Fimbriae and the Hemagglutinating Adhesin HA-Ag2 Mediate Adhesion of Porphyromonas gingivalis to Epithelial Cells

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The mechanisms by which Porphyromonas gingivalis, a gram-negative anaerobic bacterium, is pathogenic for the periodontium remain largely hypothetical. Invasion of host tissues by P. gingivalis is believed to require adhesion of the bacterium to host cells. The aim of this study was to use monoclonal antibodies (MAbs) to characterize the bacterial cell surface component(s) acting as a ligand binding to a receptor on epithelial cells. Surface antigens of P. gingivalis ATCC 33277 were obtained as a glass bead-EDTA extract (GBE), and antisera against the GBE was produced in rabbits. Epithelial cell membrane proteins (ECMP) were prepared from a homogenate of the SK-MES-1 cell line with Triton X-100. The antigen/ligand profile of GBE was resolved by crossed immunoelectrophoresis and by using ECMP in the first-dimension gel. The migration of one immunoprecipitate (IP) was retarded, indicating a ligand-receptor interaction between a surface antigen of P. gingivalis and a complementary binding site on the epithelial cell membrane. The corresponding IP in the GBE/anti-GBE immunoelectrophoresis profile was excised from replicate gels to immunize mice for production of MAbs specific for the bacterial ligand. Five MAbs were obtained and tested for reactivity with GBE in immunoblots and for inhibition of the interaction between GBE and ECMP. Immunoblot revealed polypeptides at 28, 42, 43, and 49 kDa. Inhibition tests were positive for all five MAbs. These results are conclusive evidence that the MAbs recognize functional epitopes involved in the adherence of P. gingivalis to epithelial cells and that the adhesins are likely associated with fimbriae and the hemagglutinating adhesin HA-Ag2.

Porphyromonas (previously Bacteroides) gingivalis, an anaerobic, gram-negative organism, is widely implicated in the pathogenesis of periodontal disease. Colonization of the subgingival space by this pathogen is a prerequisite for infection. It is mediated by adhesion of the microorganism to epithelial cells and to other cells of the oral microbial flora (34). Many molecular structures are implicated in bacterial adherence, including the fimbriae and extracellular vesicles, potential molecular adhesins such as lectins, hemagglutinins and their protease activity (26), and lipopolysaccharides (20, 21).

The interactions between P. gingivalis and eukaryotic or prokaryotic cell surfaces have already been demonstrated (33). Adhesins responsible for attachment to epithelial cells remain poorly known. Hemagglutination is a feature that distinguishes P. gingivalis from other asaccharolytic, black-pigmented species (32). It has been evaluated by using human and animal erythrocytes. Okuda and Takazoe also established for the first time in 1974 (24) that the pili (fimbriae) are responsible for hemagglutinating activity in P. gingivalis. It has since been shown the fimbriae are distinct from the structure that causes hemagglutination but that the two form a complex (2).

The object of the present study was to identify and characterize immunochemically the surface component(s) of the bacterial surface that mediates adhesion of P. gingivalis ATCC 33277 to epithelial cells. The technique of crossed immunoelectrophoresis (CIE) was used for preliminary characterization of the bacterial ligand against which monoclonal antibodies (MAbs) were produced. Inhibition of P. gingivalis adhesion to epithelial cells was evaluated by flow cytometry.

MATERIALS AND METHODS

Culture and epithelial membrane extracts of SK-MES-1 (ATCC HTB 58) cells. Cells were grown in Dulbecco's modified Eagle's medium supplemented with fetal calf serum (10%), l-glutamine (2 mM), and antibiotics (penicillin and streptomycin at 100 IU and 100 μg/ml, respectively). Epithelial cell membrane proteins (ECMP) were prepared by homogenization as described by Coligan et al. (5), in 0.5% Triton X-100.

Solubilization of erythrocyte membranes. Triton X-100-solubilized erythrocyte (TSEM) were prepared as previously described (22).

