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Porphyromonas gingivalis Infection of Oral Epithelium Inhibits Neutrophil Transepithelial Migration

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Periodontal diseases are inflammatory disorders caused by microorganisms of dental plaque that colonize the gingival sulcus and, subsequently, the periodontal pocket. As in other mucosal infections, the host response to plaque bacteria is characterized by an influx of polymorphonuclear leukocytes (PMNs) to the gingival crevice. Neutrophil migration through the epithelial lining of the gingival pocket is thought to be the first line of defense against plaque bacteria. In order to model this phenomenon in vitro, we used the oral epithelial cell line KB and human PMNs in the Transwell system and examined the impact of *Porphyromonas gingivalis*-epithelial cell interactions on subsequent PMN transepithelial migration. We demonstrate here that *P. gingivalis* infection of oral epithelial cells failed to trigger transmigration of PMNs. Furthermore, it significantly inhibited neutrophil transmigration actively induced by stimuli such as *N*-formylmethionyl leucyl phenylalanine, interleukin-8 (IL-8), and the intestinal pathogen enterotoxigenic *Escherichia coli*. The ability of *P. gingivalis* to block PMN transmigration was strongly positively correlated with the ability to adhere to and invade epithelial cells. In addition, *P. gingivalis* attenuated the production of IL-8 and the expression of intercellular adhesion molecule 1 by epithelial cells. The ability of *P. gingivalis* to block neutrophil migration across an intact epithelial barrier may critically impair the potential of the host to confront the bacterial challenge and thus may play an important role in the pathogenesis of periodontal disease.

An important early step of the inflammatory response to bacterial infection is the recruitment of polymorphonuclear leukocytes or neutrophils (PMNs) which migrate from the microvasculature into the lamina propria at the site of infection (27, 35). In bacterial infections of surfaces lined by epithelia, such as the intestinal, urinary, or respiratory mucosa, PMNs further migrate through the epithelial cell barrier towards the source of infection (38, 42, 44). Transepithelial migration of neutrophils represents the hallmark of active mucosal inflammation and is believed to play an important role in the clearance of mucosal infections (2, 24, 43).

Periodontal diseases are a group of inflammatory disorders caused by microorganisms of dental plaque that colonize the gingival sulcus and, subsequently, the periodontal pocket (15). As in other mucosal infections, a common histological feature of periodontal diseases is the recruitment of neutrophils, which are found to accumulate in the gingival connective tissue, the junctional epithelium, and the periodontal pocket (28). Neutrophil migration into the gingival crevice is thought to be the first line of defense against plaque bacteria (5, 28). The protective role of neutrophils in periodontal disease is emphasized by the fact that patients with diminished PMN numbers or impaired PMN function often suffer from severe periodontal breakdown (23, 41). Furthermore, neutrophil chemotactic defects are frequently encountered in subjects affected by prepubertal, juvenile, and rapidly progressive periodontitis, which are considered to be aggressive forms of periodontal disease (13, 41).

Porphyromonas gingivalis is implicated as an important etiologic agent in periodontal disease (15, 16, 25). A large array of virulence factors identified for *P. gingivalis* are thought to enable the microorganism to induce periodontal tissue damage (for a review see reference 8). *P. gingivalis* may also employ a variety of host defense evasion mechanisms, including alteration of PMN function (26, 31, 37, 39). In addition, soluble products released from *P. gingivalis* have been shown, by a PMN locomotion assay, to inhibit PMN chemotaxis in vitro (40).

PMN migration into the gingival crevice is the result of a complex series of events, including bacterium-epithelium as well as epithelium-PMN interactions. Such events cannot be addressed by classical in vitro chemotactic assays. In order to better model the neutrophil influx into the gingival crevice in vitro, we utilized an oral epithelial cell line and ^{51}Cr-labelled PMNs in the Transwell system and examined the role of *P. gingivalis*-epithelial cell interactions in PMN transepithelial migration. We demonstrate here that *P. gingivalis* infection of oral epithelial cells has a profound inhibitory effect on neutrophil transepithelial migration induced by a variety of stimuli. In addition, we show that *P. gingivalis* attenuates the production of interleukin-8 (IL-8) and the expression of intercellular adhesion molecule 1 (ICAM-1) by oral epithelial cells. The ability of *P. gingivalis* to block neutrophil migration across an intact epithelial barrier may critically impair the potential of the host to confront the bacterial challenge and thus may play an important role in the pathogenesis of periodontal disease.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. gingivalis* 381 and DPG3 were kindly provided by R. J. Genco (State University of New York at Buffalo, Buffalo). DPG3 is a nonfimbriated mutant of 381, constructed by insertional inactivation of the *fimA* gene (20). Strains OMGS 1743 and OMGS 712 are clinical isolates of *P. gingivalis* originating from a periodontal abscess and a deep periodontal pocket, respectively (collection of the Department of Oral Microbiology, Göteborg University, Göteborg, Sweden). W50 is a laboratory strain originally obtained from G. Sundqvist, University of Umeå, Umeå, Sweden. The bacteria were grown on brucella agar plates (BBL Microbiology Systems, Cockeysville, Md.) enriched with 5% defibrinated horse blood, 0.2% hemolyzed

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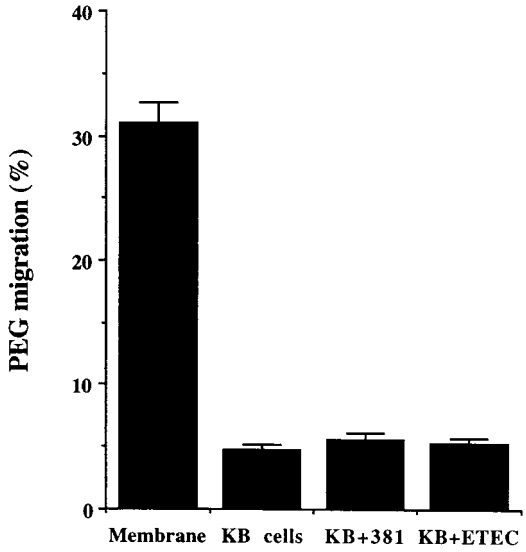

FIG. 1. Migration of [^{14}C]PEG across Transwell membranes and inverted KB epithelial cell layers during a 2-h period. KB layers were infected with *P. gingivalis* 381 (5×10^8 bacteria/ml) or ETEC (1×10^8 bacteria/ml) for 6 h, as described in Materials and Methods. Data represent the means and SDs of two experiments with triplicate samples.

blood, and 0.0005% menadione in jars with 95% H_2 and 5% CO_2 at 37°C. DPG3 was cultured on medium containing 2.5 μg of erythromycin/ml.

