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Porphyromonas gingivalis Invasion of Gingival Epithelial Cells

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***Porphyromonas gingivalis*, a periodontal pathogen, can invade primary cultures of gingival epithelial cells. Optimal invasion occurred at a relatively low multiplicity of infection (i.e., 100) and demonstrated saturation at a higher multiplicity of infection. Following the lag phase, during which bacteria invaded poorly, invasion was independent of growth phase. *P. gingivalis* was capable of replicating within the epithelial cells. Invasion was an active process requiring both bacterial and epithelial cell energy production. Invasion was sensitive to inhibitors of microfilaments and microtubules, demonstrating that epithelial cell cytoskeletal rearrangements are involved in bacterial entry. *P. gingivalis*, but not epithelial cell, protein synthesis was necessary for invasion. Invasion within the epithelial cells was not blocked by inhibitors of protein kinase activity. Invasion was inhibited by protease inhibitors, suggesting that *P. gingivalis* proteases may be involved in the invasion process. Low-passage clinical isolates of *P. gingivalis* invaded with higher efficiency than the type strain. Serum inhibited invasion of the type strain but had no effect on the invasion of a clinical isolate. Invasion of gingival epithelial cells by *P. gingivalis* may contribute to the pathology of periodontal diseases.**

Periodontal diseases are a group of infections that lead to inflammation of the gingiva, destruction of the periodontal tissues, and, in severe cases, loss of alveolar bone with eventual exfoliation of teeth. A variety of bacterial species are associated with the onset and progression of disease (33). In particular, evidence implicating a pathogenic role for a group of gram-negative anaerobes including the species *Porphyromonas gingivalis* has accumulated (31, 33). The virulence factors of *P. gingivalis* have been studied in some detail and include extracellular proteolytic enzymes that can degrade host tissues and immune response mediators, toxic metabolites and cellular constituents, and adherence factors that promote colonization (13, 14, 18, 29, 30, 38). Nevertheless, the full extent of the interaction between the organism and the host on molecular and cellular levels remains to be determined.

Many of the bacteria that initiate infections at mammalian epithelial barriers are capable of intracellular invasion of the epithelial cells. Invasive species are found in a variety of genera, associated with both acute and chronic infections, and include *Salmonella*, *Shigella*, *Escherichia*, *Yersinia*, *Haemophilus*, *Listeria*, *Brucella*, *Campylobacter*, and *Actinobacillus* spp. (6, 9, 10, 19, 21, 35). Invasion is considered an important virulence factor, affording protection from the host immune system and contributing to tissue damage. Invasive bacteria have evolved a variety of mechanisms for host cell entry. In general, however, the bacteria first attach to the epithelial cell membrane and then induce a series of structural and biochemical changes that facilitate bacterial penetration. The host cell signaling events that precede invasion can involve intracellular [Ca^{2+}] fluxes, protein phosphorylation, and protein synthesis and ultimately result in rearrangements of the cellular cytoskeleton to accommodate the membrane invaginations that bring the bacteria into the cell (1, 7, 24).

Invasion of *P. gingivalis* was first demonstrated by our group by use of primary cultures of gingival epithelial cells (16). *P. gingivalis* has also been shown to invade cells of multilayered pocket epithelium (26) and transformed epithelial cells (5). However, the molecular and physiological mechanisms of entry have not been characterized. The goals of this study were to determine the optimal conditions for invasion and to utilize inhibitors of bacterial and epithelial cell functions to elucidate the nature of the internalization process.

MATERIALS AND METHODS

Bacteria and culture conditions. *P. gingivalis* 33277 is the type strain originally obtained from the American Type Culture Collection. *P. gingivalis* MP4-504 and 4612 are low-passage clinical isolates from periodontal lesions (15). Bacteria were maintained as frozen stock cultures and grown anaerobically at 37°C in Trypticase soy broth supplemented with 1 g of yeast extract per liter, 5 mg of hemin per liter, and 1 mg of menadione per liter. *Streptococcus gordonii* G9B is a laboratory strain maintained as frozen stock cultures. *S. gordonii* was grown anaerobically at 37°C in Trypticase peptone broth supplemented with 5 g of yeast extract per liter and 0.5% glucose.

Culture of gingival epithelial cells. Primary cultures of gingival epithelial cells were generated as described previously (16, 20). Briefly, healthy gingival tissue was collected from patients undergoing surgery for removal of impacted third molars. Specimens were cut into small pieces and incubated with 0.4% dispase (B-M Biochemicals, Indianapolis, Ind.) overnight at room temperature. The surface epithelium was separated and placed in sterile phosphate-buffered saline (PBS) containing 0.05% trypsin and 0.53 mM EDTA to dissociate the intact epithelium into single-cell suspensions. Cells were collected by centrifugation, suspended in keratinocyte growth medium (KGM; Clonetics, San Diego, Calif.), and incubated at 37°C in 5% CO_2. At confluence, the cells were trypsinized and reseeded at 7×10^4 cells per well in 24-well culture dishes with KGM that did not contain antibiotics. The cells were incubated for a further 48 h until they were at approximately 10^5 cells per well prior to use in an invasion assay (see below).