Bacterial culture and bacterial membrane extracts. P. gingivalis ATCC 33277 was grown in Todd-Hewitt medium supplemented with hemin (10 μg/ml) and vitamin K₁ (1 μg/ml) in an anaerobic atmosphere (80% N₂, 10% H₂, 10% CO₂) for 24 to 48 h. Outer membranes (OM) were obtained from extracellular vesicles prepared as described by Deslauriers et al. (6). Glass bead-EDTA extract (GBE), containing all of the OM antigens, was prepared as described by Parent et al. (25). Briefly, the cells were harvested by centrifugation at 10,000 × g for 20 min at 4°C and then subjected to attrition with glass beads 150 to 212 μm in diameter in 50 mM Tris-5 mM EDTA buffer, pH 8.6, for 48 h at 4°C. GBE was the supernatant obtained after centrifugation at 10,000 × g. Part of the GBE was treated with 20% Triton X-100. The solution was shaken for 48 h at 4°C, and the insoluble material was eliminated by 2 h of ultracentrifugation at 100,000 × g at 4°C. GBE-Triton X-100 was the supernatant thus obtained. Native fimbriae were prepared and purified as described by Yoshimura et al. (37). The protein content of bacterial and eukaryotic extracts were measured by the bicinchoninic acid method of Lowry et al. (13).

Assays of P. gingivalis adherence to SK-MES-1 epithelial cells and scanning electron microscopy. Bacteria from a 48-h culture were harvested by centrifugation at 10,000 × g for 10 min. They were washed in phosphate-buffered saline (PBS) without antibiotics and then resuspended to an optical density at 600 nm corresponding to 10⁷ CFU/ml. For experiments involving adherence to SK-MES-1 cells, the bacteria were used at a ratio of 200 bacteria per eukaryotic cell. After a 15-min contact time, the epithelial cells were detached by using a trypsin-EDTA mixture (500 and 200 mg/ml, respectively) (Sigma) prewarmed to 37°C and then harvested by centrifugation at 260 × g for 5 min. For scanning electron microscopy, cells (SK-MES-1 and bacteria) were fixed in a 2.5% glutaraldehyde solution in 0.2 M cacodylate buffer, pH 7.2, for 1 h. After rinsing and dehydration through a graded series of aqueous ethanol solutions, the cells were critical point dried. They were then coated with a thin layer of gold-palladium and observed with a JEOL JSM 6400 scanning electron microscope.

Polyspecific polyclonal antiserum. Antisera against the surface antigens of P. gingivalis were produced by subcutaneous injection of young rabbits (less than 2 kg) with 2 mg of GBE every 3 weeks for 3 months (22).
and then incubated for 1 h with biotin-conjugated anti-mouse antibodies. They were washed several times for 10 min each time in TBS–0.05% Tween 20, 0.1% bovine serum albumin (BSA), 0.1% gelatin, and 0.05% Tween 20 (TBT).

Culture supernatants from positive hybridomas diluted 1:2 with TBS containing 3% gelatin and incubated overnight with the catalyst H2O2, the reaction was stopped by addition of 2 N HCl. The plates were washed in PBS–0.1% BSA. The preparations were then washed in PBS and analyzed by flow cytometry (FACScan; Becton Dickinson).

CIE and crossed immunodiffusion electrophoresis (CIAE). CIE was done by the techniques of Mouton et al. (22) and Chandad and Mouton (4). In the first dimension, 100 μg of GBE was separated by electrophoresis in an agarose gel. In the second dimension, the migration of these bacterial antigens in an agarose gel containing anti-GBE serum led to their specific immunoprecipitation as precipitation arcs. An intermediate agarose gel without protein was added to enhance resolution. The CIE gel served as a negative control. For CIAE (positive controls), ECMG (100 μg) or TSEM (200 μg) were incorporated into the first-dimension gel in which the bacterial extracts were electrophoresed.

**Fusion and production of MAb. (i) Immunizations.** The immunoprecipitate (IP) retarded by ECMG in CIAE was identified, and the corresponding IP was excised from replicate gels. Three IPs, homogenized with a pestle designed for Eppendorf microtubes, were used to inject each mouse. The homogenate was prepared with 200 μl of Freund’s complete adjuvant for the first immunization and with the same amount of Freund’s incomplete adjuvant for subsequent injections. Three intraperitoneal injections were given at 3-week intervals.