The enterotoxigenic *Escherichia coli* (ETEC) strain 258909-3 (14) was kindly provided by A.-M. Svennerholm (Göteborg University) and grown overnight on colonization factor antigen (CFA) agar plates (11) at 37°C.

Salmonella typhi Ty2 (culture collection of the University of Göteborg, CCUG 35699) was grown overnight on Luria-Bertani agar at 37°C.

Cell culture. The KB cell line (derived from a human oral epidermoid carcinoma) was obtained from the American Type Culture Collection (ATCC CCL 17). KB cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Paisley, United Kingdom) supplemented with 200 mM L-glutamine, 10% Cosmic Calf serum (HyClone Laboratories, Logan, Utah), and 100 mg of penicillin-streptomycin/ml in a 5% CO_2 atmosphere at 37°C. Subcultivation was performed twice a week, and cells used in the experiments were never older than 25 passages.

Bacterial-epithelial cell association and invasion assay. A previously described invasion assay (33) was modified as follows. KB cells were transferred to 24-well plates at a density of approximately 10^5 cells/well, resulting in confluent cultures 24 h later. Bacteria were collected from agar plates, washed twice in phosphate-buffered saline (PBS), pH 7.2, and suspended in culture medium devoid of serum and antibiotics (DMEM) at a concentration of 10^8 bacteria/ml. The bacterial concentrations were determined spectrophotometrically according to species-specific standard curves and subsequently confirmed by counting of viable cells. KB cell layers were washed in PBS, infected with 500 μl of the microbial suspension (infection ratio, ≈150 bacteria/cell), and incubated at 37°C for 90 min. Nonadherent bacteria were removed by being washed five times with PBS, and cell-associated bacteria were quantitated after lysis of the cell layers in 1 ml of distilled water and subsequent plating on agar. Internalized bacteria were assessed in parallel experiments after antibiotic application to kill the extracellular bacteria (500 μg of metronidazole/ml plus 500 μg of gentamicin/ml for 3 h for *P. gingivalis*-infected cell layers and 200 μg of gentamicin/ml for 2 h for ETEC- and *S. typhi*-infected cell layers).

Preparation of inverted epithelial layers. KB cells were suspended in culture medium at a concentration of 1.3×10^6 cells/ml, and 200 μl of the suspension was seeded on inverted Transwell inserts (12-mm-diameter polycarbonate membranes with 3-μm-diameter pores; Costar, Cambridge, Mass.). The cells were allowed to attach to the Transwell membranes in a humidified 5% CO_2 atmosphere at 37°C for 4 h and were then transferred to 12-well plates in fresh medium. After 2 days of incubation, confluent cell layers were obtained, as determined by Giemsa staining and transmission electron microscopy.

The permeability of the inverted KB-cell layers was studied by [^{14}C]polyethylene glycol ([^{14}C]PEG) migration (4). Transwell membranes with or without KB cells were transferred to new 12-well plates, and 0.5 ml of DMEM containing [^{14}C]PEG (0.4 μCi/ml) was added to the upper compartment while 1.5 ml of DMEM was added to the lower compartment. The amount of [^{14}C]PEG passing through the epithelial layer was assessed after 2 h of incubation by measuring the amount of radioactivity in the DMEM of the lower compartment with a beta counter (1215 Rackbeta; LKB Wallac). To examine the effect of bacterial infection on cell layer integrity, inverted cell layers were infected with *P. gingivalis* 381 (5×10^8 bacteria/ml) or ETEC 258909-3 (10^8 bacteria/ml) for 6 h, as described in "PMN transepithelial migration radioassay," below, and tested for [^{14}C]PEG migration. Figure 1 shows the results of [^{14}C]PEG migration across KB-cell layers. *P. gingivalis* and ETEC infection of the KB cells had no effect on cell layer permeability. Likewise, bacterial infection had no effect on cell layer viability as determined by trypan blue exclusion (data not shown).

PMN isolation. Neutrophils were isolated from the blood of healthy volunteers. Briefly, heparinized blood was placed on top of neutrophil isolation medium (NIM; Cardinal Associates Inc., Santa Fe, N.Mex.) and centrifuged at $400 \times g$ for 30 min at room temperature. The layer containing neutrophils was resuspended in Krebs-Ringer phosphate buffer containing glucose (KRG) (10 mM glucose, 1.2 mM Mg^{2+} [pH 7.3]) and pelleted by centrifugation at $400 \times g$ for 10 min at 4°C. Residual erythrocytes were removed by hypotonic lysis, and neutrophils were finally recovered at >95% purity, as determined by Write's Giemsa staining, and with 98% viability, as assessed by trypan blue exclusion. Neutrophils were resuspended in KRG at a concentration of 10^7 cells/ml and kept at 4°C.

PMN radiolabelling with ^{51}Cr. Neutrophils were radiolabelled with ^{51}Cr by using a modification of the method described by Gallin and coworkers (12). Neutrophils suspended in KRG were incubated with ^{51}Cr (5 μCi per 10^6 cells) for 60 min at 37°C. The neutrophils were then washed twice in KRG, resuspended in DMEM at a concentration of 2×10^6 cells/ml, and used immediately for the transmigration assay.

