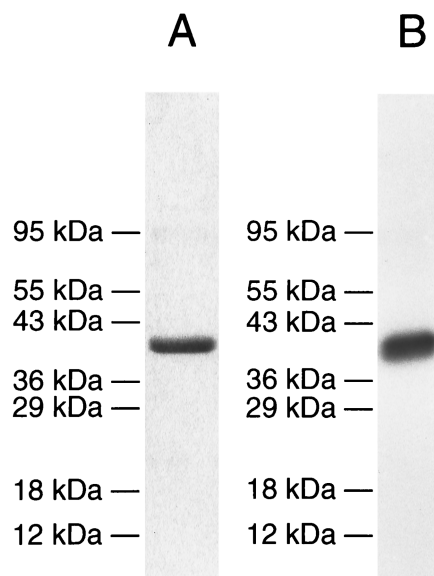


FIG. 1. SDS-polyacrylamide gel electrophoresis of purified fimbriae and autoradiograph of iodinated fimbriae. (A) SDS-polyacrylamide gel stained with Coomassie blue. (B) Autoradiograph of iodinated fimbriae. Molecular mass is indicated.

agent. Prebleed rabbit serum and a blot without primary antibodies were used as negative controls. The results showed that the antibodies against FimA peptides 42 to 61, 49 to 68, 69 to 90, 81 to 98, 99 to 110, 226 to 245, and 287 to 306 reacted positively with purified fimbriae (data not shown).

**Inhibition of binding of purified *P. gingivalis* fimbriae to KB cells by antipeptide antibodies.** Inhibition of fimbrial binding to oral epithelial KB cell line ATCC CCL17 (American Type Culture Collection, Rockville, Md.) was carried out. The culture was maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum. The ability of fimbrial peptide antibodies to inhibit fimbrial binding to KB cells was examined by preincubating purified iodinated fimbriae with antipeptide antibodies at room temperature for 1 h. Normal rabbit serum was used as a negative control. Epithelial cells were grown to confluent monolayers, the medium was removed, and cells were washed with the same medium without serum. Iodinated fimbriae (100,000 cpm) with and without antipeptide antibodies were added in equal volumes of medium without fetal calf serum and were incubated at 37°C for 1 h. The unbound iodinated fimbriae were aspirated, and cells were washed with medium without fetal bovine serum. The washed cells were solubilized in 0.5 M NaOH plus 1% SDS at 37°C for a few minutes, and the radioactivity was counted in a Beckman gamma counter. When antipeptide antibodies were incubated with iodinated fimbriae prior to their addition to KB cells, it was found that, in the presence of the antibodies against FimA peptides 49 to 68 and peptides 69 to 90, binding of fimbriae to Kb cells was reduced to 30 and 26%, respectively (Table 2).

**Inhibition of adherence of *P. gingivalis* cells to KB cells by antipeptide antibodies.** Adherence of *P. gingivalis* and its inhibition in the presence of antipeptide antibodies were assessed in an oral epithelial cell invasion model described earlier (16). The oral epithelial KB cell line ATCC CCL17 was maintained in DMEM supplemented with 10% fetal bovine serum and gentamicin. Cells were seeded into 24-well plates 24 h earlier and were allowed to grow to a density of  $5 \times 10^4$  cells/well.

TABLE 2. Effects of antipeptide serum on binding of iodinated purified fimbriae

Inhibitor	Percent binding
None	94.61 ± 9.02
Normal rabbit serum	94.98 ± 9.08
Antifimbria antibodies	29.10 ± 0.79
Antibody against peptides 49–68	30.27 ± 3.01
Antibody against peptides 69–90	26.02 ± 2.89
Antibody against peptides 226–245	101.6 ± 6.60
Antibody against peptides 287–306	94.42 ± 2.64

Bacterial cultures grown to late log phase were centrifuged, were washed in PBS, and were resuspended in DMEM containing 1 mM MgCl<sub>2</sub> and 0.2 mM CaCl<sub>2</sub> at a final concentration of 10<sup>6</sup> cells per ml. The bacterial suspensions (1.0 ml) were added to confluent epithelial monolayers and were incubated at 37°C in 5% CO<sub>2</sub> for 2 h. After incubation, unattached bacteria were removed following the washing of the monolayers twice in PBS. KB cells were lysed in 1 ml of sterile distilled water per well and were incubated for 10 min. Lysates were serially diluted, were plated on abscisic acid plates, and were incubated anaerobically at 37°C for 7 days. All assays were performed in triplicate.

The ability of antipeptide antibodies to inhibit the adherence of *P. gingivalis* to epithelial cells was examined by preincubating *P. gingivalis* with antipeptide antibodies which were found to be inhibitory in a purified fimbria binding inhibition assay. Normal rabbit serum, as well as carboxy-terminal antibodies, were used as negative controls. Freshly grown *P. gingivalis* cells were resuspended in 0.05 M PBS (pH 7.2) at a concentration of 10<sup>9</sup> cells/ml and were incubated anaerobically at 37°C for 60 min in 1:500 dilution of sera raised against fimbrial peptide. Normal rabbit serum was used as a negative control. After 100 µl of each *P. gingivalis* culture was plated on brain heart infusion blood agar 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 binding of *P. gingivalis* to KB cells. Antipeptide antibodies that inhibited the binding of purified fimbriae to epithelial cells were also found to inhibit the binding of *P. gingivalis* to KB cells. In the presence of antibodies against FimA peptides 49 to 68 and 69 to 90, *P. gingivalis* attachment to epithelial cells was reduced to 0.52 and 0.056%, respectively, compared to 5.06% attachment levels without inhibitor (Table 3). However, the antibody to FimA peptides 226 to 245 failed to block fimbrial binding, yet inhibited *P. gingivalis* binding by 60%. This might suggest that antipeptide antibodies react with another protein on the *P. gingivalis* cell surface.

