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Interleukin-8 and Intercellular Adhesion Molecule 1 Regulation in Oral Epithelial Cells by Selected Periodontal Bacteria: Multiple Effects of *Porphyromonas gingivalis* via Antagonistic Mechanisms

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Interaction of bacteria with mucosal surfaces can modulate the production of proinflammatory cytokines and adhesion molecules produced by epithelial cells. Previously, we showed that expression of interleukin-8 (IL-8) and intercellular adhesion molecule 1 (ICAM-1) by gingival epithelial cells increases following interaction with several putative periodontal pathogens. In contrast, expression of IL-8 and ICAM-1 is reduced after *Porphyromonas gingivalis* ATCC 33277 challenge. In the present study, we investigated the mechanisms that govern the regulation of these two molecules in bacterially infected gingival epithelial cells. Experimental approaches included bacterial stimulation of gingival epithelial cells by either a brief challenge (1.5 to 2 h) or a continuous coculture throughout the incubation period. The kinetics of IL-8 and ICAM-1 expression following brief challenge were such that (i) secretion of IL-8 by gingival epithelial cells reached its peak 2 h following *Fusobacterium nucleatum* infection whereas it rapidly decreased within 2 h after *P. gingivalis* infection and remained decreased up to 30 h and (ii) IL-8 and ICAM-1 mRNA levels were up-regulated rapidly 2 to 4 h postinfection and then decreased to basal levels 8 to 20 h after infection with either *Actinobacillus actinomycetemcomitans*, *F. nucleatum*, or *P. gingivalis*. Attenuation of IL-8 secretion was facilitated by adherent *P. gingivalis* strains. The IL-8 secreted from epithelial cells after *F. nucleatum* stimulation could be down-regulated by subsequent infection with *P. gingivalis* or its culture supernatant. Although these results suggested that IL-8 attenuation at the protein level might be associated with *P. gingivalis* proteases, the Arg- and Lys-gingipain proteases did not appear to be solely responsible for IL-8 attenuation. In addition, while *P. gingivalis* up-regulated IL-8 mRNA expression, this effect was overridden when the bacteria were continuously cocultured with the epithelial cells. The IL-8 mRNA levels in epithelial cells following sequential challenge with *P. gingivalis* and *F. nucleatum* and vice versa were approximately identical and were lower than those following *F. nucleatum* challenge alone and higher than control levels or those following *P. gingivalis* challenge alone. Thus, together with the protease effect, *P. gingivalis* possesses a powerful strategy to ensure the down-regulation of IL-8 and ICAM-1.

Increasing attention has been drawn to the role of gingival epithelial cells in the innate immune response of local gingival tissues. The epithelial cells express chemokines that attract and activate leukocytes and express adhesion molecules that mediate leukocyte migration. The expression of these molecules that initiate and maintain inflammatory reactions can be regulated by the interaction of bacterial pathogens with epithelial cells. Interleukin-8 (IL-8), a neutrophil chemoattractant and activator (1), is induced in gingival epithelial cells by several periodontal microbes, such as *Fusobacterium nucleatum*, *Actinobacillus actinomycetemcomitans*, and *Eikenella corrodens* (4, 14, 17). Increased IL-8 production is thought to play a role in the transmigration of neutrophils from the submucosa to the sulcular space (38), even though constitutive IL-8 expression in noninflamed gingival epithelium has been reported (9, 18). Intercellular adhesion molecule 1 (ICAM-1) is the ligand for lymphocyte function-associated antigen 1 (LFA-1) or Mac-1 expressed on leukocytes (5, 23). In human gingival epithelium, ICAM-1 expression is restricted to the junctional and sulcular epithelium (3, 13, 18), forming a gradient with the highest ICAM-1 level on epithelial cells facing the tooth surface. This gradient is thought to play a role in directing the migration of leukocytes toward the sulcular space (18, 37, 38).

Our previous report showed that IL-8 and ICAM-1 are up-regulated in gingival epithelial cells following challenge with *A. actinomycetemcomitans* (17). However, both IL-8 and ICAM-1 are down-regulated by *Porphyromonas gingivalis* (4, 17, 22). The actual role and outcome of these regulatory processes in the pathogenesis of periodontal diseases in vivo are unknown. However, it is thought that up-regulation of IL-8 and ICAM-1 in gingival epithelial cells by microorganisms such as *A. actinomycetemcomitans* and *F. nucleatum* may stimulate the host

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immune response by recruiting leukocytes to the site of infection. In contrast, *P. gingivalis*, which attenuates the expression of IL-8 and ICAM-1, may delay the host defense mechanisms and evade the immune system, thus creating more damage to the surrounding tissue (4, 17). The exact mechanism of the attenuated expression of IL-8 and ICAM-1 is not clear, although *P. gingivalis* invasiveness and proteases have been reported to play a role (4, 25, 43).

The present study was undertaken to investigate the regulation of IL-8 and ICAM-1 at the molecular level in gingival epithelial cells in response to challenge with several periodontal bacteria. Our results revealed the kinetics of the regulation of these two molecules at the protein and mRNA levels. The regulation of IL-8 and ICAM-1 mRNA in epithelial cells challenged with *P. gingivalis* appears to be governed by antagonistic mechanisms.

MATERIALS AND METHODS

Cell cultures. HOK-18A and HOK-16B-BaP-T1 cells, obtained from N.-H. Park (University of California Los Angeles, Los Angeles, Calif.) are immortalized oral keratinocyte cell lines derived from primary normal human oral keratinocyte cells (27, 33). The cell culture procedures were performed as described in previous reports with some modifications (17). Briefly, HOK-18A cells were grown in Dulbecco's modified Eagle's medium-F12 (3:1, vol/vol) (GIBCO/BRL, Grand Island, N.Y.) supplemented with 10% fetal bovine serum, 0.5 ng of human epidermal growth factor per ml, 5 μg of bovine insulin per ml, 0.4 μg of hydrocortisone per ml, 0.1 nM choleratoxin, 0.5 μg of transferrin per ml, 2 nM 3,3′,5-triiodo-L-thyronine, 25 μg of gentamicin per ml, and 250 ng of amphotericin B per ml. HOK-16B-BaP-T1 cells were grown in Dulbecco's modified Eagle's medium containing 4.5 g of D-glucose per liter and supplemented with 10% fetal bovine serum and 0.4 μg of hydrocortisone per ml. Since HOK-16B-BaP-T1 cells normally express low levels of IL-8 and ICAM-1, they were used for bacteria that showed an up-regulating effect on these molecules, i.e., *A. actinomycetemcomitans* and *F. nucleatum*. In contrast, HOK-18A cells, which express higher levels of IL-8 and ICAM-1, were mainly used to study the kinetics of IL-8 secretion following *P. gingivalis* infection, which down-regulated IL-8 and ICAM-1.