PMN transepithelial migration radioassay. At the beginning of each experiment, inverted KB-cell layers were transferred and kept in new trays containing medium free of serum and antibiotics (DMEM) for 1 h. Bacteria were prepared as for the cell association and invasion assay and suspended in DMEM at a concentration of 10^8 bacteria/ml. Transwell inserts were lifted from each well and placed, cell layer facing up, in a moist chamber. Two hundred fifty microliters of the bacterial suspension was placed on top of the epithelial layers (infection ratio, ≈150 bacteria/cell) and incubated for 90 min at 37°C. Nonadherent bacteria were removed by immersing the inserts three times in culture trays containing PBS. The cell layers were inverted and transferred into new 12-well culture plates containing 0.5 ml of DMEM in the upper reservoir and 1.5 ml in the lower reservoir. After a further 4.5-h incubation period, the medium in the upper compartment was removed and replaced with 500 μl of ^{51}Cr-labelled neutrophil suspension (10^6 cells/well). Positive controls for PMN transmigration were included in all experiments by adding, at the time of neutrophil application, the neutrophil chemoattractants *N*-formylmethionyl leucyl phenylalanine (fMLP) (10^{-7} M) or IL-8 (150 ng/ml; Sigma) to the lower reservoir. Transepithelial migration of neutrophils was quantitated by measuring the radioactivity of the lower reservoir with a gamma counter (Cobra II Autogamma) after a 2-h incubation period. The radioactivity associated with the PMNs was determined by subtracting the radioactivity of the residual medium obtained after removal of the PMNs by centrifugation from the total radioactivity added. The medium-associated radioactivity never exceeded 2% of the total. The PMN transmigration radioassay was validated in pilot experiments by concomitant evaluation of PMN migration by light microscopy and PMN counting in a Bürker chamber.

Effect of *P. gingivalis* infection of KB cells on fMLP-, IL-8-, and ETEC-induced transepithelial neutrophil migration. *P. gingivalis* suspensions of 0.5×10^8, 1×10^8, and 5×10^8 bacteria/ml were used to infect inverted KB-cell layers, as described for the transmigration assay. PMN migration across infected and noninfected cell layers was compared in the presence of an fMLP or IL-8 gradient. In addition, PMN transmigration was assessed across cell layers infected by either ETEC alone (10^8 bacteria/ml) or a combination of ETEC and *P. gingivalis* in the above concentrations.

Detection of IL-8 secretion by KB cells. KB cells were seeded in 24-well culture plates and allowed to grow to confluence (≈24 h). During the last 12 h, the cells were incubated in serum- and antibiotic-free medium (DMEM). At time zero, the medium was removed and 500 μl of *P. gingivalis*, ETEC, or *S. typhi* suspension in DMEM was used to infect the KB-cell layers for 90 min (infection ratio, ≈150 bacteria/cell). After being washed with PBS to remove nonadherent bacteria, the cells were incubated in 500 μl of DMEM containing 5 μg of gentamicin/ml (to prevent growth of ETEC and *S. typhi* strains) in 5% CO_2 at 37°C. Eight hours after infection, supernatants were collected, centrifuged at $1,000 \times g$ for 15 min, and kept at −20°C. Supernatants from KB cells stimulated with IL-1α (5 ng/ml; Sigma) and tumor necrosis factor alpha (TNF-α) (50 ng/ml; Sigma) served as positive controls. The IL-8 content of the samples was analyzed by enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well plates (Maxisorp Immunoplate; Nunc) were coated with polyclonal goat anti-human IL-8 antibody (10 μg/ml, AB-208-NA; R&D Systems, Abingdon, United Kingdom) and subsequently incubated with the samples, laid in duplicate. Serial dilutions of recombinant IL-8 (8 to 0.06 ng/ml; R&D Systems) were included in each plate and served as references. Captured IL-8 was detected by a polyclonal rabbit anti-human IL-8 antibody (1 μg/ml, P-801; Endogen, Boston, Mass.), followed by a goat anti-rabbit immunoglobulin G antibody conjugated with alkaline phosphatase (1:2,000) (D487; Dakopatts, Copenhagen, Denmark). Captured alkaline phosphatase was visualized with a *P*-nitrophenyl phosphate substrate (Sigma). Absorbance was measured at 405 nm with an ELISA counter (Titertek Multiskan MCC). The lowest detection level of the assay was 125 pg/ml. Samples were obtained from two independent experiments conducted in triplicate.

