

Invasion of Human Oral Epithelial Cells by *Prevotella intermedia*

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Invasion of oral epithelial cells by pathogenic oral bacteria may represent an important virulence factor in the progression of periodontal disease. Here we report that a clinical isolate of *Prevotella intermedia*, strain 17, was found to invade a human oral epithelial cell line (KB), whereas *P. intermedia* 27, another clinical isolate, and *P. intermedia* 25611, the type strain, were not found to invade the cell line. Invasion was quantified by the recovery of viable bacteria following a standard antibiotic protection assay and observed by electron microscopy. Cytochalasin D, cycloheximide, monodansylcadaverine, and low temperature (4°C) inhibited the internalization of *P. intermedia* 17. Antibodies raised against *P. intermedia* type C fimbriae and against whole cells inhibited invasion, but the anti-type-C-fimbria antibody inhibited invasion to a greater extent than the anti-whole-cell antibody. This work provides evidence that at least one strain of *P. intermedia* can invade an oral epithelial cell line and that the type C fimbriae and a cytoskeletal rearrangement are required for this invasion.

Periodontal disease, a chronic bacterial infection of the tissues supporting the teeth, affects approximately forty-nine million people in the United States (5). *Prevotella intermedia*, a gram-negative anaerobe, has been implicated as a putative periodontal pathogen due to its isolation from lesions of patients with early periodontitis, advanced periodontitis, and acute necrotizing ulcerative gingivitis (see reference 17 for a complete review). Periodontal disease may be a bigger health risk than previously thought, since recent epidemiological data strongly suggest that periodontitis is an important risk factor for coronary heart disease (2, 6, 23).

Invasion of epithelial cells is an important step in the pathogenesis of many infections (13). Several etiologic agents of disease, including *Brucella*, *Listeria*, *Salmonella* and *Shigella* spp., invade nonphagocytic cells (7, 13, 16). The ability to survive intracellularly allows bacteria to evade the immune system and possibly to disseminate. The ability to persist within the host cell has been demonstrated to be vital for the virulence of these pathogens (12).

Two other putative periodontal pathogens—*Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*—have previously been reported to invade oral epithelial cells (9, 20, 24, 27). Hence, invasion could be a common virulence factor among bacteria associated with periodontitis. *P. intermedia* (formerly *Bacteroides melaninogenicus* subsp. *intermedius*) has previously been found in the oral epithelium and connective tissue of gnotobiotic rats by microscopic observation after infection with *P. intermedia* (1). *P. intermedia* has also been shown to invade human coronary artery endothelial and smooth muscle cells in vitro (8) and has been found in atheromatous plaques (18).

Using the standard antibiotic protection assay as modified for oral black-pigmented anaerobes (11, 20), we investigated the invasion of oral epithelial cells and the requirements for invasion by three isolates of *P. intermedia*. These were *P. intermedia* 17, a clinical isolate from a human periodontal pocket, *P. intermedia* 27, a clinical isolate from a periapical lesion, and *P. intermedia* ATCC 25611, the type strain (15). These strains

can be differentiated by the type of fimbriae that each expresses on the cell surface (22). The fimbriae of *P. intermedia* are classified solely on the basis of diameter: *P. intermedia* 17 possesses type C (8-nm-diameter) fimbriae, which are not found in the other strains, whereas strains 27 and 25611 possess type D (5-nm-diameter) and type A (1- to 2-nm-diameter) fimbriae, respectively.

P. intermedia strains were grown in Todd-Hewitt broth (Difco) supplemented with 0.5% yeast extract, 0.075% cysteine, hemin (5 µg/ml), and menadione (0.05 mg/ml) in an anaerobic chamber (Coy, Ann Arbor, Mich.) with an atmosphere composed of 5% CO₂, 10% H₂, and 85% N₂. *E. coli* MC1061 was grown in Luria-Bertani (LB) medium consisting of Bacto Tryptone (10 g/liter), Bacto yeast extract (5 g/liter), and NaCl (10 g/liter) under aerobic conditions. KB cells (ATCC CCL-17) were maintained in minimum essential medium (Mediatech, Herndon, Va.) supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, Utah), 200 mM L-glutamine (Sigma Chemical Co., St. Louis, Mo.), and 100 mg of penicillin-streptomycin/ml (Sigma). For the invasion assay, approximately 10⁵ KB cells seeded in wells of 24-well tissue culture plates (Sarstedt, Newton, N.C.) were washed three times with phosphate-buffered saline (PBS) and then infected by the addition of a resuspended overnight culture of 10⁷ *P. intermedia* cells in 1.0 ml of antibiotic-free medium at 37°C. After 90 min of aerobic incubation, the media were removed from infected cells and the cells were washed three times with PBS. Medium containing gentamicin (300 µg/ml) and metronidazole (200 µg/ml) was then added to each well, and the plates were incubated for an additional 60 min aerobically at 37°C. Control wells without KB cells were also included to establish that the antibiotic treatment was effective in killing the extracellular bacteria of all strains used in this study. Finally, the media were removed, and the cells were washed three times with PBS and lysed by the addition of sterile distilled water and subsequent incubation for 20 min at 37°C under aerobic conditions. Dilutions of the cell lysates infected with *P. intermedia* were plated in triplicate on tryptic soy agar (Difco) plates supplemented with 5.0% sheep blood, 0.5% yeast extract, hemin (5 µg/ml), and menadione (5 µg/ml). Plates of *P. intermedia* were cultured under anaerobic conditions, while the dilutions of the lysates of *E. coli* MC1061 were plated on LB agar and cultured at 37°C aerobically. CFU of invasive bacteria were then enumerated.