Bacterial strains and cytokine. The following laboratory strains were utilized: *A. actinomycetemcomitans* Y4 from K. Miyasaki (University of California Los Angeles); *F. nucleatum* 12230, a clinical isolate from the upper trachea, obtained by S. Finegold (Los Angeles, Calif.); *P. gingivalis* ATCC 33277 (American Type Culture Collection, Rockville, Md.); *P. gingivalis* strain W50 from J. Sandros (Gøteborg University, Gøteborg, Sweden); and the *P. gingivalis* protease mutant strain V2296 (*kgp*, Lys-gingipain) and its corresponding wild-type strain, W83, from H. M. Fletcher (Loma Linda University, Loma Linda, Calif.) (10). The protease (Arg-gingipain)-defective mutants, MT10 (*rgpA*) and G-102 (*rgpB*), were derived from wild-type *P. gingivalis* strain 381 (35, 36). *Escherichia coli* HB101 was obtained from Y. Han (Case Western Reserve University, Cleveland, Ohio).

Infection of oral epithelial cells. Oral epithelial cells were seeded into 24- or 6-well Costar tissue culture plates at a density of 10^5 cells/ml in volumes of 0.5 ml (24-well) or 2 ml (6-well) per well. Cultures were grown to confluence before being infected with bacteria. The preparation of *A. actinomycetemcomitans*, *P. gingivalis*, and *F. nucleatum* was described in our previous reports (14, 17). Briefly, bacteria were inoculated in 5 ml of appropriate broth medium and grown at 37°C under 80% N_2–10% H_2–10% CO_2 in an anaerobic chamber (Coy Laboratory Production, Ann Arbor, Mich.) overnight. Typically, 1 ml of the bacterial culture was then transferred to 9 ml of fresh broth medium and allowed to grow to an optimal optical density so that the bacteria were in the exponential phase of growth. *E. coli* HB101 was grown in Luria-Bertani medium at 37°C to the logarithmic growth phase. The bacteria were washed three times with phosphate-buffered saline (pH 7.2) and resuspended at concentrations equivalent to various multiplicities of infection (MOI) in the antibiotic-free medium used to grow the specific cell lines. Bacteria were added in 200-μl volumes to the cell monolayers in 24-well culture plates or in 1-ml volumes to the cell monolayers in 6-well culture plates and were centrifuged onto the monolayers at 900 × *g* for 5 min at room temperature. The bacteria were cocultured with epithelial cells at 37°C for 1.5 to 2 h to allow interaction between the bacteria and epithelial cells. After incubation, the monolayers were washed three times to remove extracellular bacteria and the cultures were further incubated for 2 to 30 h in fresh medium containing antibiotics (described below) to kill the remaining extracellular bacteria. Alternatively, bacteria were cocultured with the epithelial-cell monolayers for the entire incubation period; i.e., bacteria were continuously cocultured with the epithelial cells throughout the incubation without removal of the bacteria or change of medium. The antibiotics used in the media were as follows: for infection with *A. actinomycetemcomitans* and *F. nucleatum*, 0.1 mg of gentamicin per ml; for infection with *P. gingivalis*, 0.1 mg of metronidazole per ml and 0.5 mg of gentamicin per ml. After incubation, the supernatants were collected for IL-8 detection by enzyme-linked immunosorbent assay (ELISA). Epithelial-cell viability was determined by trypan blue exclusion after the supernatant was collected. Supernatant from epithelial cells in 24-well plates were used for ELISA to measure the amount of secreted IL-8. Epithelial cells in six-well plates were harvested for Northern blot analyses. The epithelial-cell viability was >90% for all the kinetics studies described above when optimal epithelial-cell lines and bacterial doses were used.

Invasion inhibition studies. The bacterial invasion inhibitor sodium azide (Sigma, St. Louis, Mo.) (50 mM in PBS) was used to block *P. gingivalis* invasion of gingival epithelial cells (21). Preliminary experiments indicated that the inhibitor had no effect on bacterial viability at the concentrations and under the conditions utilized. The inhibitor was preincubated with *P. gingivalis* for 4 h and then removed by washing prior to coculture of the epithelial cells with *P. gingivalis*. The extent of inhibition of invasion was determined by parallel invasion assays using the standard antibiotic protection method (6, 31).

Northern blot analysis. Cellular RNA was isolated using RNA STAT-60 (Tel-Test "B," Inc., Friendswood, Tex.). Total RNA (8 to 20 μg) was size fractionated on 1.5% formaldehyde–agarose gels, transferred to nitrocellulose filters, and probed with a ^{32}P-labeled cDNA fragment specific for human ICAM-1 or IL-8. The ICAM-1 probe targeted a 1,400-bp fragment in the coding region. This fragment was released by *Xho*I digestion from plasmid pICAM-1 (kindly provided by B. Seed, Boston, Mass.), which carries the full-length human ICAM-1 cDNA. The IL-8 probe targeted a 420-bp fragment released by *Eco*RI digestion from plasmid pBhIL8 (kindly provided by M. Kagnoff, La Jolla, Calif.), which carries the full-length human IL-8 cDNA. A ^{32}P-labeled cDNA fragment of human glyceraldehyde-3-phosphate dehydrogenase was used as the control probe to verify that an equal amount of RNA from each cell line was used in each analysis. The signals were visualized by autoradiography using a PhosphorImager system (Molecular Dynamics, Sunnyvale, Calif.). The images of specific bands were quantitated using an ImageQuant software program (Molecular Dynamics).

ELISA for IL-8. The procedures for ELISA for IL-8 were described in previous studies (17, 18). Standard ELISA was performed using polyclonal goat anti-human IL-8 antibodies (R&D Systems, Minneapolis, Minn.) as capturing antibodies, polyclonal rabbit anti-human IL-8 antibodies (Endogen Inc., Cambridge, Mass.) as detecting antibodies, and horseradish peroxidase-labeled polyclonal goat anti-rabbit immunoglobulin G (Biosource International, Camarillo, Calif.) as a second-step antibody.