Invasion assay. Invasion of bacteria was quantitated by the standard antibiotic protection assay (36) as modified for *P. gingivalis* (5, 25). Bacteria were harvested, washed, and resuspended in KGM without antibiotics. Autoaggregation of all strains was negligible under these conditions. The numbers of organisms were determined in a Klett-Summerson photometer and confirmed retroactively by viable counting on blood agar supplemented with hemin and menadione. *P. gingivalis* was reacted (without centrifugation) with gingival epithelial cells for 90 min (unless otherwise noted) at 37°C under normal aerobic conditions. External, nonadherent bacteria were removed by washing three times in PBS, and external adherent bacteria were then killed by incubating for 1 h with 300 μg of gentamicin per ml and 200 μg of metronidazole per ml. These concentrations of antibiotics were sufficient to completely kill 10^9 bacteria per ml in 1 h. The clinical isolates, MP4-504 and 4612, were equally sensitive to the combined antibiotics at the concentrations specified above. The antibiotics did not affect

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the morphology of the epithelial cells or alter their ability to exclude trypan blue. Controls for antibiotic killing were included in all experiments. After exposure to the antibiotic, the cells were washed in PBS (three times), and internal bacteria were released by lysis of the cells in sterile distilled water for 20 min (control experiments demonstrated that exposure to water for 20 min did not affect bacterial viability). Dilutions of the lysate were plated on blood agar supplemented with hemin and menadione and cultured anaerobically. CFU of invasive organisms were then enumerated. Invasion was expressed as the percentage of the initial inoculum recovered after antibiotic treatment and epithelial cell lysis.

Inhibitors of bacterial and epithelial cell functions. The effects of a variety of inhibitors of procaryotic and eucaryotic cell functions on *P. gingivalis* invasion were investigated. Chemicals were obtained from Sigma Chemical Co., St. Louis, Mo. The following inhibitors, in the solvent and at the final concentration indicated, were used. Cytochalasin D, 1 μg/ml in dimethyl sulfoxide (DMSO); nocodazole, 10 μg/ml in DMSO; staurosporine, 1 μM in DMSO; 2,4-dinitrophenol (DNP), 500 μM in KGM; carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), 500 μM in ethanol; sodium azide, 50 mM in PBS; cycloheximide, 100 μg/ml in ethanol; chloramphenicol, 5 μg/ml in KGM; rifampin, 0.25 μg/ml in acetone; nalidixic acid, 5 μg/ml in 1 N NaOH; and a cocktail of protease inhibitors containing aprotinin, 2 μg/ml in H_2O, leupeptin, 0.5 μg/ml in H_2O, phenylmethylsulfonyl fluoride, 0.1 mM in methanol, pepstatin, 0.7 μg/ml in methanol, and benzamidine, 1 mM in methanol. Cytochalasin D and staurosporine were preincubated with the epithelial cells for 30 min prior to addition of the bacteria and remained present throughout the invasion assay. Nocodazole was preincubated with the epithelial cells for 1 h on ice and then at 37°C for 30 min prior to reacting with the bacteria. The drug was present during the invasion assay. Cycloheximide was preincubated with the epithelial cells for 4 h before bacterial addition and was present during the assay. DNP, CCCP, and sodium azide were preincubated with the epithelial cells for 4 h and then removed by washing three times in KGM prior to bacterial addition. DNP, CCCP, and sodium azide were also preincubated with the bacteria for 4 h and then removed by washing prior to the assay. Chloramphenicol, rifampin, and nalidixic acid were preincubated with the bacteria for 4 h before reaction with the epithelial cells and were present during the assay. Protease inhibitors were preincubated with the bacteria for 30 min prior to the assay. Several controls for the effects of these compounds were included. All potential inhibitors were tested at the concentration used for possible adverse effects on the epithelial cells, when compared with cells without the inhibitor, by examining the morphology of the cells and the confluency of the monolayer and by trypan blue exclusion. Cytochalasin D, nocodazole, staurosporine, cycloheximide, and the protease inhibitors were examined for any toxic effects on *P. gingivalis* as determined by viable counting. DNP, CCCP, sodium azide, chloramphenicol, rifampin, and nalidixic acid do affect *P. gingivalis* viability or growth; however, ethanol, acetone, and NaOH, which were used as solvents, were tested at the appropriate concentrations for any reduction in *P. gingivalis* numbers.

Electron microscopy. Transmission electron microscopy was performed on normal gingival epithelial cell monolayers (3.5×10^5 cells per 35-mm petri dish), monolayers incubated with *P. gingivalis* (100 *P. gingivalis* bacteria per epithelial cell), and monolayers incubated with *P. gingivalis* in the presence of either cytochalasin D or nocodazole by the procedures described above. The incubation time for all samples with or without *P. gingivalis* was 90 min. Following respective incubations in 35-mm petri dishes and multiple PBS rinses, the specimens were fixed in a solution containing 3% (vol/vol) glutaraldehyde, 4% (wt/vol) sucrose, and 0.2% (wt/vol) ruthenium red in 0.1 M Na cacodylate, at room temperature, for 1 h. Cells were postfixed in 1% OsO_4 in cacodylate buffer containing 0.05% ruthenium red, at room temperature, for 50 min. Cells were then dehydrated through a graded series of ethanol and embedded in Medcast resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a JEOL 1200 EX II transmission electron microscope.

Normal human serum. Whole blood was obtained from several healthy humans (laboratory volunteers). After clotting, serum was obtained by centrifugation and pooled. Modulation of invasion by serum was examined by preincubating a series of dilutions of pooled serum with *P. gingivalis* for 1 h prior to reacting with the epithelial cells. The serum did not cause agglutination of bacteria and remained present throughout the invasion assay.

RESULTS

Effects of inoculum concentration on invasion. The invasion efficiency of *P. gingivalis* 33277 over a range of inoculum concentrations (multiplicity of infection [MOI]) was measured. Invasion rates at an MOI of 10 to 1,000 were relatively high, ranging between 6.8 and 12.8%, with an optimum at an MOI of 100 (i.e., 10^7 bacteria with 10^5 epithelial cells). Invasion efficiency was markedly lower (although the actual number of bacteria recovered did not decline) at an MOI of 10,000 (Fig. 1). All subsequent experiments employed an MOI of 100. As a control, for the remote possibility that the epithelial cells were phagocytic, internalization of the oral commensal *S. gordonii* G9B was investigated. While *S. gordonii* adhered to the gingival epithelial cells (laboratory observations), no *S. gordonii* could be recovered intracellularly from these cells at any MOI (10 to 10,000). Furthermore, *Escherichia coli* DH5α showed less than 0.001% invasion at an MOI of 100.