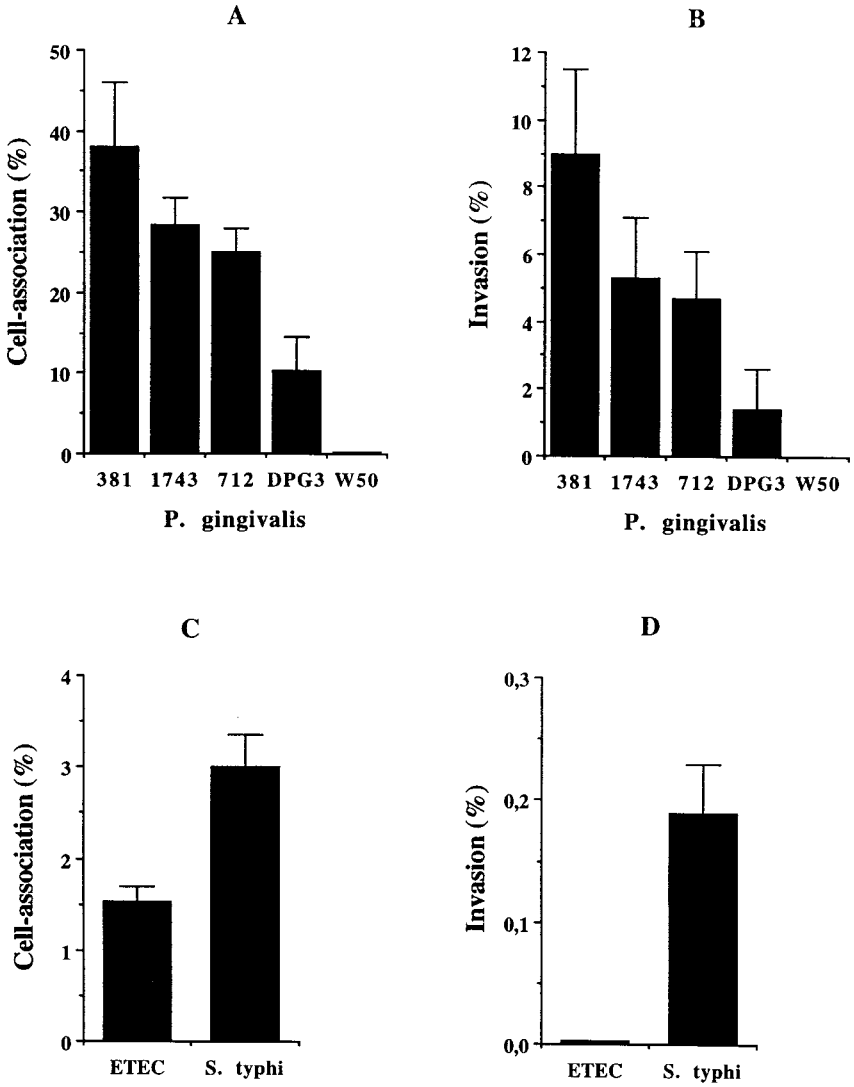

FIG. 2. KB-cell association with and invasion by *P. gingivalis* (A and B) and ETEC and *S. typhi* (C and D). KB epithelial cell layers were infected for 90 min at a concentration of 10^8 bacteria/ml, and numbers of cell-associated bacteria were determined after removal of nonadherent bacteria. Internalized bacteria were quantitated by an antibiotic protection assay. Data are expressed as percentages of the total numbers of bacteria added and represent mean values and SDs of three experiments with triplicate samples.

Effect of IL-8 neutralization on ETEC-induced PMN transmigration. The role of IL-8 release by KB cells on neutrophil transmigration induced by ETEC was investigated by the use of an IL-8-neutralizing monoclonal antibody (AB-208-NA; R&D Systems). Inverted KB layers were infected with ETEC as described for the transmigration assay, and anti-IL-8 antibody (30 μg/ml) was added to the lower compartment of the Transwell inserts 30 min prior to neutrophil application. Five hundred microliters of a PMN suspension (10^6 cells/well) containing anti-IL-8 antibody (30 μg/ml) was subsequently added to the upper compartment, and transmigration was quantitated as described above.

Detection of ICAM-1 expression by KB cells. Inverted KB-cell layers were infected with *P. gingivalis* (5×10^8 bacteria/ml), ETEC (10^8 bacteria/ml), or *S. typhi* (10^8 bacteria/ml), as outlined for the transmigration assay. After 4 and 8 h of infection, the epithelial cells were detached from the membranes by incubating the Transwell inserts in new culture plates containing 1.5 ml of 10 mM EDTA in DMEM in 5% CO_2 at 37°C for 20 min. The cells were washed in PBS by centrifugation at $400 \times g$ for 10 min and resuspended in 80 μl of ice-cold DMEM containing 0.3% bovine serum albumin. After incubation on ice for 15 min, the cells were reacted for 30 min with 20 μl of monoclonal anti-ICAM-1 antibody conjugated with phycoerythrin (anti-CD54-PE, 84H10; Immunotech, Marseille, France). Following two washes in ice-cold PBS, the samples were fixed in 4% paraformaldehyde for 10 min at room temperature and analyzed with a FACScan flow cytometer (Becton Dickinson). A total of 10,000 cells were counted for each sample.

Role of adhesion molecules in transepithelial neutrophil migration. To study the role of ICAM-1 in neutrophil migration across KB cells, inverted cell layers were preincubated for 30 min with a monoclonal anti-ICAM-1 antibody (anti-CD54, 84H10; Immunotech) before assaying neutrophil migration in the presence of a transepithelial gradient of fMLP. The medium in the upper reservoir of the Transwell inserts was removed and replaced with 450 μl of DMEM containing anti-ICAM-1 antibody (50 μg/ml), irrelevant control anti-HLA-I monoclonal antibody (50 μg/ml, M736; Dakopatts), or DMEM alone. Subsequently, 15 μl of fMLP (10^{-5} M) was added to the lower reservoir and 50 μl of a neutrophil suspension of 2×10^7 cells/ml was added to the upper reservoir. Neutrophil transmigration was assessed after 2 h of incubation at 37°C, as previously described.

The role of β_2 integrins in neutrophil migration across KB-cell layers was examined by a 30-min incubation at room temperature of ^{51}Cr-labelled neutrophils with medium containing either anti-β_2 integrin antibody (10 μg/ml, anti-CD18 monoclonal antibody 05-249; Upstate Biotechnology Inc., Lake Placid, N.Y.) or anti-HLA-I antibody (10 μg/ml). Five hundred microliters of PMN suspension was added to the upper compartment of the Transwell inserts, and transmigration was quantitated as described above.

RESULTS

Characterization of *P. gingivalis* attachment and invasion of KB-cell layers. We examined the ability of the different *P. gingivalis* strains to attach to and invade KB cells throughout a

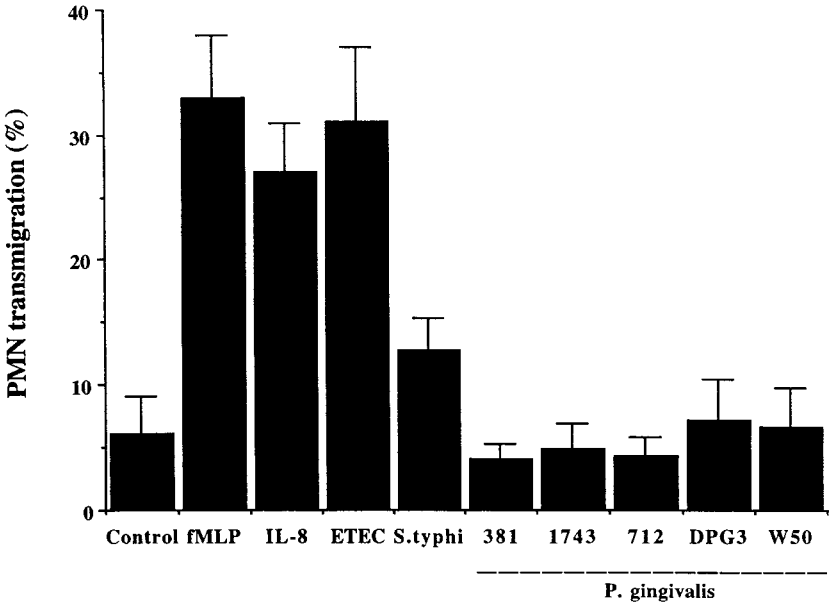

FIG. 3. Effect of *P. gingivalis*, ETEC, and *S. typhi* infection of KB cells on neutrophil transepithelial migration. PMN migration (2 h) was measured across inverted KB-cell layers infected with an inoculum of 10^8 bacteria/ml, as described in Materials and Methods. Unstimulated cell layers served as a negative control. Positive controls for PMN transmigration were established by imposing transepithelial gradients of the chemoattractants fMLP (10^{-7} M) or IL-8 (150 ng/ml). Data are expressed as percentages of total neutrophils added and represent mean values and SDs of four experiments with triplicate samples.