RESULTS

Kinetics of IL-8 secretion by gingival epithelial cells following *F. nucleatum* or *P. gingivalis* infection. Epithelial cells were cocultured with *F. nucleatum* or *P. gingivalis* for 2 h. During the subsequent incubation, extracellular bacteria were removed and fresh medium with antibiotics was added to kill the remaining extracellular bacteria so that any effect of bacterial infection on IL-8 induction or reduction was exerted during the coculturing period due to bacterium–epithelial-cell interactions and/or during the subsequent incubation period due to the activity of invaded or attached bacteria. The production of secreted IL-8 during sequential 4-h intervals after infection was measured by ELISA (Fig. 1). The results showed that IL-8 secretion by gingival epithelial cells increased from 2 to 14 h following *F. nucleatum* infection and decreased thereafter up to 26 h. In contrast, IL-8 secretion into the supernatant rapidly decreased 2 h after *P. gingivalis* infection and remained suppressed during the remaining incubation period. As shown in Fig. 1, these effects appeared to be dose dependent, since the

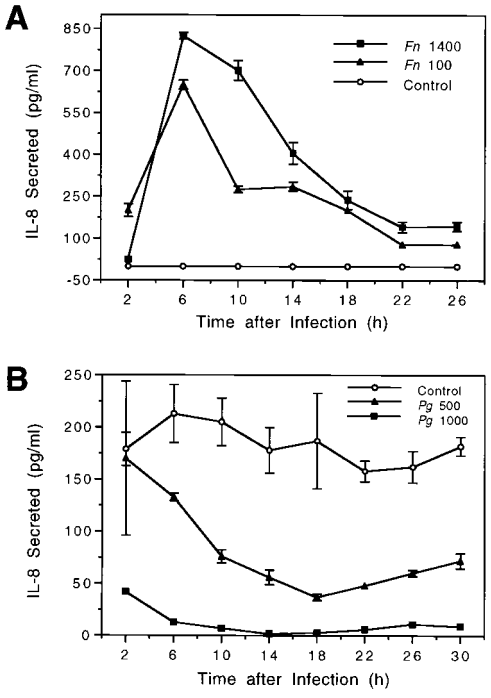

FIG. 1. Kinetics of IL-8 secretion by gingival epithelial cells following bacterial infection. (A) Confluent HOK-16B-BaP-T1 cell monolayers were infected with *F. nucleatum* 12230 (*Fn*) at a MOI of 1,400:1 or 100:1. (B) Confluent HOK-18A cell monolayers were infected with *P. gingivalis* 22377 (*Pg*) at a MOI of 1:1,000 or 1:500. At 2 h after infection, the cultures were washed and further incubated in the presence of gentamicin (0.5 mg/ml) for *F. nucleatum* or metronidazole (0.1 mg/ml) plus gentamicin (0.5 mg/ml) for *P. gingivalis* for up to 26 to 30 h. The MOIs were confirmed retroactively in parallel experiments. IL-8 secretion was determined for each time point by removing the medium and subsequently incubating the cells in freshly added medium containing the same concentration of antibiotics. Data points represent mean and standard error of the mean of the results of triplicate assay determinations from one representative experiment.

higher the bacterial load, the stronger the IL-8-regulatory effect.

Kinetics of IL-8 and ICAM-1 mRNA levels after infection with *A. actinomycetemcomitans*, *F. nucleatum*, or *P. gingivalis*. After 2 h of bacterium–epithelial-cell coculture, extracellular bacteria were removed by washing the epithelial-cell cultures and fresh medium containing antibiotics was added for further incubation. At different time points, supernatants were collected for the measurement of accumulated IL-8 and RNA was isolated from the epithelial-cell cultures for analysis. The results presented in Fig. 2 demonstrate that all three bacteria induced IL-8 (4.0- to 91.0-fold) and ICAM-1 (1.2- to 7.2-fold) mRNA production between 2 and 4 h and that this was followed by reduced levels (Fig. 2, top and middle panels). The level of mRNA was proportional to the number of bacteria used in the coculture (Fig. 2B and C). *F. nucleatum* appeared to be more potent in inducing IL-8 and ICAM-1, as indicated by the same level of induction at a lower MOI compared to the other two microorganisms.

The accumulated IL-8 in the supernatant continued to increase following *A. actinomycetemcomitans* and *F. nucleatum* challenge, whereas it decreased in response to *P. gingivalis* challenge (Fig. 2, bottom panel). The accumulated cell surface ICAM-1 expression could not be tested in this experiment due to the harvesting of cellular RNA. Separate experiments were performed to measure the cell surface ICAM-1 expression following *F. nucleatum* challenge. The results (data not shown) were similar to those of previous studies in which epithelial cells challenged with *A. actinomycetemcomitans* showed increased cell surface ICAM-1 expression (17).

The decrease in secreted IL-8 production is facilitated by *P. gingivalis* attachment. To determine whether *P. gingivalis* attachment and invasion plays a role in attenuating IL-8 secretion, we examined the effects of the poorly adherent and poorly invasive *P. gingivalis* strains W50 and W83 with those of invasive strains 381 and 33277. The data indicate that *P. gingivalis* W50 and W83 did not attenuate IL-8 production (Table 1), suggesting that attachment and invasion is important for mediating the decrease in secreted IL-8 levels under these experimental conditions. Two protease knockout mutant strains were also used to examine the effect of proteases on IL-8 attenuation. The mutant strains derived from *P. gingivalis* 381 did not affect IL-8 regulation compared with their wild-type strains. Pretreatment with sodium azide, which inhibited *P. gingivalis* invasion but not attachment, also did not affect IL-8 attenuation. These results suggest a more important role of attachment than invasion in IL-8 response.

In the studies described above, the bacterium–epithelial-cell coculture time was only 1.5 to 2 h. Therefore, the IL-8 attenuation effect was exerted either during the coculture period or by the attached bacteria after removal of nonattached bacteria or both. To examine these possibilities, we analyzed IL-8 attenuation using two approaches. In one approach, the bacteria were incubated with the epithelial cells for 2 h, and this was followed by washing and the addition of fresh medium containing antibiotics. The supernatants were collected for IL-8 analysis at both the end of the 2-h coculture time and the end the incubation (at 6 h) after the addition of fresh medium. In the other approach, the bacteria were continuously cocultured with the epithelial cells throughout the incubation period (0 to 18 h). The results presented in Table 2 show that when IL-8 production was measured at the end of the 2-h coculture period, all three *P. gingivalis* strains had attenuated it. After the cultures were washed and fresh medium was added, the level of IL-8 accumulated in the supernatant during the 2- to 6-h incubation period was decreased only for *P. gingivalis* 381. When these *P. gingivalis* strains were cocultured with the epithelial cells throughout the entire incubation period (18 h), all the strains attenuated IL-8. The results indicate that the continuous presence of bacteria along with epithelial cells, either by attachment or by their presence in the cocultures, played a key role in this IL-8 attenuation.