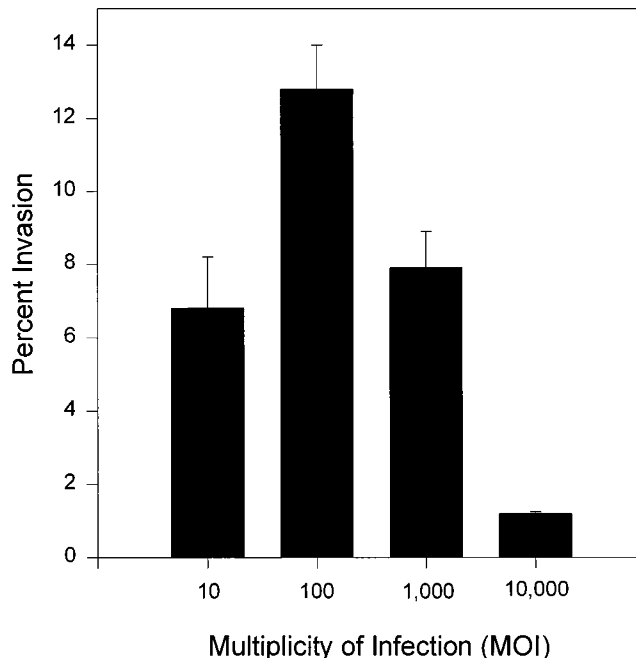

FIG. 1. Relationship between invasion of *P. gingivalis* 33277 and MOI (10^5 epithelial cells). Invasion was calculated from CFU recovered intracellularly as a percentage of total bacteria inoculated and incubated with normal epithelial cells for 90 min. Error bars represent standard errors ($n = 6$).

Effects of incubation time on invasion. The numbers of *P. gingivalis* 33277 bacteria recovered intracellularly after 30, 60, 90, 120, and 300 min of reaction time with the epithelial cells were determined (Fig. 2). Invasion efficiency increased with incubation time up to 90 min and remained constant up to 120 min. However, when the time of interaction was extended to 300 min, which is sufficient to allow doubling of *P. gingivalis* in a laboratory culture, the numbers of recovered bacteria increased further. Additional experiments were conducted to address the possibility of intracellular replication. Bacteria were incubated with the epithelial cells for 90 min, and the extracellular bacteria were killed with antibiotics. After washing to remove the antibiotics, the cells were maintained for a further 240 min to allow replication of intracellular bacteria. Antibiotics were added again to kill any released extracellular bacteria. Following epithelial cell lysis, 32% ± 5% (mean ± standard deviation; $n = 3$) of the initial inoculum of *P. gingivalis* was recovered. Thus, *P. gingivalis* appears to be capable of replication within the epithelial cells, increasing in numbers from a mean of 1.1×10^6 to a mean of 3.2×10^6 over a 240-min period.

Bacterial growth phase and invasion. To determine whether invasion efficiency is dependent on bacterial growth phase, *P. gingivalis* 33277 was harvested from broth during the lag, mid-log, early stationary, and late stationary phases of growth. The numbers were adjusted, and the bacteria were reacted with epithelial cells at an MOI of 100 for 90 min. *P. gingivalis* in the lag phase invaded poorly (2.2% ± 0.3%), whereas invasion was more efficient from the log to the late stationary phase (mid-log phase, 15.0% ± 2.1%; early stationary phase, 10.2% ±

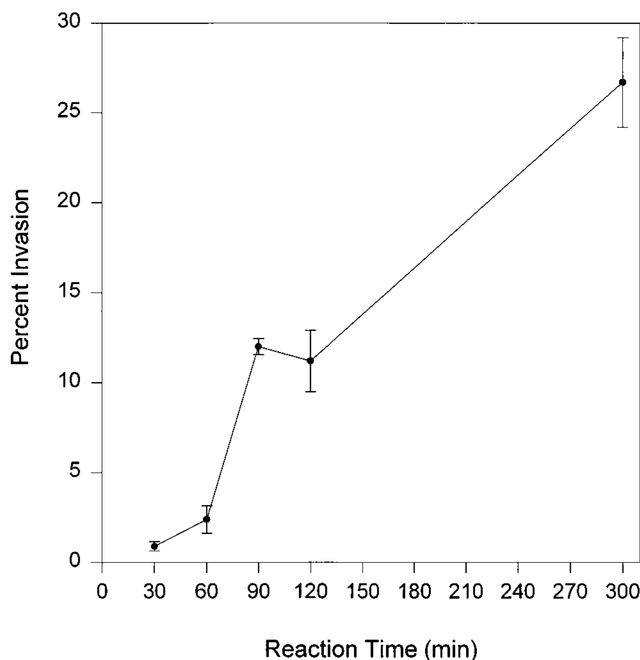

FIG. 2. Kinetic parameters of *P. gingivalis* 33277 invasion of normal gingival epithelial cells. Percent invasion was calculated from CFU recovered intracellularly after different incubation times. Error bars represent standard deviations ($n = 3$).