90-min time course. Among the strains tested, strain 381 demonstrated the highest adhesive (38% of the inoculum) and invasive (9%) capacity (Fig. 2A and B). The clinical isolates OMGS 712 and OMGS 1743 followed closely, reaching 65 to 75% and 55 to 65% of the adhesive and invasive ability, respectively, of strain 381. The role of fimbriae on *P. gingivalis* adhesion and invasion of epithelial cells was shown in experiments involving the fimbria-deficient mutant DPG3, which adhered about four times less efficiently than the parental strain 381, while its ability to invade KB cells was reduced approximately sevenfold. Finally, the laboratory strain W50 was found to adhere very poorly (0.2%) and was unable to enter the epithelial cells.

The enteropathogens *S. typhi* and ETEC were both found to be able to adhere to KB cells, albeit to a much lesser extent than *P. gingivalis* (Fig. 2C). However, they differed vastly in their invasive capacity (Fig. 2D). Attachment of *S. typhi* to KB cells was twice as efficient as that of ETEC, yet its invasion capacity was almost 100 times higher.

***P. gingivalis* does not stimulate transepithelial neutrophil migration.** We used oral epithelial cells and ^{51}Cr-labelled PMNs in the Transwell system to model neutrophil migration into the periodontal pocket in response to plaque bacteria. Transepithelial gradients of the chemotactic peptide fMLP and the chemokine IL-8 served as positive controls and induced 33% (standard deviation [SD], 5.1%) and 27% (SD, 3.9%), respectively, of the added neutrophils to transmigrate (Fig. 3). Infection of KB-cell layers with ETEC elicited a transmigration response comparable to that provoked by fMLP (31%; SD, 6.0%). In contrast, *S. typhi* induced modest neutrophil migration across KB layers, which is in accordance with previous studies involving intestinal epithelia (21). We subsequently investigated the ability of *P. gingivalis* to stimulate PMN transepithelial migration. Five different *P. gingivalis* strains, with different adhesion and invasion capacities, were used to infect inverted KB-cell layers. As shown in Fig. 3, none of the tested strains stimulated PMN transmigration over the level of the uninfected control cell layers. On the contrary, a tendency for reduced PMN migration was observed across KB layers infected by the three most adhesive and invasive *P. gingivalis* strains.

***P. gingivalis* blocks fMLP-, IL-8-, and ETEC-induced transepithelial neutrophil migration.** The above findings suggest that *P. gingivalis* may exert a negative effect on transepithelial neutrophil migration. In order to further test this notion, we studied the impact of *P. gingivalis* infection of epithelial cells on an actively stimulated PMN transmigration. Hence, neutrophil migration was induced through uninfected and *P. gingivalis*-infected KB-cell layers by imposing a transepithelial chemotactic gradient (fMLP or IL-8). As shown in Fig. 4, *P. gingivalis* inhibited both fMLP- and IL-8-induced neutrophil transmigration, in a dose-dependent fashion. Interestingly, this property was also strain dependent and closely related to the strain's ability to adhere to and enter KB cells. Thus, a concentration of 5×10^8 bacteria of strain 381/ml exhibited the strongest effect by arresting 66% of the fMLP- and 70% of the IL-8-induced migration. At the same concentration, the two clinical isolates displayed inhibitory effects of slightly lower magnitude (55 to 60% for both fMLP and IL-8) while DPG3 blocked only 30% of the fMLP- and 38% of the IL-8-induced migration. The low-adhering, noninvading strain W50 did not exhibit any significant blocking effect, even at the highest concentration tested.

In order to examine the ability of *P. gingivalis* to influence bacterially induced neutrophil transmigration, epithelial cell layers were infected with either a pure ETEC inoculum or with a combination of ETEC and *P. gingivalis*. Neutrophil transmigration across the latter cell layers was inhibited at a level comparable to that observed for fMLP and IL-8 (Fig. 4).

***P. gingivalis* affects IL-8 secretion by KB cells.** IL-8 has been shown to be secreted by epithelial cells in response to bacterial infection (10, 17) and is thought to play an important role in transepithelial migration of neutrophils (3). We examined the ability of KB cells to secrete IL-8 in response to cytokine and