(ii) Fusion. MAbs were obtained from the spleen of a BALB/c mouse immunized with IPs. Spleen lymphocytes were fused with Sp2/O-Ag14 myeloma cells at a 1:5 ratio by using 50% (wt/vol) polyethylene glycol 1450–10% (vol/vol) dimethyl sulfoxide (Sigma Chemical Co.) and dispensed into 96-well culture plates at 100,000 cells per well on a feeder layer of peritoneal macrophages (5,000 cells/well).

(iii) Screening. After 10 to 14 days of growth, the hybridoma culture supernatants were tested by enzyme-linked immunosorbent assay (ELISA) to detect anti-P. gingivalis antibodies. Briefly, a 96-well microplate (Maxisorb; Nunc Co.) was coated with 100 μg of GBE at a concentration of 5 μg/ml. Culture supernatant from each hybridoma (100 μl) was added, and the reactivity was tested by using peroxidase-conjugated anti-mouse antibody diluted 1:2,000 in PBT followed. Reactivity was detected by adding o-phenylene-diamine dihydrochloride (Sigma) and the catalyst H2O2. The reaction was stopped after 10 min by adding 2 N HCl, and the plates were read at 490 nm. The negative control (background) corresponded to the absorbance obtained after incubation of the ECMG from SK-MES-1 with anti-GBE serum and then peroxidase-conjugated anti-rabbit immunoglobulins. All values were obtained after subtraction of the background. The positive control corresponded to the absorbance obtained after interaction of the GBE and ECMG in the absence of MAbs.

**Flow cytometry test for adhesion inhibition.** A 24-well macroplate was seeded with 100,000 epithelial cells per well and 400 bacteria per cell (optimal conditions established in preliminary adhesion experiments). After 1 h of incubation with MAbs partially purified by Centrufuge 100 (Amicon Co.) with a molecular mass cutoff of 100 kDa. Microtiter plates (Maxisorb; Nunc Co.) were coated with 100 μl of ECMG from SK-MES-1 cells without Triton X-100 (0.5 μg/ml). After 1 h of incubation at room temperature with orbital shaking, the plates were placed at 4°C overnight. The wells were washed three times with PBS and then blocked for 1 h in PBS–1% BSA. Culture supernatants concentrated by four centrifugations in Centrufuge 100 and containing prepurified MAbs were serially diluted 1:3 to 1:155 in PBS containing 0.1% BSA and 0.05% Tween 20 (PBT). They were preincubated for 1 h at 37°C and 0.5-μg/ml GBE. A 100-μl volume of each mixture was added to the coated plates, which were then incubated for 1 h. Adhesion was detected by ELISA using 100 μl of rabbit anti-GBE serum diluted 1:1,000 in PBT. A 1-h incubation with peroxidase-conjugated goat anti-rabbit immunoglobulins (heavy-chain-specific immunoglobulin G; Dako) diluted 1:2,000 in PBT followed. Reactivity was detected by adding o-phenylene-diamine dihydrochloride and the catalyst H2O2. The reaction was stopped after 10 min by adding 2 N HCl, and the plates were read at 490 nm. The negative control (background) corresponded to the absorbance obtained after incubation of the ECMG from SK-MES-1 with anti-GBE serum and then peroxidase-conjugated anti-rabbit immunoglobulins. All values were obtained after subtraction of the background. The positive control corresponded to the absorbance obtained after interaction of the GBE and ECMG in the absence of MAbs.

**Flow cytometry test for adhesion inhibition.** A 24-well macroplate was seeded with 100,000 epithelial cells per well and 400 bacteria per cell (optimal conditions established in preliminary adhesion experiments). After 1 h of incubation with MAbs partially purified by Centrifuge 100 (as mentioned above) and serially diluted 1:3 to 1:27, the cells were trypsinized and centrifuged at 1,500 rpm. They were fixed on ice for 20 min with 500 μl of 70% cold ethanol. The pellet was washed in PBS–0.1% BSA and then blocked for 1 h in the same buffer. After centrifugation, the fixed bacteria were detected by using 50 μl of rabbit anti-GBE serum diluted 1:1,000 in PBS–0.1% BSA, followed by fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (heavy chain specific) diluted 1:1,000 in PBS–0.1% BSA. The preparations were then washed in PBS and analyzed by flow cytometry (FACSscan; Becton Dickinson).
RESULTS

Adherence of *P. gingivalis* to SK-MES-1 epithelial cells. Scanning electron microscopic observations (Fig. 1) showed *P. gingivalis* cells attached to SK-MES-1 epithelial cells.