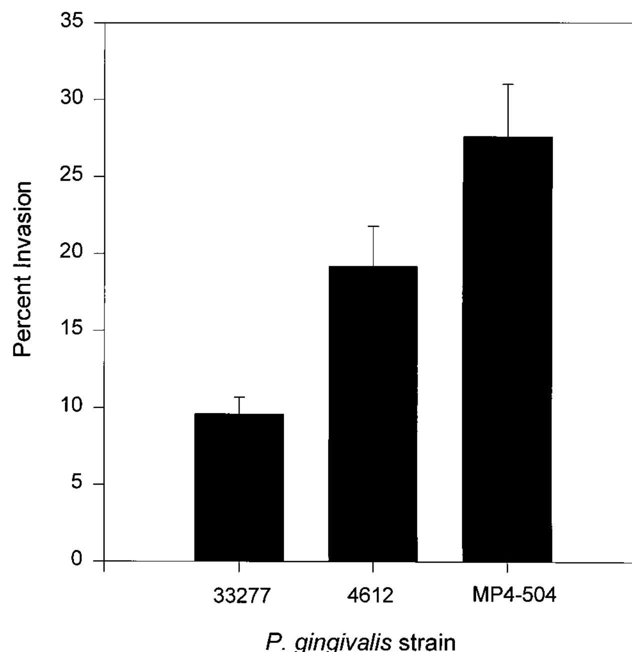

FIG. 3. Invasion of normal gingival epithelial cells by *P. gingivalis* isolates. Error bars represent standard errors ($n = 4$).

3.4%; late stationary phase, 13.9% ± 0.4% [all values are CFU recovered intracellularly from epithelial cells as a mean percentage of input bacterial number, 10^7, ± standard deviation; $n = 4$]).

Metabolic requirements for invasion. Inhibition of invasion by compounds that impede various metabolic functions of eucaryotic and procaryotic cells revealed *P. gingivalis* and epithelial cell metabolic requirements for invasion (Table 1). Cytochalasin D, an inhibitor of actin polymerization, and nocodazole, an inhibitor of microtubule formation, substantially reduced invasion, indicating that both microfilament and microtubule activity are required for invasion. Staurosporine, a broad-spectrum inhibitor of protein kinases, did not lower invasion, suggesting that protein phosphorylation is not involved in the epithelial cell signaling pathways that result in bacterial entry into the cell. De novo synthesis of epithelial cell proteins also does not appear to be required for invasion since cycloheximide, an inhibitor of eucaryotic protein synthesis, did not prevent invasion. Epithelial cell energy metabolism is important for the invasion process. DNP and CCCP, which uncouple oxidative phosphorylation and reduce proton motive force, along with sodium azide, which also reduces proton motive force and inhibits cytochrome oxidase, abrogated invasion. *P. gingivalis* energy metabolism is likewise required for invasion. DNP, CCCP, and sodium azide, when reacted with the bacteria, all abolished invasion. Bacterial DNA, RNA, and protein synthesis are necessary for invasion, since nalidixic acid, rifampin, and chloramphenicol prevented *P. gingivalis* invasion.

TABLE 1. Effects of metabolic inhibitors on *P. gingivalis* invasion

Inhibitor	Target[a]	% Inhibition[b]
Cytochalasin D	GEC	93 ± 1.4
Nocodazole	GEC	88 ± 1.7
Staurosporine	GEC	7 ± 4.3
Cycloheximide	GEC	2.4 ± 0.7
DNP	GEC	94 ± 1.2
CCCP	GEC	98 ± 0.2
Azide	GEC	100[c]
DNP	*P. gingivalis*	75 ± 10.6
CCCP	*P. gingivalis*	100
Azide	*P. gingivalis*	100
Chloramphenicol	*P. gingivalis*	98 ± 1.2
Rifampin	*P. gingivalis*	100
Nalidixic acid	*P. gingivalis*	100
Protease inhibitors	*P. gingivalis*	78 ± 1.4

[a] Inhibitors were preincubated with either the gingival epithelial cells (GEC) or *P. gingivalis* as described in Materials and Methods.
[b] Reduction in invasion in the presence of inhibitors as compared with that of a control with no inhibitor present. Values are means ± standard errors ($n = 6$).
[c] 100, no bacteria were recovered intracellularly.

Effect of protease inhibitors on invasion. The cocktail of protease inhibitors, aprotinin, leupeptin, phenylmethylsulfonyl fluoride, pepstatin, and benzamidine, at concentrations predetermined not to be detrimental to the gingival epithelial cell monolayers (see Materials and Methods), inhibited *P. gingivalis* invasion by 78% (Table 1).

Invasion of clinical isolates. The invasive capacity of low-passage clinical isolates of *P. gingivalis* was compared with that of the type strain. Figure 3 shows that strains MP4-504 and 4612, which have been subcultured fewer than five times since isolation from periodontal lesions, invaded more than twice as well as strain 33277. Invasion may, therefore, be a property that confers a selective advantage under in vivo conditions.

Effects of serum on invasion. Pooled human serum was used as a surrogate for gingival crevicular fluid to investigate possible modulation of invasion. Serum at a 1:10 dilution inhibited invasion of strain 33277 (Table 2). Inhibitory activity was lost at dilutions of 1:100 and greater. None of the concentrations of serum tested altered the invasive ability of the clinical strain MP4-504.

TABLE 2. Effects of serum on invasion of *P. gingivalis*

P. gingivalis strain	% Invasion[a]			
	Control[b]	In the presence of serum[c] diluted:		
		1:10	1:100	1:1000
33277	11.6 ± 1.5	2.0 ± 0.3	10.1 ± 2.1	13.6 ± 2.8
MP4-504	26.4 ± 0.1	27.9 ± 1.6	31.7 ± 1.7	25.1 ± 1.2

[a] CFU recovered intracellularly from epithelial cells as a percentage of input bacterial number (10^7) (mean ± standard error; $n = 6$).
[b] In the absence of serum.
[c] *P. gingivalis* was incubated with pooled human serum at the dilution indicated for 1 h prior to reacting with the epithelial cells.