IL-8 secreted from epithelial cells can be attenuated by *P. gingivalis* supernatant. The above data revealed that although *P. gingivalis* up-regulated the mRNA levels of IL-8 and ICAM-1, both molecules were down-regulated at the protein level. This attenuation was related to the physical association of bacteria with epithelial cells. To determine if soluble proteases released from *P. gingivalis* into the culture supernatant are capable of degrading IL-8 from HOK-18A cells, we incubated *P. gingivalis* culture supernatants with IL-8 secreted from

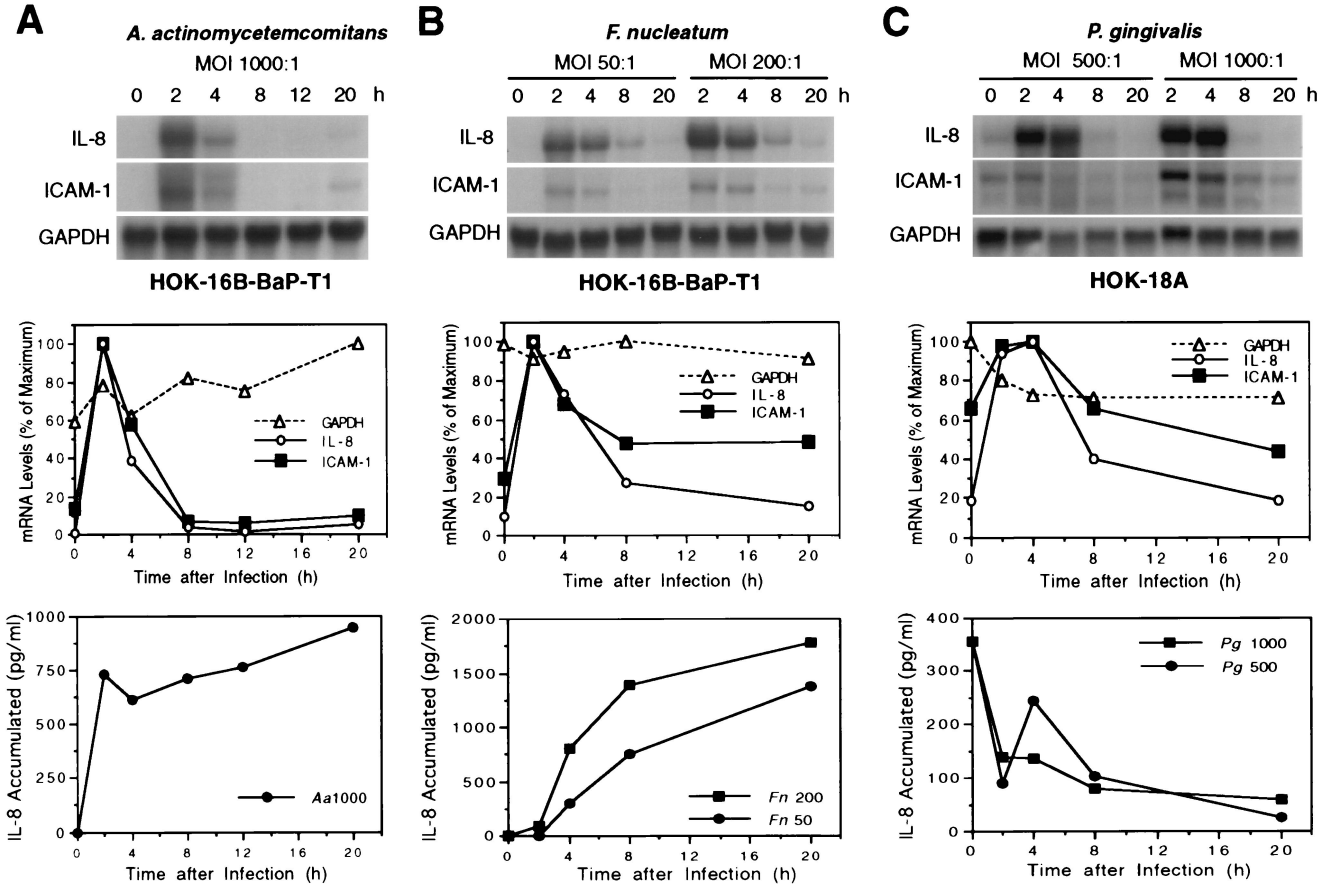

FIG. 2. Kinetics of IL-8 and ICAM-1 mRNA levels in gingival epithelial cells following bacterial infection. (A and B) Confluent HOK-16B-BaP-T1 cell monolayers were infected with *A. actinomycetemcomitans* Y4 at a MOI of 1,000:1 (A) or *F. nucleatum* 12230 at MOIs of 50:1 and 200:1 (B). (C) Confluent HOK-18A cell monolayers were infected with *P. gingivalis* 22377 at MOIs of 500:1 and 1,000:1. At 2 h after infection, the cultures were washed and further incubated for up to 20 h in the presence of gentamicin (0.5 mg/ml) (*A. actinomycetemcomitans* and *F. nucleatum*) or metronidazole (0.1 mg/ml) plus gentamicin (0.5 mg/ml) (*P. gingivalis*). At various intervals after infection, epithelial cells were harvested and RNA was isolated. A 20-μg sample of total RNA was subjected to Northern blot analysis. The MOIs were confirmed retroactively. The results are shown in the top panels. The levels of mRNA from the analysis are plotted and shown in the middle panels. Data presented in the middle panel for panel B were from a MOI of 50:1, and those in the middle panel for panel C were from a MOI of 1000:1. Supernatants were also collected at each time point for accumulated IL-8 measurement using ELISA (data shown in the bottom panel).

epithelial cells. The results showed that supernatants from all *P. gingivalis* strains used in this study, i.e., both wild-type strains and their protease mutants, were capable of degrading IL-8 secreted from HOK-18A cells (Table 3). In contrast, supernatant from *E. coli* HB101 or *F. nucleatum* did not appear to degrade IL-8. The supernatants of mutant strains, MT10 (*rgpA*) and G-102 (*rgpB*), appeared less potent in degrading IL-8 than did that of mutant strain V2296 (*kgp*), suggesting that Arg-gingipain may play a more important role than Lys-gingipain in this degradation. We also incubated IL-8 with *P. gingivalis* cells directly and found that bacterial cells were much more potent than their supernatant in degrading IL-8 (data not shown). We reasoned that *P. gingivalis* protease activities should be potent enough to reduce the IL-8 levels secreted in response to a prior infection with *F. nucleatum*. Therefore, we challenged epithelial cells with *P. gingivalis* at either 4 or 8 h after infection with *F. nucleatum*. The results presented in Fig. 3 show that *P. gingivalis* cells were capable of degrading secreted IL-8 which had been induced by *F. nucleatum* and had accumulated in the culture supernatant.