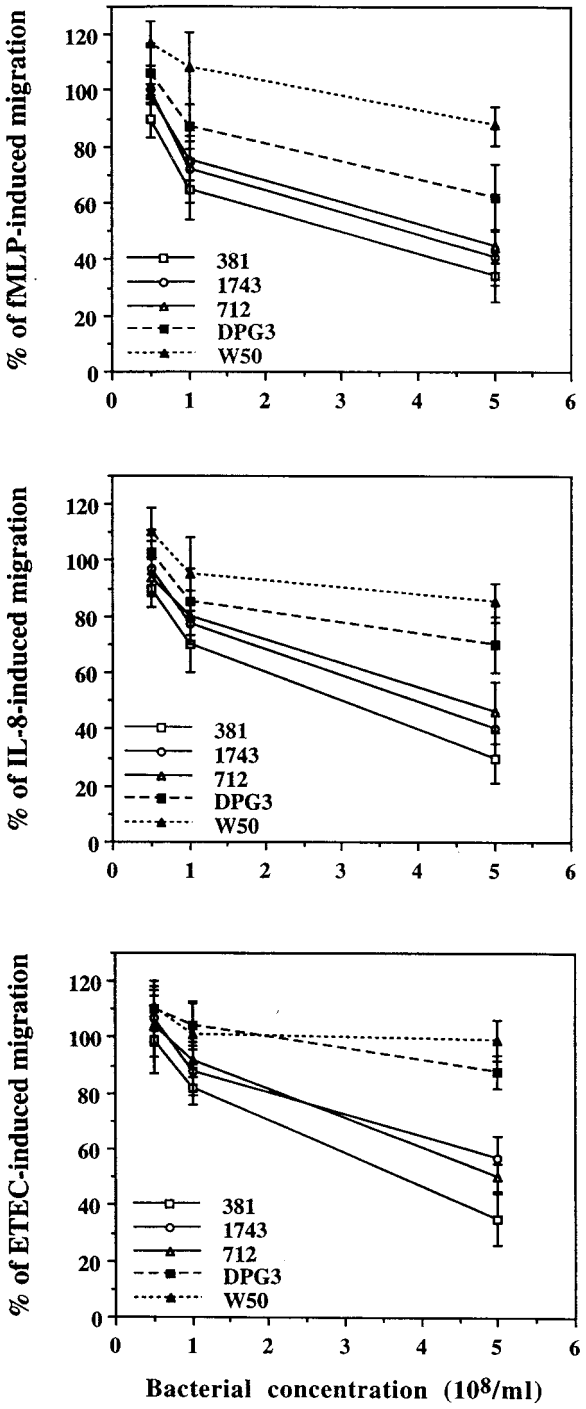

FIG. 4. Effect of *P. gingivalis* infection of KB cells on transepithelial neutrophil migration induced by fMLP (10^{-7} M), IL-8 (150 ng/ml), and ETEC (10^8 bacteria/ml). Inverted KB layers were infected with *P. gingivalis* suspensions of 0.5×10^8, 1×10^8, and 5×10^8 bacteria/ml for 6 h, as described in Materials and Methods. PMN transmigration was induced during a subsequent 2-h period by imposing transepithelial chemotactic gradients of fMLP or IL-8. The effect on ETEC-induced PMN migration was assessed by measuring PMN transmigration through ETEC-infected versus ETEC- and *P. gingivalis*-coinfected KB layers. Data are expressed as percentages of migration induced by the stimulant alone and represent mean values and SDs of four experiments with triplicate samples.

TABLE 1. IL-8 secretion by KB cells[a]

Bacterial or other stimulus	IL-8 (pg/ml)[b]	
	Mean	SD
Culture medium (control)	235	34
IL-1α (5 ng/ml)	6,205	263
TNF-α (50 ng/ml)	4,315	128
P. gingivalis 381	<125[c]	
P. gingivalis 1743	<125	
P. gingivalis 712	<125	
P. gingivalis DPG3	127	18
P. gingivalis W50	139	22
ETEC 258909-3	545	46
+ 381	<125	
+ 1743	<125	
+ 712	<125	
+ DPG3	128	17
+ W50	131	19
S. typhi Ty2	1,232	73
+ 381	<125	
+ 1743	<125	
+ 712	<125	
+ DPG3	127	21
+ W50	136	26

[a] KB-cell layers were infected as described in Materials and Methods. After 8 h in culture, the concentration of IL-8 in the supernatants was determined.
[b] Data are from two separate experiments with triplicate samples.
[c] Below the detection level of the method.

bacterial stimuli. Both IL-1α and TNF-α induced secretion of IL-8 by the KB cells (Table 1). Infection with *S. typhi* and ETEC also triggered IL-8 production by KB cells; however, the response was greater for the invasive pathogen *S. typhi*. Conversely, culture supernatants harvested from *P. gingivalis*-infected cell layers contained lower levels of IL-8 than those obtained from untreated epithelial layers (Table 1). In addition, IL-8 levels detected in supernatants from KB layers coinfected with ETEC-*P. gingivalis* or *S. typhi*-*P. gingivalis* were found to be reduced to below the level of the untreated controls. We examined whether this IL-8-reducing effect contributed to the inhibition of the ETEC-induced PMN transmigration by *P. gingivalis*. Application of an anti-IL-8 antibody inhibited ETEC-induced PMN transmigration by 17% (SD, 3.4), while it completely blocked the transepithelial migration of neutrophils induced by IL-8 added to the medium at a concentration of 10 ng/ml (data not shown). Hence, it appears that the *P. gingivalis*-induced attenuation of IL-8 secretion by KB cells can only partially explain its inhibitory effect on ETEC-provoked neutrophil transepithelial migration.

***P. gingivalis* attenuates ICAM-1 expression by KB cells.** ICAM-1-mediated adhesion has been shown to be involved in neutrophil migration across endothelial and uroepithelial cell layers (4, 18). We therefore examined the expression of ICAM-1 by the KB cell line by using immunofluorescent staining. KB cells constitutively expressed ICAM-1 molecules on their surface (>99% positive cells). Infection of KB cells by ETEC or *S. typhi* for 8 h did not appear to affect ICAM-1 expression (Table 2). *P. gingivalis* infection, however, induced a striking effect on the expression of ICAM-1 by KB cells. Within 4 h of incubation with strain 381, ICAM-1 expression was reduced by more than 50%; it peaked at an 85% reduction at 8 h (Fig. 5). A similar effect was induced by strains 1743 and 712, which both suppressed ICAM-1 expression to 17% of the normal control levels (Table 2). Interestingly, *P. gingivalis* W50,

TABLE 2. ICAM-1 expression by KB cells[a]

Bacterial stimulus[b]	Mean fluorescence intensity	Difference from control	SE of difference
Culture medium (control)	1,417		
ETEC 258909-3	1,375	42	75
S. typhi Ty2	1,288	129	56
P. gingivalis 381	216[c]	1,201	72
P. gingivalis 1743	230[c]	1,187	107
P. gingivalis 712	242[c]	1,175	96
P. gingivalis DPG3	597[c]	820	55
P. gingivalis W50	1,216	201	58

[a] Data are from three individual experiments.
[b] Inverted KB-cell layers were infected as described in Materials and Methods. At 8 h after infection, ICAM-1 expression was determined by flow cytometry analysis.
[c] Significantly different from control, as calculated by a paired *t* test ($P < 0.01$).

which was not found to markedly influence PMN transmigration, did not significantly alter ICAM-1 expression either. A decreased effect, in comparison to that achieved by the wild-type strain, 381, was also observed for the fimbria-deficient mutant DPG3 (58% reduction).