Electron microscopy. Figure 4A shows normal morphology of a human gingival epithelial cell with ruthenium red deposit along the plasma membrane. Ruthenium-red-coated, surface-associated *P. gingivalis* bacteria appeared as opaque structures, without discernible outer membranes (Fig. 4B, C, E, and F and 5), while internalized organisms lacked a ruthenium red coating (Fig. 4B, C, D, and E) and clearly demonstrated the presence of gram-negative outer membranes (Fig. 4D). Endocytic vacuoles were not found surrounding internalized *P. gingivalis* organisms (Fig. 4B, C, D, and E). In addition, evidence of internalized *P. gingivalis* undergoing division was noted (Fig. 4E). There was no electron microscopic evidence to support internalization of *P. gingivalis* in cells treated with either cytochalasin D or nocodazole (Fig. 5A and B). An interesting observation was noted in Fig. 4F, where what appears to be epithelial cell cytoskeletal contact with surface-adhering *P. gingivalis* is visible.

DISCUSSION

Invasion of mammalian epithelial cells is a strategy adopted by a variety of pathogenic bacteria (7, 9). An intracellular location may sequester bacteria from the immune system, allow replication in a nutritionally rich environment, and can presage either cell-to-cell spread of the bacteria or destruction of the epithelial cell (7, 9, 27, 37). The role of bacterial invasion in periodontal diseases has been investigated only recently. Many of the clinical manifestations of periodontal disease are chronic and episodic in nature, characteristics that could be explained at least partially by bacterial invasion. Furthermore, *Actinobacillus actinomycetemcomitans* and *P. gingivalis*, important pathogens in various forms of periodontitis, can invade KB cells, a human oral epithelial cell line (5, 19, 25). Our preliminary electron microscopic observations demonstrated that *P. gingivalis* can invade primary cultures of human gingival epithelial cells (16). Sandros et al. (26) have also shown invasion of *P. gingivalis* in multilayered human pocket epithelial cells in culture. Our present electron microscopic observations, utilizing noncornified normal gingival epithelial cells incubated with *P. gingivalis* for only 90 min, support the finding of Sandros et al. (26) showing that internalized *P. gingivalis* bacteria are found associated directly with the epithelial cell cytoplasm and not encapsulated by endocytic vacuoles. However, while Sandros et al. (26) described the presence of intracellular *P. gingivalis* frequently surrounded by endosomal membranes, in which they observed the release of numerous *P. gingivalis*-associated outer membrane vesicles, we could not document such observations. Ruthenium red staining prior to processing for transmission electron microscopy permitted us to discern internal from external organisms and pseudovacuolated organisms (Fig. 5A). We cannot, however, rule out the possibility that vacuolization does occur early in invasion of *P. gingivalis*. In the present study, we have quantitated and characterized the invasion of primary cultures of gingival epithelial cells by *P. gingivalis.*

Bacterial invasion was measured by the standard antibiotic protection assay (36), modified by the inclusion of metronidazole along with gentamicin to facilitate killing of extracellular, noninvading *P. gingivalis* (25). Optimal invasion of the type strain of *P. gingivalis* (strain 33277) under these conditions was around 13% of the initial inoculum. This is of the same order of magnitude as that reported for many of the gastrointestinal tract pathogens, such as *Salmonella* and *Shigella* spp. and enteroinvasive *E. coli*, organisms whose pathogenicity depends on invasion (19, 32). The reported invasion efficiency of *P. gingivalis* 33277 for KB cells is lower, under 0.1% (5). Primary cultures of gingival epithelial cells have several advantages over cell lines, such as KB cells, for the study of invasion. The gingival epithelial cells are nontransformed and so will not have altered expression of surface receptors or internal physiology as can result from neoplasticity. Furthermore, immunohistochemical analysis (laboratory observations) with monoclonal antibodies to differentiation marker keratins K-13 and K-19 demonstrated that the epithelial cells, grown under the conditions described, were noncornified, which is a characteristic shared with junctional epithelial cells. Thus, primary cultures of noncornified gingival epithelial cells represent a relevant model for the study of *P. gingivalis* invasion.

The optimal MOI for *P. gingivalis* was 100, a value similar to that described for other classically invasive pathogens such as *Salmonella*, *Shigella*, and *Listeria* spp. (10, 19, 32). In general, the ability to invade efficiently at a relatively low MOI is indicative of specific uptake pathways. Should a high MOI be required, it is possible that the mammalian cells are simply overwhelmed by the bacterial challenge and the organisms enter by nonspecific mechanisms. Invasion efficiency of *P. gingivalis* was markedly reduced at an MOI of 10,000. Although many factors could influence the efficiency of invasion at different bacterial concentrations, the gingival epithelial cells may possess specific surface receptors for *P. gingivalis* that are required to initiate invasion. Once these receptors are fully occupied by the bacteria, increasing the number of bacteria will not further increase invasion.

The time course of the interaction between *P. gingivalis* and the epithelial cells demonstrated that about 90 min is required to complete the invasion process for most of the bacteria. A 2-h incubation of bacteria with epithelial cells did not result in increased invasion. However, when the reaction time was extended to 5 h, significantly higher numbers of internalized bacteria were recovered. In addition, killing of the external bacteria after 90 min of incubation, followed by an additional 4 h of incubation, also resulted in a notably higher recovery of internal *P. gingivalis*. It appears likely, therefore, that the bacteria are dividing within the epithelial cells. Our transmission electron microscope observations support this (Fig. 4E), as do reports of other invasive organisms, including enteric pathogens such as *Salmonella* spp. and oral pathogens such as *Actinobacillus actinomycetemcomitans*, that are also capable of intracellular replication (9, 28).