Effect of bacterium–epithelial-cell coculture time on IL-8 mRNA expression. IL-8 and ICAM-1 mRNA levels peaked between 2 and 4 h after a 2-h challenge with *P. gingivalis* (Fig. 2). We next determined the kinetics of the mRNA levels when bacteria were continuously cultured for 4 to 6 h with the epithelial cells and compared them to the kinetics in the groups using the 2-h challenge followed by washing and addition of fresh medium for further incubation to 4 to 6 h. The results in Fig. 4A and B showed that IL-8 mRNA was detected at lower levels when the continuous challenge with *P. gingivalis* was used than when the 2-h challenge was used. IL-8 mRNA levels after continuous challenge were even lower than the levels after no infection. Thus, *P. gingivalis* 381, ATCC 22377, and W50 up-regulated IL-8 and ICAM-1 mRNA when the epithelial-cell cultures were washed at the 2-h time point and down-regulated IL-8 below the baseline level when the bacteria were continuously cocultured with the epithelial cells. However, whereas 381 and ATCC 33277 are adherent and invasive, W50 is not. This suggests that the attachment and invasion phenotype is not required for the IL-8 mRNA regulation. The above

TABLE 1. IL-8 secretion from gingival epithelial cells following *P. gingivalis* challenge[a]

Bacterium added	Amt of secreted IL-8 (pg/ml)	% Attachment[b]	% Invasion[c]
None	950 ± 97		
P. gingivalis 381	128 ± 31	3.1563 ± 2.6456	1.4231 ± 0.9102
Mutant MT-10	151 ± 34	2.3943 ± 0.5823	1.9674 ± 0.8827
Mutant G-102	332 ± 79	6.0650 ± 3.2536	4.6520 ± 3.6773
P. gingivalis W50	944 ± 82	0.0038 ± 0.0016	0.0083 ± 0.0074
P. gingivalis W83	927 ± 63	0.0042 ± 0.0025	0.0005 ± 0.0003
Mutant V2296	1,041 ± 69	0.0292 ± 0.0241	0.009 ± 0.0085
None	457 ± 44		
P. gingivalis ATCC 33277			
Not treated	74 ± 74	0.807 ± 0.577	0.617 ± 0.537
Azide treated	82 ± 26	0.826 ± 0.536	0.134 ± 0.051

[a] Confluent HOK-18A cell monolayers were infected with *P. gingivalis* 381, W50, or W83 and their mutants for 2 h at MOIs of 1,200 or with *P. gingivalis* 33277 and sodium azide treated for 1.5 h at MOIs of 1,500. The cultures were then washed and further incubated for 12 h in the presence of metronidazole (0.1 mg/ml) and gentamicin (0.5 mg/ml). IL-8 secretion was determined by ELISA. The MOIs were confirmed retroactively in parallel experiments by plating bacteria onto appropriate blood agar plates and counting bacteria colonies after incubation. Values represent mean ± standard error of the mean of the results of at least two independent experiments in triplicate (IL-8 measurement) or duplicate (attachment and invasion analyses) assays.

[b] Defined as the percentage of bacteria added that bound to HOK-18A cells. Cultures were subjected to standard attachment assays following the 1.5- to 2-h coculture incubation.

[c] Defined as 100 × number of bacterial CFU recovered following antibiotic treatment/number of bacteria added.

phenomenon did not occur when *F. nucleatum* was used to challenge the epithelial cells (Fig. 4C). In fact, *F. nucleatum* induced more IL-8 mRNA when continuously cocultured with the epithelial cells.

Regulation of IL-8 mRNA levels following sequential challenge of epithelial cells with *P. gingivalis* and *F. nucleatum*. To determine how IL-8 mRNA regulation is affected by sequential challenge of epithelial cells with *P. gingivalis* and *F. nucleatum*, we carried out kinetic studies examining IL-8 mRNA levels at 4 h after stimulation. The results showed that when the cells were challenged first with *F. nucleatum* and then with *P. gingivalis* 33277 and when they were challenged first *P. gingivalis* 33277 and then with *F. nucleatum*, the IL-8 mRNA levels were similar (Fig. 5). In both cases, the IL-8 mRNA level was still higher than the levels associated with control (mock) and *P. gingivalis* infection alone but lower than the level associated with *F. nucleatum* alone.

TABLE 2. IL-8 secretion from HOK-18A cells following *P. gingivalis* challenge

Bacterium added	Amt of secreted IL-8 (pg/ml)[a]		
	0–2 h[b] (continuous) (MOI, 1,000:1)	2–6 h[c] (washed) (MOI, 1,000:1)	0–18 h[d] (continuous) (MOI, 500:1)
None	292 ± 37	1,111 ± 18	1,212 ± 111
P. gingivalis 381	134 ± 2	163 ± 7	54 ± 4
P. gingivalis W50	24 ± 6	1,241 ± 121	26 ± 12
P. gingivalis W83	54 ± 15	1,343 ± 86	13 ± 2

[a] Values represent mean ± standard error of the mean of the results of duplicate or triplicate assay determinations from one representative experiment. At the end of infection and/or incubation, supernatants were collected for IL-8 determination by ELISA.

[b] Confluent epithelial-cell monolayers infected with *P. gingivalis* for 2 h.

[c] Epithelial cells infected with *P. gingivalis* for 2 h followed by washing to remove extracellular bacteria and further incubation to 6 h in the presence of metronidazole (0.1 mg/ml) and gentamicin (0.5 mg/ml).

[d] Epithelial cells infected with *P. gingivalis* for 18 h continuously without removal of bacteria.