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TABLE 1. Invasion of KB cells by *P. intermedia* strains

Strain	No. of CFU recovered ^a
<i>P. intermedia</i> 17	$4.1 \times 10^4 \pm 2.7 \times 10^4$
<i>P. intermedia</i> 27	328 \pm 272
<i>P. intermedia</i> 25611	152 \pm 57
<i>P. gingivalis</i> 381 ^b	$9.6 \times 10^5 \pm 1.0 \times 10^5$
<i>E. coli</i> MC1061 ^c	278 \pm 133

^a Values represent the means \pm the standard deviations for triplicate samples of lysates from the infection of 10^5 KB cells by 10^7 bacteria ($n = 4$).

^b *P. gingivalis* 381 was used as the positive control.

^c *E. coli* MC1061 was used as the negative control.

Viability of invaded cells prior to lysis was verified by trypan blue exclusion.

P. intermedia 17 showed a significantly greater ability to be internalized than the other two *P. intermedia* strains and a non-invasive *E. coli* strain (Table 1). Although the level of invasion of strain 17 was approximately 10-fold less than that of the positive control, *P. gingivalis*, the level of invasion was approximately 100-fold greater than that of the negative control. Conversely, the ability of *P. intermedia* 27 and *P. intermedia* 25611 to enter the KB monolayer was no greater than that of the negative control, *E. coli* MC1061. Therefore, the data indicate that of the three strains tested, only strain 17 invades KB cells.

Factors that potentially inhibit cellular invasion—temperature and the presence of cycloheximide (Sigma), cytochalasin D (Sigma), and monodansylcadaverine (MDC) (Sigma)—by *P. intermedia* were investigated. To test the effects of temperature, the invasion assay was performed as described above except that the incubations were done at 4°C. Cycloheximide (100 μ g/ml in ethanol) was preincubated with the KB cells for 4 h prior to the addition of the bacteria and was present during the assay. Cytochalasin D (1 μ g/ml in dimethyl sulfoxide) was preincubated with the KB cells for 0.5 h before addition of the bacteria and was also present during the assay. MDC (100 μ M in PBS) was preincubated with the KB cells for 1.0 h prior to the addition of the bacteria and was present during the assay. These inhibitors were also tested at the appropriate concentration for adverse effects on the viability of KB cells by trypan blue exclusion and by examining the confluency of the monolayer. In addition, dimethyl sulfoxide and ethanol were also tested for possible toxicity to *P. intermedia* by plating and enumerating CFU.

All four of the inhibitors tested greatly reduced invasion (Table 2). The number of CFU of *P. intermedia* 17 incubated at 4°C was reduced by 90.3%. This indicates that invasion requires metabolic activity of *P. intermedia*, the KB cell, or both. Cycloheximide inhibits protein synthesis in mammalian cells but not prokaryotes. Since exposure of KB cells to cycloheximide reduced strain 17 invasion by 98%, it is likely that de novo host protein synthesis is required. Cycloheximide also inhibited KB cell invasion by *A. actinomycetemcomitans* (24). Cytocha-

TABLE 2. Effects of inhibiting factors on *P. intermedia* 17 invasion of KB cells

Inhibitor	% Inhibition ^a
4°C	90.3 \pm 3.3
Cycloheximide	98.0 \pm 3.3
Cytochalasin D	98.0 \pm 0.4
MDC	95.5 \pm 5.4

^a Percent inhibition was the reduction of invasion in the presence of inhibitors compared to the invasion without inhibitors present in assays performed in parallel. Values are means \pm standard deviations ($n = 4$).