**CIE and CIAE.** The *P. gingivalis* CIE profile used as a negative control in our experiments showed at least eight IPs (Fig. 2A). We observed much greater retardation of one IP than the others in the CIAE profile. This positive finding, noted for both ECMP (Fig. 2B) and TSEM (Fig. 2C), thus shows an important affinity between a *P. gingivalis* surface antigen (the ligand) and a complementary binding site on the ECMP (the receptor). Consequently, this IP was used for immunization of mice for the production of anti-ligand MAbs.

**Characterization of MAbs.** To have enough antigen for subsequent immunization of the mice, the mice were arbitrarily immunized with three IPs per injection. After cloning and subcloning, five MAbs were obtained after immunization with the three IPs corresponding to the ligand. All were of the immunoglobulin M isotype. The various MAbs were tested for the ability to recognize surface antigens by Western blotting of OMs and GBE. Results obtained with the five MAbs were compared with those obtained by using two MAbs already characterized as antifimbrial (7) and anti-HA-Ag2 (2) (Fig. 3A). All of the MAbs obtained reacted with polypeptides of 28, 42, and 49 kDa; 28- and 42-kDa bands were detected on OM blots, and 42- and 49-kDa bands were detected on GBE blots.
MAbs 9, 105, and 245 also detected a 43-kDa band in OMs. Furthermore, all of the MAbs reacted specifically with the 28- and 42-kDa subunits of purified fimbriae (Fig. 3B).

With GBE, MAbs 105, 245, 14, and 75 detected a high-molecular-mass band (100 kDa). All of the MAbs detected a ladder-like pattern on GBE blots. A 70-kDa band was especially reactive. The MAbs also recognized several unidentified bands of low molecular weight.

Inhibition of the ligand-receptor interaction by MAbs. All five MAbs were tested for the ability to inhibit the interaction of \textit{P. gingivalis} with epithelial cells.

The equation $100 - \left\{ \frac{\text{absorbance in presence of GBE and MAb} - \text{absorbance without GBE or MAb}}{\text{absorbance in presence of GBE}} \right\} \times 100$ was used to calculate the percentage of inhibition.

Of all the MAbs obtained inhibited the interaction between GBE and ECMP, the degree being inversely proportional to the dilution factor of the MAb (Fig. 4). A control test using hybridoma culture medium supernatant concentrated by Centriprep 100 showed no inhibition of the GBE-ECMP interaction.

Inhibition of \textit{P. gingivalis} adhesion to epithelial cells by MAbs. The equation $100 - \left\{ \frac{\text{fluorescence of 400 bacteria in presence of MAb} - \text{fluorescence without bacteria or MAb}}{\text{fluorescence of 400 bacteria}} \right\} \times 100$ was used to calculate the percentage of inhibition.

The same inhibition profiles were obtained by flow cytometry for all of the MAbs. The results obtained with the five MAbs confirmed the inhibition of \textit{P. gingivalis} adhesion to epithelial cells. Figure 5 shows flow cytometry histograms demonstrating the inhibition of \textit{P. gingivalis} binding to epithelial cells by MAb 75. Culture supernatants diluted 1:27 showed an inhibition of 11.5%; this increased slightly to 13.7% at 1:9 and 30.3% at 1:3 and reaching 60.9% when the supernatant was undiluted. Binding inhibition was incomplete (60.9%) when the supernatant was used neat and in antibody excess compared with the number of bacteria. Inhibition was reduced when the MAb concentration in the medium decreased.

A control test using hybridoma medium concentrated by Centriprep 100 showed no inhibition of \textit{P. gingivalis} adhesion to epithelial cells.