DISCUSSION

Our previous studies demonstrated that secreted IL-8 and cell surface ICAM-1 protein expression are increased in gingival epithelial cells challenged with *A. actinomycetemcomitans* or *F. nucleatum* whereas both proteins are down-regulated when the cells are challenged with *P. gingivalis* (14, 17). Similar findings have also been reported by other investigators (4, 22). The present studies further investigated the mechanisms underlying the regulation of IL-8 and ICAM-1 expression. Both *A. actinomycetemcomitans* and *F. nucleatum* up-regulated IL-8 at the protein and mRNA levels. Interestingly, whereas *P.*

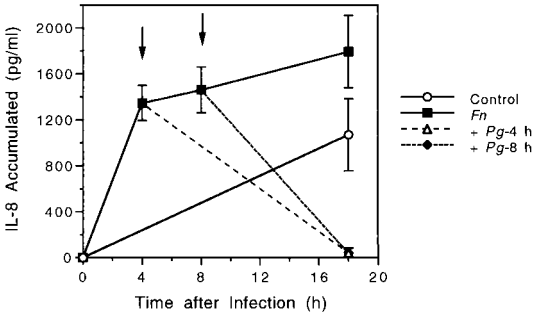

FIG. 3. IL-8 secretion from HOK-18A following sequential challenge of *F. nucleatum* and *P. gingivalis*. (○) Control group without bacteria. Supernatants were collected at 18 h for IL-8 ELISA. (■) *F. nucleatum* (MOI, 300:1) was added to cell monolayers and supernatants were collected for IL-8 measurement at the 4-, 8-, or 18-h time point. ↓, *P. gingivalis* 381 (MOI, 500:1) was added to cell monolayers 4 h (△) or 8 h (◆) after the addition of *F. nucleatum* (MOI, 300:1), and the supernatant was collected at 18 h.

TABLE 3. Degradation of secreted IL-8 by *P. gingivalis* supernatant[a]

Bacterium added	% of IL-8 remaining
None	100
P. gingivalis 381	66 ± 3[b,c]
Mutant MT-10	82 ± 10
Mutant G-102	94 ± 11
P. gingivalis W83	58 ± 14[d]
Mutant V2296	60 ± 11
E. coli HB101	106 ± 4
F. nucleatum ATCC 12230	99 ± 11

[a] Secreted IL-8 from confluent HOK-18A culture medium was collected, and bacterial supernatant was collected from cultures in logarithmic growth. Equal volumes (100 μl) of the HOK-18A culture medium and *P. gingivalis*, *F. nucleatum*, or *E. coli* supernatant were combined in each well of a 24-well plate and incubated for 18 h at 37°C. The remaining IL-8 was determined by ELISA. Values represent mean ± standard error of the mean of the results of at least three independent experiments in triplicate assays, except for *F. nucleatum* ATCC 12230, for which the values represent results of two independent experiments in a triplicate assay).

[b] $P = 0.183$ (when result is compared with that for MT-10).

[c] $P = 0.043$ (significant difference [$P < 0.05$] by the t test) (when result is compared with that for G-102).

[d] $P = 0.939$ (when result is compared with that for V2296).

gingivalis down-regulated IL-8 at the protein level, it could both up- and down-regulate mRNA levels. The up-regulation versus down-regulation was dependent on the duration of the *P. gingivalis* interaction with epithelial cells. Time course studies demonstrated the kinetic profile of IL-8 and ICAM-1 mRNA expression in gingival epithelial cells in response to a 2-h bacterial challenge followed by washing and incubation with fresh medium. All three bacteria, *A. actinomycetemcomitans*, *F. nucleatum*, and *P. gingivalis*, up-regulated IL-8 and ICAM-1 mRNA with similar kinetics. Although *P. gingivalis* stimulation of epithelial cells down-regulated IL-8 and ICAM-1 at the protein level, the mRNA induction patterns of these two molecules were almost identical to what was seen with stimulation by *A. actinomycetemcomitans* and *F. nucleatum*. This suggests that all three bacteria induce an epithelial-cell response that potentially utilizes identical pathways to activate IL-8 and ICAM-1 transcription. The kinetics of IL-8 secretion in response to *F. nucleatum* appeared identical to the response to *A. actinomycetemcomitans* (17). *P. gingivalis*, on the other hand, rapidly attenuated the production of secreted IL-8 from epithelial cells at 2 h after infection, even as the IL-8 mRNA accumulation reached its peak at 2 h. This suggests that this down-regulation is exerted at the translational and/or post-translational level.

Based on the potent protease activities possessed by *P. gingivalis* (2, 4, 11, 12, 20, 25, 43), the most likely cause is the degradation of ICAM-1 and IL-8 proteins by proteases. Consistent with this hypothesis, the greater the bacterial load to the epithelial cells, the stronger this effect. Our findings suggest that *P. gingivalis* proteases may be responsible for the low levels of IL-8 and ICAM-1 protein expression during the period after the epithelial cells were washed. The results shown in Table 1 suggest a role for *P. gingivalis* attachment in that after the epithelial cells were washed, the attached *P. gingivalis* bacteria were able to execute IL-8 degradation. This degradation was not affected by the presence of antibiotics, as shown in our previous study, in which we demonstrated that antibiotic-killed *P. gingivalis* is still capable of degrading IL-8 (17). The use of nonadherent and noninvasive *P. gingivalis* strains, W50 and W83 (Table 1), ensured that these bacteria could be easily washed away; i.e., they were not present after washing to degrade IL-8. Furthermore, the deletion of one (*rgpA*, *rgpB*, or *kgp*) protease gene in *P. gingivalis* did not dramatically affect IL-8 attenuation, indicating that no individual protease was exclusively or dominantly responsible for the degradation. The gingipain proteases released from *P. gingivalis* have shown potent activity in degrading IL-8 in purified form (25) and may account for the degradation from crude supernatant, as demonstrated by our study (Table 3); however, bacterial cell-associated protease activity appears to be more important in IL-8 protein degradation.