The invasive properties of *P. gingivalis* were stable over most of the growth curve, although invasion was significantly lower during the lag phase. This may be a reflection of lower viability or metabolic activity of the organisms during the lag phase and is consistent with the finding that *P. gingivalis* energy metabolism and protein synthesis are necessary for invasion. The de novo synthesized proteins then remain functionally active as the bacteria progress through their growth cycle.

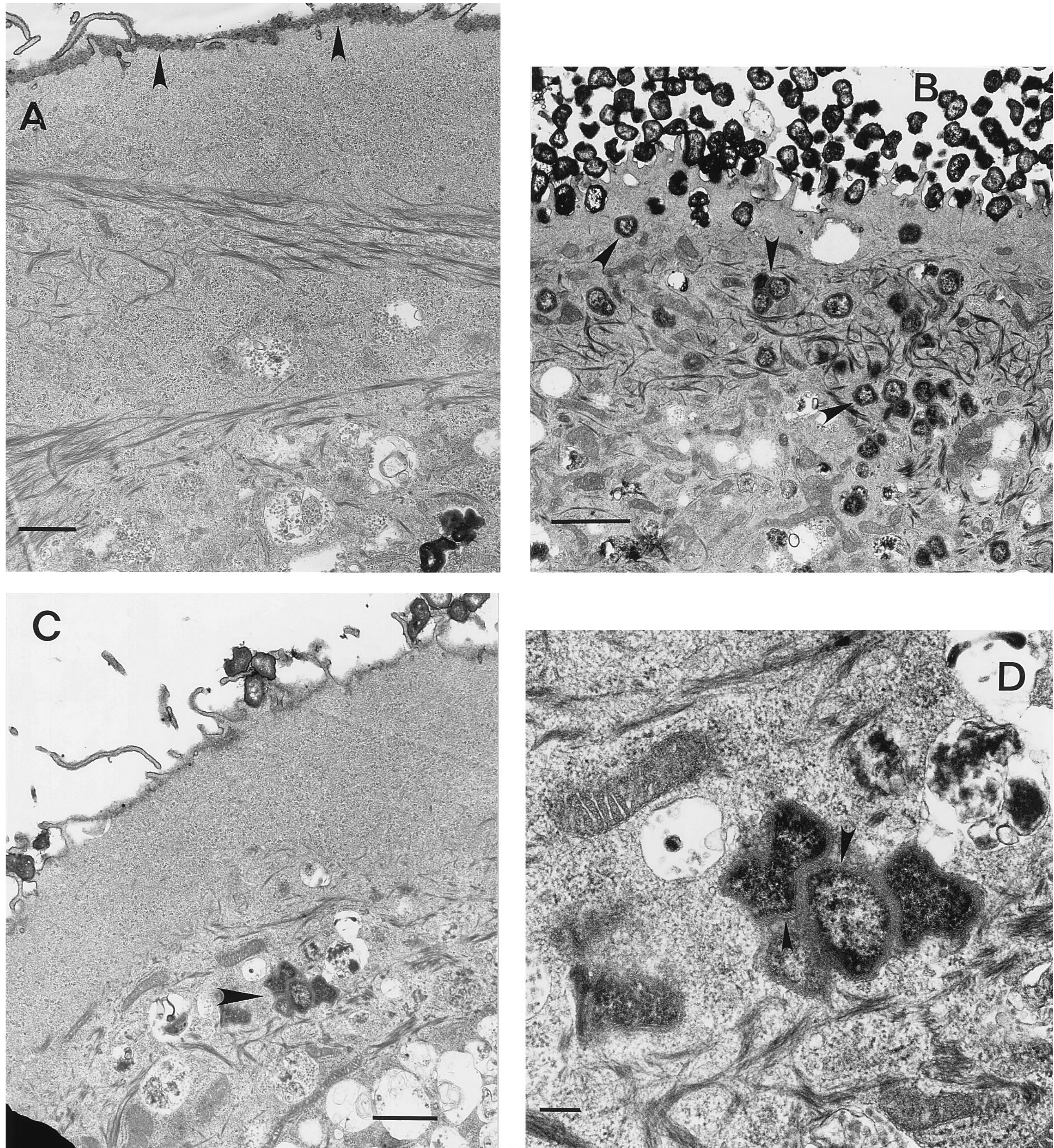

FIG. 4. Transmission electron micrographs of *P. gingivalis* 33277 invasion of normal gingival epithelial cells after 90-min incubations. (A) Normal gingival epithelial cell morphology with deposits of ruthenium red along the cell surface (arrowheads). (B) Example of many *P. gingivalis* organisms inside a gingival epithelial cell (arrowheads), without endocytic vacuoles surrounding them. Note the difference in opacity between external, ruthenium-red-coated organisms and invading organisms. (C) Another example of internalized *P. gingivalis* organisms (arrowhead). Note the absence of endocytic vesicles surrounding the bacteria. (D) Higher magnification of the group of internalized *P. gingivalis* organisms depicted in panel C. Note the discernible double-layered outer membranes (arrowheads). (E) Example of an internalized *P. gingivalis* microorganism undergoing division (arrowhead). (F) Ruthenium-red-stained *P. gingivalis* microorganisms along the epithelial cell surface. Note the apparent contact between microfilamentous cellular components and surface-adhering *P. gingivalis* (arrowheads). Bars: panels A, C, E, and F, 1 μm; panel B, 2 μm; panel D, 200 nm.