PMN migration across KB cells is β_2 integrin dependent. The role of β_2 integrin-mediated adhesion of PMN to KB cells during transepithelial migration was studied in inhibition experiments with an anti-CD18 antibody. Pretreatment of neutrophils with the antibody blocked the fMLP-induced neutrophil transmigration by ≈80% (Fig. 6), confirming the β_2 integrin dependence of transepithelial PMN migration (4, 30).

Limited involvement of ICAM-1 in PMN migration across KB cells. The involvement of ICAM-1 molecules in PMN transepithelial migration was studied in similar antibody-mediated blocking experiments. Pretreatment of KB-cell layers with anti-ICAM-1 antibody (50 μg/ml) caused only limited inhibition (19%) of the fMLP-stimulated PMN transmigration (Fig. 6), suggesting that alternative β_2 integrin ligands are involved in the transmigration process.

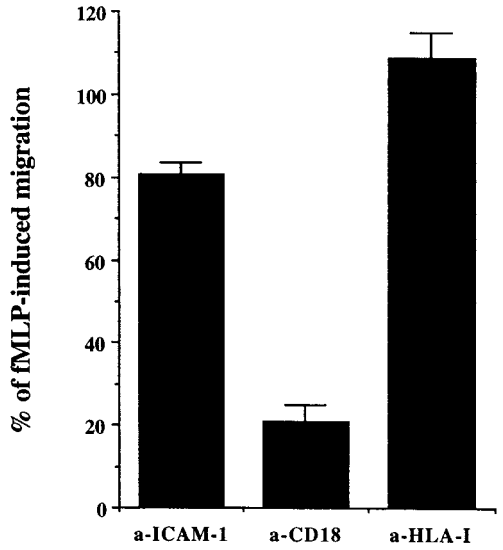

FIG. 6. Effect of monoclonal antibodies on PMN migration across KB-cell layers. PMNs were preincubated with anti-CD18 (a-CD18) (10 μg/ml) and KB cells were preincubated with anti-ICAM-1 (a-ICAM-1) (50 μg/ml) antibodies, in separate experiments, before assaying PMN transmigration induced by a transepithelial gradient of fMLP (10^{-7} M). Anti-HLA-I (a-HLA-I) was used in both cases as irrelevant control antibody. Data are expressed as percent PMN transmigration induced by fMLP in the absence of antibodies and represent mean values and SDs of three experiments with triplicate samples.

DISCUSSION

This study revealed a series of interesting features of the infectious potential of *P. gingivalis* interacting with oral epithe-

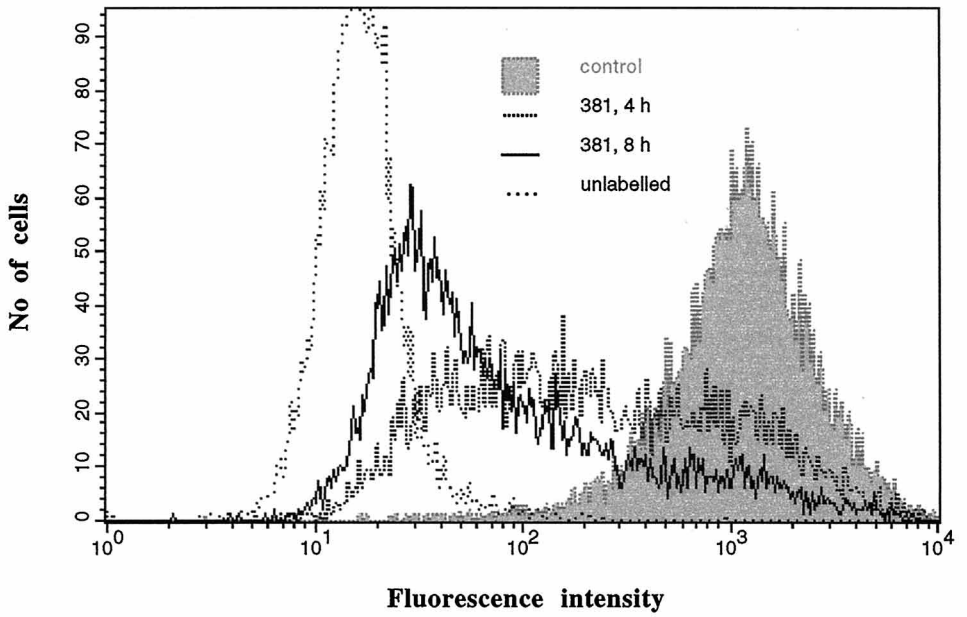

FIG. 5. Effect of *P. gingivalis* infection on ICAM-1 expression by KB cells, as determined by flow cytometry analysis. Inverted KB-cell layers were infected with *P. gingivalis* 381 (5×10^8 bacteria/ml), as described in Materials and Methods. Expression of ICAM-1 was determined after 4 and 8 h. The data shown are from one representative experiment of three performed.

lial cells. Transepithelial migration of neutrophils was not triggered by the bacteria, and an actively induced neutrophil transmigration by both nonbacterial and bacterial stimuli was effectively inhibited by a superimposed *P. gingivalis* infection. This ability to block PMN transmigration was found to be dependent on the concentration of the inoculum and was strongly associated with the ability of *P. gingivalis* to adhere to and invade epithelial cells. In addition, *P. gingivalis* infection induced an alteration of epithelial cell function, including reduction of IL-8 production and ICAM-1 expression which, in turn, may exercise influence on a number of host-protective responses. Considering the undisputed role of the neutrophil as the primary protective cell against dental plaque in the development of the periodontal lesion, we propose that these particular properties of *P. gingivalis* constitute a virulence mechanism of significant bearing on the pathogenesis of periodontal disease.