Darveau et al. (4) showed that *P. gingivalis* added to epithelial-cell cultures halted ongoing IL-8 accumulation induced by *F. nucleatum* stimulation without the loss of previously secreted IL-8. Their data suggest that *P. gingivalis* interaction with epithelial cells counteracts the induction of IL-8 by *F. nucleatum*, but does not affect already secreted IL-8 in the supernatant. The data presented in Fig. 3, however, do not correspond to their finding. It might be that the use of different MOIs of *P. gingivalis* or the use of primary gingival epithelial cells in their system versus the use of cell lines in our system accounts for this discrepancy. It appears likely that *P. gingivalis* interacts with epithelial cells with different affinities depending on the strain. For strains that can attach well to the epithelial cells, their secreted or vesicle-associated proteases can establish a high concentration on the cell surface, thus degrading the IL-8 as it is secreted from the epithelial cells and degrading the cell surface ICAM-1. For strains of *P. gingivalis* that do not attach well (W50 and W83), continuous coculture with the epithelial cells may allow their released soluble protease as well as surface-associated proteases to degrade the accumulated IL-8 in the epithelial cell supernatant (Tables 2 and 3) (4, 43). These data suggest that the proteolytic activity, rather than attachment itself, is likely to be responsible for the IL-8 protein degradation.

It is of interest that *P. gingivalis* can either up- or down-regulate IL-8 mRNA in epithelial cells (Fig. 4). At 2 h after *P. gingivalis* challenge, the bacteria were removed, the culture was washed, and fresh medium was added for further incubation until the 4- to 6-h time point. At this time, the IL-8 and ICAM-1 mRNA levels increased. However, when *P. gingivalis*, regardless of whether the strains were adherent (381 and ATCC 33277) or nonadherent (W50), was cocultured with the cells continuously for 4 to 6 h, the ICAM-1 mRNA levels were not as elevated and the IL-8 mRNA concentrations decreased. These observations suggest that when *P. gingivalis* interacts with the epithelial cells, a signal is triggered that leads to the up-regulation of IL-8 and ICAM-1 mRNA. At the same time, another signal is triggered that leads to the down-regulation of IL-8 mRNA, and ICAM-1 mRNA to a lesser extent. This latter signal may occur through induced degradation of mRNA. Sequential addition of *F. nucleatum* and then *P. gingivalis* to epithelial-cell cultures resulted in the attenuation of IL-8 mRNA levels compared to challenge with *F. nucleatum* alone

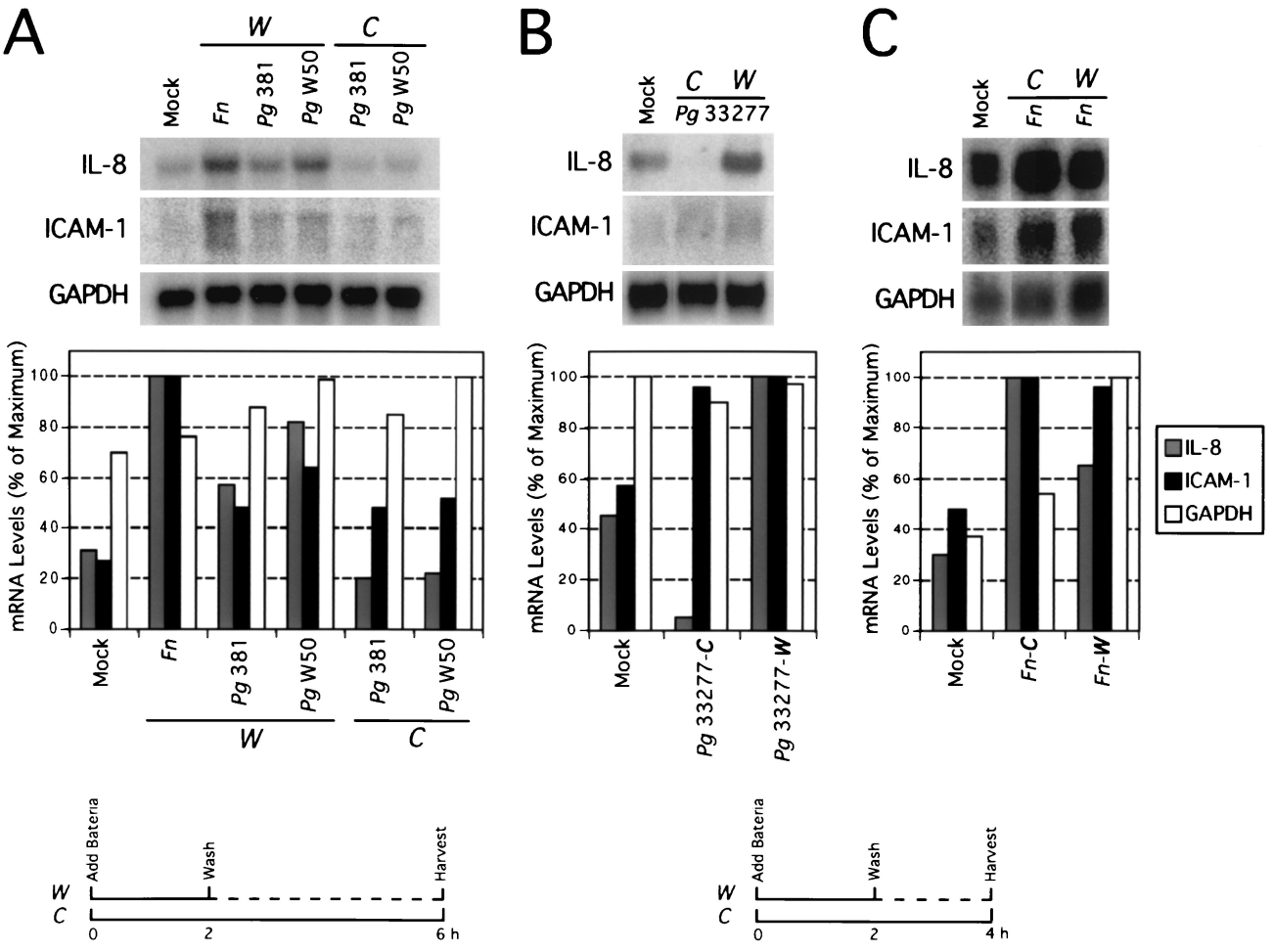

FIG. 4. IL-8 and ICAM-1 mRNA levels in gingival epithelial cells following bacterial challenge. Confluent HOK-18A cell monolayers were infected with *F. nucleatum* or various *P. gingivalis* strains at a MOI of 500:1. The bacterium–epithelial-cell coculture period was either 2, 4, or 6 h. RNA was isolated at 4 or 6 h after infection, and 8 to 12 μg of total RNA was subjected to Northern blot analysis. For the 2-h coculture group, the culture was washed and fresh medium containing appropriate antibiotics was added at 2 h and the cultures were further incubated for another 2 to 4 h before harvesting. For the 4- or 6-h coculture group, the bacteria were continuously cocultured with the epithelial cells to 4 or 6 h (see diagram in the bottom panel). (A) Epithelial cells were challenged with *F. nucleatum* 12230, *P. gingivalis* 381, or *P. gingivalis* W50 for 2 h (washed) or 6 h (continuous), and RNA was isolated at 6 h. (B and C) Epithelial cells were challenged with *P. gingivalis* 33277 (B) or *F. nucleatum* 12230 (C) for 2 h (washed) or 4 h (continuous) and RNA was isolated at 4 h. The levels of mRNA from the Northern blot analysis (shown in the top panel) are plotted and shown in the middle panel. Mock, no bacteria were added to the cultures. *Fn*, *F. nucleatum*; *Pg*, *P. gingivalis*; *W*, 2-h coculture group that was washed at the 2-h time point; *C*, 4- or 6-h coculture group, bacteria continuously cocultured with the epithelial cells.