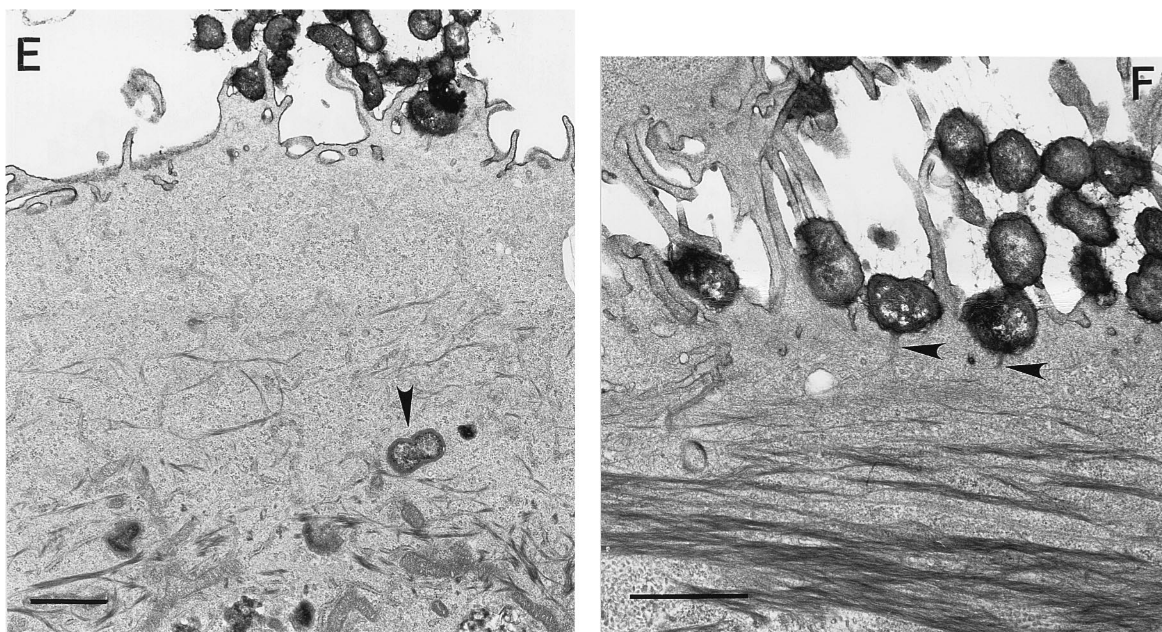

FIG. 4—*Continued.*

The interactions or cross-talk between bacteria and epithelial cells that results in bacterial internalization often involves bacterial induction of eucaryotic signaling pathways, eventually producing the membrane invaginations that engulf the bacteria in a process termed directed phagocytosis (27). Eucaryotic cytoskeletal rearrangements are usually required to effect this process (1, 9). Treatment of the epithelial cells with cytochalasin D, which inhibits actin polymerization, or nocodazole, which depolymerizes microtubules, blocked *P. gingivalis* invasion. This was confirmed by transmission electron microscopy, in which internalization of *P. gingivalis* was not observed (Fig. 5). In addition, in the presence of cytochalasin D, we did not observe epithelial cell cytoskeletal contact with surface-adhering *P. gingivalis* microorganisms, as described when this agent was not included in the invasion assay (Fig. 4F). Thus, both microfilaments and microtubules seem to be involved in invasion of this organism. Similarly, microfilaments and microtubules are required for invasion of *Neisseria gonorrhoeae*, *Haemophilus influenzae*, *Citrobacter freundii*, and enteropathogenic *E. coli* (4, 21, 22, 35), although many bacteria, such as *Actinobacillus actinomycetemcomitans*, *Yersinia enterocolitica*, *Salmonella choleraesuis*, and *Shigella flexneri*, require only microfilament activity (8, 9, 34). We did not find evidence that the signal transduction pathways within the epithelial cells that are triggered by *P. gingivalis* involve protein phosphorylation, since invasion was not blocked by staurosporine, a wide-spectrum inhibitor of protein kinases. This is in contrast to some other pathogens; e.g., the *Yersinia inv* gene-mediated invasion exploits a host cell secondary messenger pathway involving protein phosphorylation of tyrosine residues by tyrosine protein kinases (23). *P. gingivalis* may, however, subvert host cell [Ca^{2+}] signaling pathways since the organism can induce a transient elevation of intracellular [Ca^{2+}] (unpublished data). Cycloheximide, an inhibitor of mammalian protein synthesis, did not prevent *P. gingivalis* invasion, demonstrating that de novo synthesis of epithelial cell proteins is not required for invasion. This is concordant with the finding that invasion is saturable, and hence, newly synthesized epithelial receptors do not become available for the bacteria during the course of the invasion process. In contrast, *P. gingivalis* protein, RNA, and DNA syntheses are necessary for invasion. It is not yet known which step in the invasion process (that includes both initial attachment and subsequent internalization) is dependent upon de novo protein synthesis. In addition, the role of the epithelial cells in stimulating the requisite bacterial protein synthesis is another area that requires further investigation. Invasion of *P. gingivalis* required both bacterial and epithelial cell energy metabolism, consistent with the finding that bacterial synthetic and epithelial cell signaling pathways are involved in invasion. Invasion of many other pathogens, such as *Actinobacillus actinomycetemcomitans* and *Salmonella* and *Shigella* spp., is also an active, energy-dependent event (9, 34).

P. gingivalis produces a number of extracellular proteases with different specificities (11, 18). A role for these enzymes in invasion is inferred by the ability of a mixture of protease inhibitors to significantly reduce invasion. The proteases may thus be involved in the initial attachment of *P. gingivalis* to the epithelial cells, as can occur with *P. gingivalis* adherence to *Actinomyces viscosus* (17). Alternatively, the proteases may have other effects, such as exposing cryptitopes (12) in the epithelial cell receptors.