TABLE 3. Effects of antipeptide serum on attachment of *P. gingivalis* to KB cells

Inhibitor	% Total inoculum attachment	% Binding	% Inhibition
None	5.06 ± 0.66	100	0
Antibody against peptides 49–68	0.52 ± 0.16	10.3	89.7
Antibody against peptides 69–90	0.06 ± 0.02	1.2	98.8
Antibody against peptides 226–245	2.04 ± 0.55	40.3	59.7
Antibody against peptides 287–306	4.35 ± 0.18	86.0	14.0

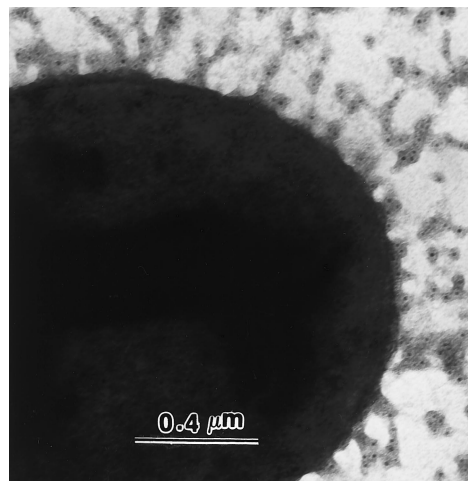


FIG. 2. Localization of fimbriae on a whole cell visualized by immunogold electron microscopy. Cells were incubated with antibody against FimA peptides 69 to 90 followed by gold-labeled goat anti-rabbit antibody. Samples were prepared by negative staining with 2% uranyl acetate.

**Immunogold labeling.** *P. gingivalis* 2561 was grown as described above. Cells were washed with PBS and transferred to nickel grids coated with Formvar film to air dry. The cells were then incubated with 10 µl of rabbit polyclonal antibodies raised against peptides (1:500 dilution of antisera in PBS containing 1% BSA) at 37°C for 1 h. Following washing five times with PBS, cells were incubated for 30 min with sheep anti-rabbit immunoglobulin G conjugated with 5-nm gold particles (1:20 AuroProbe EM; Amersham) at 37°C for 30 min. The cells were rinsed twice with PBS and negatively stained with 2% (wt/vol) uranyl acetate for 1 min. The fixed and stained cells were examined and photographed with a Hitachi H-600 electron microscope operating at 75 kV. The results showed that the antibody against FimA peptides 69 to 90 recognized the native fimbriae on the surface of whole cells (Fig. 2). No labeling was observed when normal rabbit sera or only secondary antibodies were used as controls (data not shown).

Many researchers have suggested that major *P. gingivalis* fimbriae play an important role in *P. gingivalis* adherence and invasion of oral epithelial cells (16, 30). Njoroge et al. (16) found that incubation of *P. gingivalis* with antisera raised to major fimbriae prior to infection of epithelial cells resulted in complete inhibition of adherence to these cells. In addition, adherence to and invasion of oral epithelial cells were not detected with the *fimA*-knockout strains DPG3 and MPG1. The interaction of *P. gingivalis* with epithelial cells appears to involve at least a two-stage process of initial and intimate attachment. The initial attachment of *P. gingivalis* with epithelial cells appears to be mediated by the major fimbriae. In this study, we have elucidated the region(s) of fimbriillin that is involved in the initial attachment process.

To elucidate the regions of *P. gingivalis* fimbriillin that may be involved in epithelial binding, we first identified the surface-exposed regions of fimbriillin by utilizing antibodies raised against synthetic peptides of fimbriillin. We found that a majority of the antibodies against the amino-terminal region recognized the native fimbriae. Additionally, secondary structure and hydrophilicity prediction of fimbriillin protein according to Chou and Fasman algorithms (1) showed that the amino-terminal region is surface exposed. Taken together, these studies showed that the amino-terminal region of fimbriillin is surface

exposed and may be available for binding. It should be noted here that the failure of antibodies directed against other domains of fimbrillin to recognize native fimbriae does not suggest that such domains are not surface exposed. It is probable that these synthetic peptide antibodies do not recognize the native peptide conformation in fimbriae. By utilizing antipeptide antibodies to inhibit *P. gingivalis* binding to epithelial cells, we found that antibodies against the FimA amino-terminal region (from peptides 49 to 90) were most effective in inhibiting *P. gingivalis* attachment to epithelial cells; the antibodies against FimA peptides 49 to 68 and 69 to 90 inhibited the binding to 89.7 and 98.9%, respectively.

In conclusion, our results suggest that the amino-terminal region of fimbrillin (from amino acid residues 49 to 90) is the potential epithelial binding domain of *P. gingivalis* fimbriae.

This study was supported in part by U.S. Public Health Service grant no. DE08240, DE04898, DE12320-01, and AI09268.

We thank Darlene Badgett for her excellent technical assistance and Sara Saldi for editing the manuscript.

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Editor: P. E. Orndorff