(Fig. 5). This appears to contradict the *P. gingivalis* regulation of IL-8 mRNA kinetics presented above (Fig. 2 and 4). It was expected that stimulation of epithelial cells with *P. gingivalis* followed by *F. nucleatum* at 2 h would result in the attenuation of IL-8 mRNA levels compared to challenge with *F. nucleatum* alone. However, challenge of the epithelial cells with *F. nucleatum* followed by *P. gingivalis* at 2 h was expected to induce an even higher level of IL-8 mRNA than challenge with *F. nucleatum* alone since this would allow *P. gingivalis* to up-regulate IL-8 mRNA further before the down-regulation took place. A possible explanation is that the intracellular signals in epithelial cells leading to up-regulation of IL-8 mRNA may have been exhausted by *F. nucleatum* in the first 2 h of infection, so that the *P. gingivalis* down-regulation activities appeared early.

Bacterial components play important roles in stimulating proinflammatory gene expression (15, 39, 41, 42). Released bacterial surface-associated material from several periodontal microbes, including *A. actinomycetemcomitans* and *P. gingivalis*, is capable of inducing gingival fibroblasts and human peripheral blood mononuclear cells to release cytokines (28, 29). A lipid A-associated protein of *P. gingivalis* is a potent stimulator of IL-6 production (32). Bacterial factors interacting with epithelial cells could initiate intracellular signaling events, leading to activation of genes. For example, *Helicobacter pylori* induces activation of the transcription factor AP-1 through the ERK/mitogen-activated protein kinase cascade in gastric epithelial cells (24). The bacterial immunodominant antigen CagA from *H. pylori* enters epithelial cells and becomes wired to the eukaryotic signal transduction pathways (34). Increased NF-κB and AP-1 activities, a result of activation of intracellular signaling events, in intestinal epithelial cells in response to

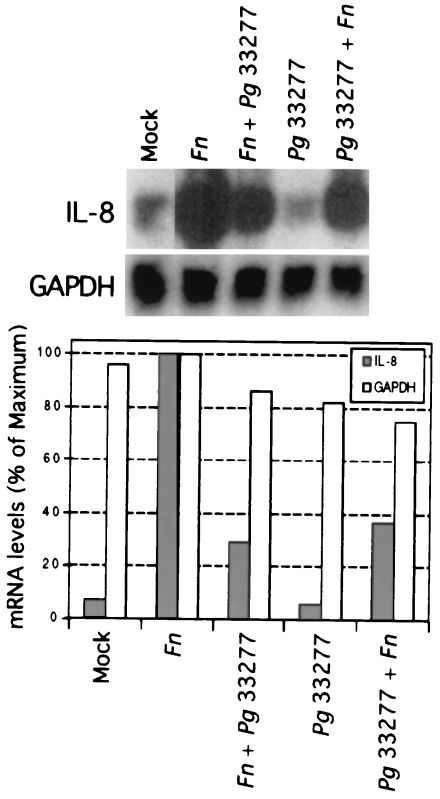

FIG. 5. IL-8 mRNA levels in gingival epithelial cells following sequential bacterial challenge. Confluent HOK-18A cell monolayers were stimulated with *F. nucleatum* 12230 (*Fn*), or *P. gingivalis* 33277 (*Pg*) alone or sequentially with *P. gingivalis* and *F. nucleatum* at MOIs of 500:1. The bacterium–epithelial-cell coculture period was 4 h. RNA was isolated at 4 h after infection, and 10 μg of total RNA was subjected to Northern blot analysis. For stimulation with one type of bacterium alone, the stimulus and the bacteria were in the culture throughout the 4-h period. For sequential stimulation, the first stimulus or bacterium was added at 0 h and remained in culture for 4 h. The second bacterium was added at 2 h. The levels of mRNA from the Northern blot results (shown in the top panel) are plotted and shown in the bottom panel. Mock, no bacteria were added to the cultures.

infection with enteroinvasive bacteria play a central role in intestinal innate immunity (8, 16). Studies also showed that *P. gingivalis* induces phosphotyrosine-dependent intracellular signaling in gingival epithelial cells, which facilitates the invasion of *P. gingivalis* (30). Although not yet investigated, this activated signal transduction may also lead to activation of genes. *P. gingivalis* lipopolysaccharide interacts with gingival fibroblasts through toll-like receptor 4 and induces the activation of several intracellular proteins including NF-κB and AP-1 (40). In our experimental setting, it is possible that one of these signal transduction events activate IL-8 and ICAM-1 genes whereas another event induces the degradation of IL-8 mRNA.

The down-regulation of IL-8 may debilitate the recruitment of neutrophils which normally play a critical role in maintaining periodontal health by defending against bacterial infection (4, 18, 22, 25, 26). The data presented here indicate that *P. gingivalis* is able to regulate the production of IL-8 mRNA at either the transcriptional or posttranscriptional level. The up-regulation of IL-8 in epithelial cells upon interaction with many microbial pathogens is a common feature, as evidenced by many observations (4, 7, 14, 18). Bacterial lipopolysaccharide, in *F. nucleatum* and *E. coli*, could be partly responsible for this IL-8 mRNA up-regulation (19). Thus, the ability of *P. gingivalis* to down-regulate IL-8 mRNA makes it a unique microorganism among the potential periodontal pathogens. The threefold effects of *P. gingivalis* on IL-8 expression, i.e., increased IL-8 mRNA levels, decreased IL-8 mRNA levels, and decreased IL-8 protein levels by protease-mediated degradation, illustrate its multiple strategies that may ultimately incapacitate local host defenses.

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