**DISCUSSION**

The binding of the gram-negative anaerobe \textit{P. gingivalis} to epithelial cells has been reported on several occasions (9, 14, 17, 23). Although the role of fimbriae has been mentioned in a number of \textit{P. gingivalis} adhesive interactions, the exact involvement of the fimbrial molecule in the adherence to epithelial cells has not been clearly established.

In parallel, the hemagglutinating adhesin HA-Ag2, which seems to be complexed with the fimbriae, confers hemagglutination activity on the bacterial cells (2). If these two adhesins, one fimbrial, the other nonfimbrial, participate in hemagglutination, we will have shown for the first time that they are also involved in adhesion to epithelial cells. They constitute what we refer to as a ligand, as opposed to the receptor on the epithelium.

To circumvent the problem of purifying the ligand prior to characterization, we used the technique of CIAE, which was used for the characterization of ligand-receptor interactions by Bjerrum et al. (1). Membrane proteins of epithelial cells from...
an established line were introduced into the first dimension of the gel in which the GBE was electrophoresed. In CIE, proteinaceous antigens retain their native form and their biological activity. Where biospecific ligand-receptor interactions exist, they are not destroyed. The CIE profiles obtained for *P. gingivalis* in the present study were not strictly identical to those obtained by Mouton et al. (22). The differences observed relate to the extraction of the surface antigens. These antigens, used for the production of rabbit anti-GBE serum, were, like the CIE profile, different. Such variations in the content of bacterial membrane extracts have already been demonstrated. Indeed, the CIE profile obtained depends on the relative concentration of antigens and corresponding antibodies in the system (25).

The adherence of *P. gingivalis* to epithelial cells (KB cells, human gingival epithelial cells) has already been shown (8, 17, 29). To definitively characterize the molecules hitherto implicated in this function, we first confirmed the recognition of functional sites by incorporating membrane proteins into the first-dimension gel, either from established cell lines or from erythrocytes. The retardation of peak migration was also obtained with membrane proteins of human epithelial gingival cells (data not shown).

The GBE preparation method results in minimal lysis of the cytoplasmic membrane, and the concentration of nucleic acids is 20 times lower than in the preparation of cell lysates (25). The presence of EDTA inhibits cellular autolysis (28). The purification of ECMP by ultracentrifugation at 150,000 × g eliminates all extraneous elements from the cytoplasmic membrane of the epithelial cells. The CIAE results obtained correspond to a ligand-receptor type of interaction between the surface antigens of *P. gingivalis* and a complementary binding site on the cytoplasmic membrane of the epithelial cells.

After selection and cloning of positive hybridomas, five MAbs were obtained. These were tested for the ability to detect antigens in Western blots of GBE and OMs. This bank of MAbs detected polypeptides of 28, 42, 43, and 49 kDa. The 42-kDa polypeptide detected in OMs and in GBE was identified as fimbrial. These structures have been purified from *P. gingivalis* 381, and it has been shown that the fimbiae from this strain are polymers of repeated monomeric 42-kDa subunits (37). The 42-kDa polypeptide detected in OM has already been shown in a sodium dodecyl sulfate-polyacrylamide gel electrophoresis profile of different OM preparations (6). The presence of this band suggests that fimbrial subunits remain bound to the OMs of *P. gingivalis* and that the fimbiae are, in the present study, involved in adhesion to epithelial cells. The smaller, 28-kDa subunit has since been acknowledged to be a fimbrial subunit (2). The 43- and 49-kDa polypeptides which we detected in OMs and the 49-kDa polypeptide detected in GBE was identified as fimbrillin. These structures have been purified from *P. gingivalis* 381, and it has been shown that the fimbiae from this strain are polymers of repeated monomeric 42-kDa subunits (37). The 42-kDa polypeptide detected in OM has already been shown in a sodium dodecyl sulfate-polyacrylamide gel electrophoresis profile of different OM preparations (6). The presence of this band suggests that fimbrial subunits remain bound to the OMs of *P. gingivalis* and that the fimbiae are, in the present study, involved in adhesion to epithelial cells. The smaller, 28-kDa subunit has since been acknowledged to be a fimbrial subunit (2). The 43- and 49-kDa polypeptides which we detected in OMs and the 49-kDa polypeptide detected in GBE correspond to the hemagglutinating adhesins described by Chandad et al. (2, 3). The 100-kDa band, together with minor bands in the 50- to 200-kDa range, mainly in the GBE, may be attributed to polymeric forms of fimbrillin which were not fully dissociated in this highly complex preparation (2, 10). The 70-kDa band detected in GBE by all of the MAbs may correspond to the minor fimbiae (67 kDa) demonstrated by Hamada et al. (10). It was
notably demonstrated that this minor fimbrial type is also present in wild-type P. gingivalis ATCC 33277, as well as in the fimA mutant MPG1. Our work requires additional experiments using antibodies against this 67-kDa fimbrial type.