In this series of experiments, the influx of neutrophils into the gingival pocket was mimicked in a Transwell system (30) with the oral epithelial cell line KB, which has been used extensively to study interactions between oral bacteria and epithelial cells (9, 19, 22, 32, 36). We have demonstrated previously that *P. gingivalis* adheres to and invades KB cells and primary cultures of pocket epithelium in a comparable fashion (33). In addition, cytokine secretion by KB cells matches that of pocket epithelial cells in response to *P. gingivalis* stimulation (unpublished data). We examined the integrity of the epithelial cell layers formed on the polycarbonate membranes and performed PMN transmigration experiments using ^{51}Cr-labelled neutrophils. Our antibody-mediated inhibition experiments demonstrated that, in this model, PMN transmigration was dependent on specific PMN-epithelial cell interactions. Therefore, we consider this assay to be suitable in the study of bacterially induced transepithelial neutrophil migration.

We used two potent PMN chemoattractants (fMLP and IL-8) and two bacterial species with high and low capacity to stimulate PMN migration (ETEC and *S. typhi*, respectively) and examined the ability of *P. gingivalis* to induce PMN transmigration. *P. gingivalis* exerted no stimulatory effect in this model; on the contrary, the bacterium blocked the neutrophil transepithelial migration that was actively induced by gradients of the chemoattractants used. Moreover, *P. gingivalis* attenuated the ability of ETEC to stimulate transmigration of neutrophils. This finding is of particular importance since the environment of the periodontal pocket constitutes a mixed infection rather than a monoinfection. Interestingly, the observed inhibitory effect correlated with the ability of *P. gingivalis* to adhere to and invade epithelial cells. Since an adhesive but noninvasive *P. gingivalis* strain, to our knowledge, is not available, the relative impact of these two properties on neutrophil migration could not be evaluated.

P. gingivalis has been shown to suppress PMN responses to the chemotactic peptide fMLP (26, 40). Van Dyke and coworkers (40) reported that *P. gingivalis* low-molecular-weight soluble products specifically inhibited both fMLP binding to the neutrophil surface and fMLP-induced neutrophil chemotaxis in a Boyden chamber assay. Although such molecules may account for the fMLP-related inhibition in our system, the ability of *P. gingivalis* to inhibit transepithelial migration induced by three different stimuli (fMLP, IL-8, and ETEC) in a virtually identical fashion points against a stimulus-specific inhibitory effect. Furthermore, in preliminary experiments, we observed that *P. gingivalis* similarly inhibited PMN transmigration induced by the nonpeptide chemoattractant leukotriene B_4. Altogether, our data suggest broad interference with the PMN transmigration process induced by alteration of epithelium- and/or neutrophil-related functions.

Epithelial secretion of IL-8, a potent chemoattractant and activator of neutrophils, is thought to be the initial signal for the acute inflammatory response that follows bacterial infection of mucosal surfaces (1, 7, 10, 17). Agace et al. (3) provided evidence that IL-8 secretion by urinary tract epithelial cells was involved in the influx of neutrophils to the urine in response to *E. coli* infection. In addition, IL-8 secretion by uroepithelial and intestinal epithelial cells was found to be involved in neutrophil transepithelial migration induced by *E. coli* and enteropathogenic *E. coli* in vitro (32). Our findings demonstrate that KB cells respond to ETEC and *S. typhi* infection by secreting IL-8. In contrast, IL-8 levels in the supernatants of *P. gingivalis*-infected cell layers were lower than those of uninfected controls. Furthermore, *P. gingivalis* dramatically reduced the IL-8 response to ETEC and *S. typhi* by KB cells. Our IL-8 neutralization experiments suggest that this IL-8 reduction had a limited contribution to the inhibitory effect of *P. gingivalis* on ETEC-induced PMN transmigration. We are currently investigating the involvement of *P. gingivalis* proteases in the reduction of IL-8 production by KB cells. Preliminary experiments with protease inhibitors and in situ hybridization for IL-8 mRNA expression suggest that secreted IL-8 is degraded by *P. gingivalis* proteases.

ICAM-1 is a member of the immunoglobulin supergene family of adhesion molecules expressed on a variety of cells, including T and B lymphocytes and endothelial as well as epithelial cells. ICAM-1 is involved in a number of cell-to-cell interactions, including transendothelial migration of neutrophils (18, 35). Recently, ICAM-1 has been shown to be upregulated on uroepithelial cells after stimulation with *E. coli* and to be involved in neutrophil transuroepithelial migration (4). In the present system, neither ETEC nor *S. typhi* exerted any significant effect on ICAM-1 expression. On the other hand, *P. gingivalis* infection resulted in a dramatic reduction of ICAM-1 expression in the epithelium. This does not appear to be due to receptor coverage by cell-associated bacteria, since ICAM-1 suppression was aggravated with time, beyond the point at which bacterial adhesion was terminated. Since ICAM-1 was only partially involved in PMN transmigration in this model, it appears conceivable that other relevant epithelial surface molecules were similarly affected. The mechanisms resulting in reduced ICAM-1 expression on KB cells after *P. gingivalis* infection are largely unknown. *P. gingivalis* soluble products, including proteases, have recently been shown to decrease the expression of a number of PMN surface receptors, such as CR1, FcγRII, and FcγRIII (37). Our own preliminary experiments with protease inhibitors suggest that *P. gingivalis* proteases are involved in the reduction of ICAM-1 expression by KB cells.

The process by which PMNs migrate across mucosal epithelia is only partially understood. PMN migration across intestinal as well as uroepithelial cells has been shown to require the PMN β_2 integrin CD11b/CD18 (4, 30). Involvement of ICAM-1 has been reported in neutrophil migration across A-498 kidney cell layers (4), while migration across intestinal T84 epithelial layers was shown to be ICAM-1 independent (6, 30). Recently, CD47, an integral membrane glycoprotein with homology to the immunoglobulin superfamily present on both intestinal epithelia and PMNs, was shown to be required for neutrophil migration across polarized intestinal epithelia (29). Our results reveal that PMN transepithelial migration across KB-cell layers is β_2 integrin dependent. ICAM-1 appeared to be less involved, suggesting that another, presently unknown,

β_2 integrin ligand is mediating neutrophil migration across KB-cell layers.

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