Low-passage clinical isolates of *P. gingivalis* showed higher levels of invasion than that of the type strain. This supports the concept that invasion of *P. gingivalis* is an important in vivo property that may be lost gradually or become less efficient as the bacteria adapt to laboratory culture. Further evidence for the in vivo significance of invasion is provided by the serum inhibition experiments. Bacteria in the gingival crevice are exposed continually to gingival crevicular fluid molecules that have the potential to modulate various bacterial activities such as adherence (2, 3). Crevicular fluid is difficult to obtain in levels sufficient for in vitro testing, and hence, pooled human serum was tested as a surrogate for crevicular fluid. Although high concentrations of serum inhibited invasion of the type strain, invasion of the clinical isolate MP4-504 was not reduced

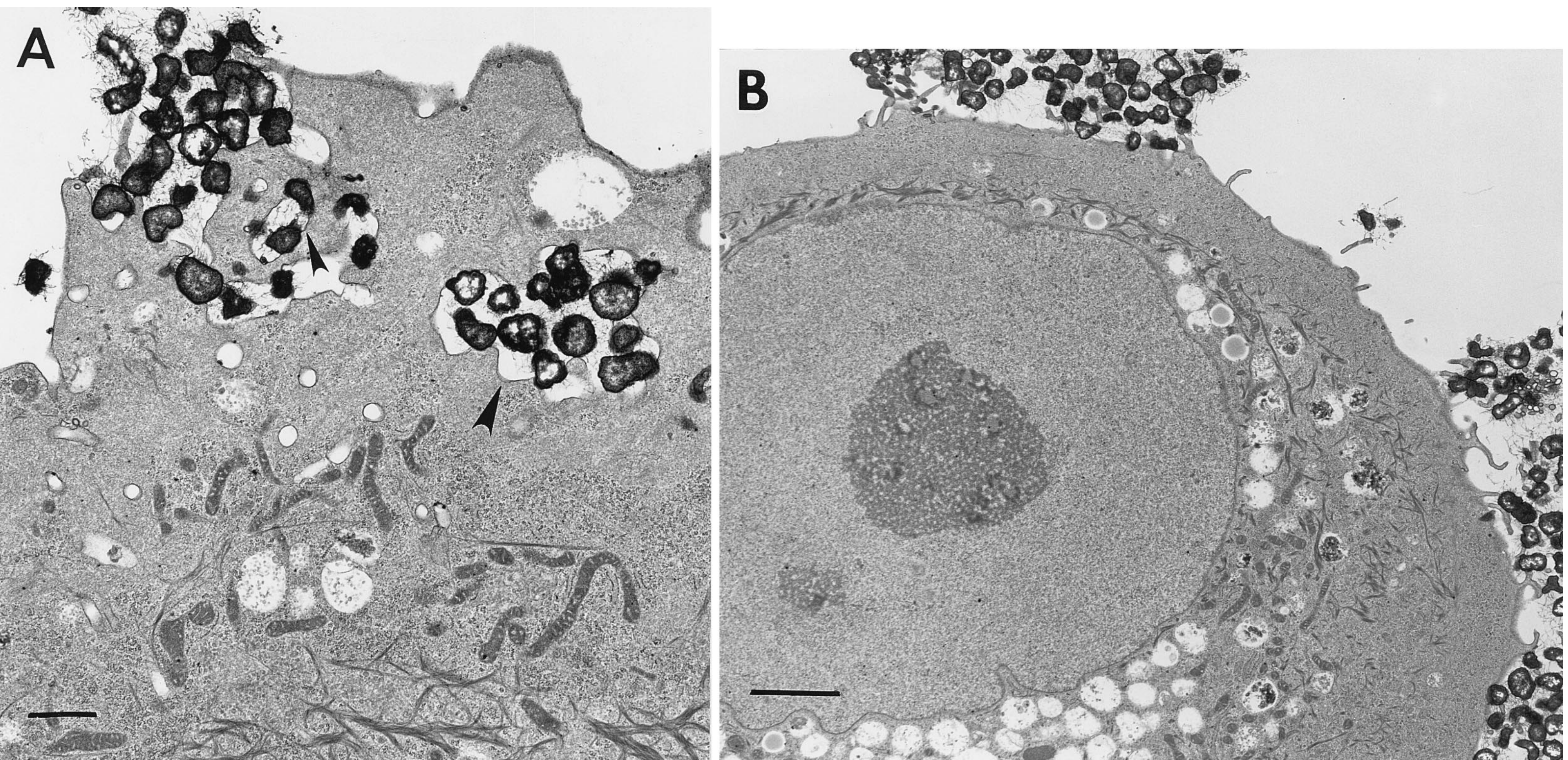

FIG. 5. Transmission electron micrographs of *P. gingivalis* 33277-normal gingival epithelial cell invasion assays in the presence of cytochalasin D or nocodazole. (A) Gingival epithelial cells treated with cytochalasin D and then incubated with *P. gingivalis*. Note the characteristic ruthenium red staining of extracellular organisms, demonstrating pseudovacuoles (arrowheads). (B) Example of gingival epithelial cells treated with nocodazole and then incubated with *P. gingivalis*. Note the absence of internalized microorganisms. Bars: panel A, 1 μm; panel B, 2 μm.

by exposure to serum (and, by extension, to crevicular fluid) molecules.

In conclusion, the periodontal pathogen *P. gingivalis* can invade primary cultures of gingival epithelial cells in high levels. The invasion mechanisms utilized by *P. gingivalis* show similarity to those of other invasive pathogens. *P. gingivalis* invasion may be important in the pathogenesis of periodontal diseases.

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