The MAbs produced in this study recognize bands with molecular masses below 28 kDa. These bands may correspond to hemagglutinins associated with Arg- and Lys-specific trypsin proteinases. Additional experiments are required to characterize these polypeptides. These numerous enzymes are secreted by the organism with a variety of different molecular masses (27, 31) and have been demonstrated to be involved in adherence to various host cells (15).

Chandad and Mouton (4) showed that the 43- and 49-kDa polypeptides from HA-Ag2 detected in OMs become 33- and 38-kDa polypeptides, respectively, in GBE. This modification in size may be due to the EDTA treatment undergone by GBE. Our results do not show these differences. However, more recent work by Chandad and Mouton (2) found 42- and 49-kDa bands, as well as 33- and 38-kDa bands, in GBE.

Overall, the MAbs obtained by CIAE after incorporation of ECMP recognized both structures suspected of adhering to erythrocytes and epithelial cells. Both types of adhesin therefore have a role in adherence to epithelial cell membranes. Different organizational models have already been invoked for these two types of adhesins (2), before their common participation in adherence to epithelial cells was clearly identified.

From the results obtained in the present study, therefore, HA-Ag2 does not have a unique role in hemagglutination and the fimbriae are not the only means of adherence to different cells and substrates. These two structures are closely associated. Fimbrillin cannot participate in the ligand-receptor interaction alone (8). Other surface molecules participate in this function. It is highly probable that fimbrillin forms a support structure for an associated adhesin, as in other gram-negative species (12, 13). Such a system was demonstrated by the work of Duncan et al. (8). Our results concur with those of Hamada et al. (11), who showed that the FimA protein of P. gingivalis is essential for interaction of the organism with human gingival tissue cells. They add the finding that the fimA function does not directly affect the expression of P. gingivalis hemagglutination. The partial inhibition of adherence obtained with epithelial cells and the fimA (MPG1) mutant confirms the participation of hemagglutinin.

The MAbs obtained in this study thus recognize epitopes corresponding to fimbrial subunits and to HA-Ag2. They cause 80 and 90% inhibition of the interaction between molecular structures of the P. gingivalis membrane and epithelial cells, confirming that epitopes of the fimbriae and of HA-Ag2 are functionally involved in bacterium-host adherence phenomena. Inhibition experiments using flow cytometry confirmed these results. They effectively enabled the visualization of the dynamic phenomenon of adherence and its inhibition by MAbs, in vitro, by use of cultured epithelial cells and living bacteria. We observed that inhibition of the interaction between the molecular structures of the bacterium and its host is stronger than inhibition of adherence of the whole bacterium to epithelial cells. Conformational changes may have taken place when membrane protein extracts were used, unmasking binding sites that are inaccessible on the whole cell. However, work by Isogai et al. (14) has shown that MAbs against fimbriae of P. gingivalis almost totally block its adherence, as measured by microscopic counts. Conversely, Lee et al. (18) obtained only 65% inhibition of P. gingivalis adherence to saliva-coated hydroxyapatite beads. The latter suggests that other components, such as lipopolysaccharides (21) and proteolytic adhesins (26, 35), intervene in adherence. However, these adhesins may play a minor role in adhesion compared with fimbriae and the HA-Ag2 hemagglutinin.

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