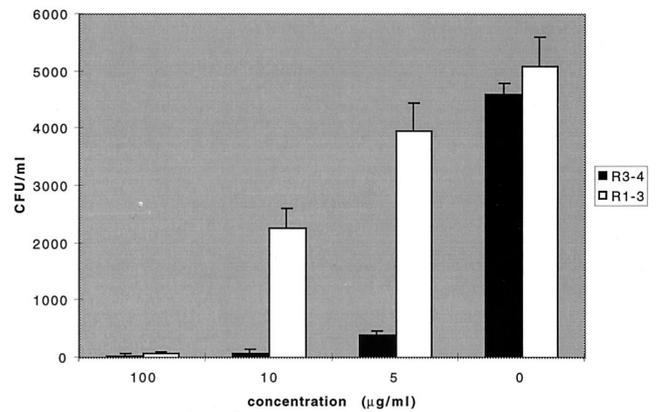
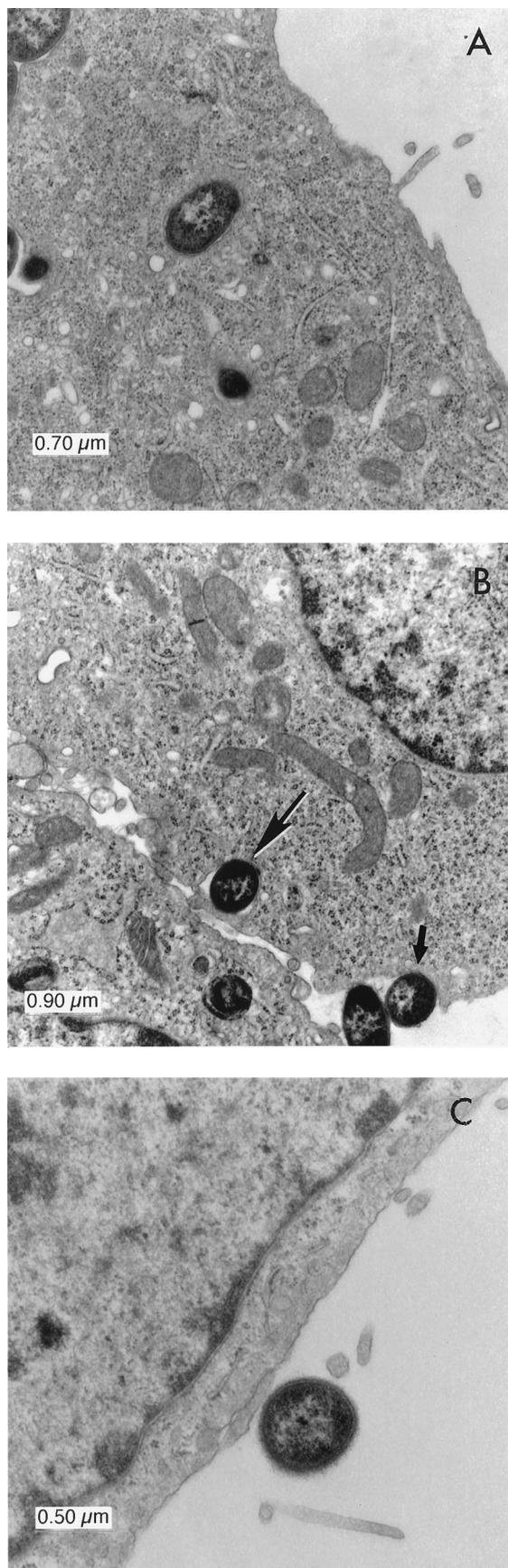


FIG. 1. Inhibition of invasion. Values represent the means of triplicate samples and reflect similar results from several experiments.

lasin D, an inhibitor of actin polymerization, reduced internalization by 98%. This inhibitor also prevented invasion in many other species of pathogenic bacteria, e.g., *A. actinomycetemcomitans*, *Listeria monocytogenes*, *P. gingivalis*, *Salmonella typhimurium*, and *Shigella flexneri* (4, 14, 16, 20, 24). A strategy common among invasive bacteria is to trigger the host cell to undergo cytoskeletal rearrangements mediated by actin polymerization (25), and internalization may occur through receptor-mediated endocytosis (RME). To confirm that invasion happens via RME, the effects of MDC were investigated, since it has been shown to reduce invasion of oral epithelial cells by *P. gingivalis* and *A. actinomycetemcomitans* (26, 28). This inhibitor of RME reduced invasion by 95.5%.

Antibodies to type C fimbriae (R3-4) and to *P. intermedia* whole cells (R1-3) were produced as previously described (21). Inhibition of invasion by specific antibodies was investigated by preincubating a series of dilutions of the affinity-purified immunoglobulin G of both antibodies with *P. intermedia* 17 for 1 h before infection and throughout the assay. The respective preimmune sera were included as negative controls. Both antibodies to *P. intermedia* 17 were found to inhibit invasion (Fig. 1). However, R3-4 had a greater inhibitory effect upon invasion than an equal concentration of R1-3. The assay to determine the effects of antibody inhibition included *P. intermedia* 17 without antibodies and included one more hour of aerobically than the standard invasion assay due to the bacterium-antibody incubation time. This extra hour may push *P. intermedia* 17 past the limit of aerotolerance and survival, which could account for the difference in the invasion efficiency as reported in Table 1. Preimmune sera at dilutions of 1:200 and 1:400 (for R3-0 and R1-0) had a negligible inhibitory effect upon invasion (data not shown).

P. intermedia 17 possesses type C fimbriae, which are lacking in other strains. The type C fimbriae are 8 nm in diameter and are present on the cell surface in varying numbers (22). *P. intermedia* 17 has been reported to bind to human buccal epithelial cells more avidly than strains 27 and 25611 (2.9 and 3.1 times, respectively). *P. intermedia* 17 also possesses strong agglutinating activity for several mammalian erythrocytes (human, monkey, mouse, rabbit, and sheep), and the hemagglutinating activity observed may be due to the presence of type C fimbriae or fimbrial components (21). The present study demonstrates that anti-type-C-fimbria antibodies had a much greater inhibitory effect upon invasion by *P. intermedia* 17 than the anti-whole-cell antibodies. A study of *P. gingivalis* also implicated fimbria involvement with invasion of gingival epithe-



lial cells (29). The type C fimbriae could promote invasion by providing a means for the bacteria to attach to the cell surface. A receptor on oral epithelial cells may be specific for fimbriae, whereby the binding may induce internalization. It is thought that the bacteria need to attach to the cell surface (19) and then induce host cell or bacterial changes to gain entry to the host cell (3, 10). Possibly, a separate ligand-receptor interaction is necessary for the internalization during the association between *P. intermedia* and the oral epithelial cell as promoted by the type C fimbriae. These results suggest that the type C fimbriae of *P. intermedia* 17 are likely to be involved in the invasion of KB cells.

To confirm that *P. intermedia* 17 cells were internalized by cultured KB cells as demonstrated in the *in vitro* assay, infected monolayers were examined by transmission electron microscopy (TEM). The bacteria were incubated with the KB cells for 90 min, at which time they were fixed in 2% glutaraldehyde at room temperature for 1 h. After the cells were centrifuged and the pellet was washed with PBS (pH 7.3), 3 drops of low-gelling 3% agarose was added and the pellet was solidified at 4°C for 10 min. The agarose-embedded pellet was then washed twice for 10 min each time in PBS, postfixed in 1% osmium tetroxide for 1 h, and washed three times in distilled H₂O for 10 min each time. The specimens were then dehydrated in a graded series of ethanol and stained overnight en bloc in 2% uranyl acetate. Following the last ethanol treatment, the specimens were infiltrated and embedded in EM Bed-812 (Electron Microscopy Sciences, Ft. Washington, Pa.). Thin sections were cut, poststained with uranyl acetate and lead citrate, and examined in a Hitachi 7000 transmission electron microscope.

Results of TEM confirmed the internalization of *P. intermedia* 17 within the KB cell monolayer (Fig. 2A). *P. intermedia* 17 cells were enclosed within membrane-bound vacuoles. Dense matrix-like materials were found closely associated with these vacuoles containing the bacterial cells, suggesting that the vacuoles were located intracellularly. In several instances *P. intermedia* 17 cells were observed to be attached to the KB cell surface, and some appeared to be involved in invagination by the KB cell (Fig. 2B). TEM also showed the absence of internalized *P. intermedia* 27 and *P. intermedia* 25611, although *P. intermedia* 27 cells were occasionally found attached to the KB cell (Fig. 2C). However, *P. intermedia* 25611 cells were rarely observed to be attached.

The use of the standard antibiotic protection assay with KB cells has been established as a reliable *in vitro* cell culture model to assess whether a periodontal pathogen is invasive. We have demonstrated that *P. intermedia* 17 can invade human oral epithelial cells *in vitro*, but *P. intermedia* 27 and 25611 cannot invade under the conditions employed here. However, KB cells do not support invasion efficiencies as high as those supported by primary cultures of gingival epithelial cells (20). Consequently, the invasion efficiency of *P. intermedia* 17 may be higher in primary cultures of human oral epithelial cells. This work also provides evidence that the type C fimbriae and a cytoskeletal rearrangement are required for invasion of oral epithelial cells by *P. intermedia*. Since invasion may be central to the infectious process, the differentiation between these strains may be important in the diagnosis and treatment not

FIG. 2. Transmission electron micrographs of KB cells infected with *P. intermedia*. (A) *P. intermedia* 17 cell internalized by a KB cell. (B) *P. intermedia* 17 cells with intimate attachment to a KB cell (small arrow) and invagination around a *P. intermedia* 17 cell (large arrow). (C) *P. intermedia* 27 cell attached to a KB cell.

only periodontal disease but also of any related systemic conditions.

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