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-tingipain 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...
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 involved in the regulation of IL-8 and ICAM-1 mRNA in epithelial cells challenged with 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 primary 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 mM 3,3′,5-triiodo-L-thyronine, 25 μg of gentamicin per ml, and 250 μg 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, 0.4 g of bovine insulin per ml, 0.4 μg of hydrocortisone per ml, and 0.5 μg of transferrin per ml. Cells were grown in Dulbecco's modified Eagle's medium with 2% fetal bovine serum and 0.4 g of bovine insulin per ml.

**Culture plates and conditions.** Cell cultures were grown on 24-well culture plates and were centrifuged onto the monolayers at 900 g for 2 min. The supernatants were collected for IL-8 secretion following infection with *P. gingivalis*. The antibiotics used in the media were as follows: for infection with *Actinomyces odontolyticus* 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 experiments 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 32P-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 XhoI 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 EcoRI digestion from plasmid pHIL8 (kindly provided by M. Kagnoff, La Jolla, Calif.), which carries the full-length human IL-8 cDNA. A 32P-labeled cDNA fragment of human glyceraldehyde-3-phosphate dehydrogenase was used as the control probe to verify equal amount of RNA from each cell line was used in each analysis. The signals were visualized by autoradiography using a phosphor Image 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 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. actinomycteumocytum* 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.

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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 of 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
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

<table>
<thead>
<tr>
<th>Bacterium added</th>
<th>Amt of secreted IL-8 (pg/ml)</th>
<th>% Attachment</th>
<th>% Invasion</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>950 ± 97</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. gingivalis 381</td>
<td>128 ± 31</td>
<td>3.1563 ± 2.6456</td>
<td>1.4231 ± 0.9102</td>
</tr>
<tr>
<td>Mutant MT-10</td>
<td>151 ± 34</td>
<td>2.3943 ± 0.5823</td>
<td>1.9874 ± 0.8827</td>
</tr>
<tr>
<td>Mutant G-102</td>
<td>332 ± 79</td>
<td>6.0650 ± 3.2536</td>
<td>4.6520 ± 3.6773</td>
</tr>
<tr>
<td>P. gingivalis W50</td>
<td>944 ± 82</td>
<td>0.0038 ± 0.0016</td>
<td>0.0083 ± 0.0074</td>
</tr>
<tr>
<td>P. gingivalis W83</td>
<td>927 ± 63</td>
<td>0.0042 ± 0.0025</td>
<td>0.0005 ± 0.0003</td>
</tr>
<tr>
<td>Mutant V2296</td>
<td>1,041 ± 69</td>
<td>0.0292 ± 0.0241</td>
<td>0.009 ± 0.0085</td>
</tr>
<tr>
<td>None</td>
<td>457 ± 44</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

P. gingivalis ATCC 33277

Not treated: 74 ± 74
Azide treated: 82 ± 26

* 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.

**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. gingivalis
epithelial cells were washed, the attached $P.\ gingivalis$ the epithelial cells were washed. The results shown in Table 1 of IL-8 and ICAM-1 protein expression during the period after

tation by

at the protein level, the mRNA induction patterns of these two

stimulation of epithelial cells down-regulated IL-8 and ICAM-1 transcription. The kinetics of IL-8 secretion

suggests that all three bacteria induce an epithelial-cell re-

appearance identical to the response

$P.\ gingivalis$ and $F.\ nucleatum$, 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 experi-

ments in a triplicate assay.

$P.\ gingivalis$ 381................................................................. 66 ± 3$^c$

Mutant MT-10................................................................. 82 ± 10

Mutant G-102................................................................. 94 ± 11

$P.\ gingivalis$ W83................................................................. 58 ± 14$^d$

Mutant V2296................................................................. 60 ± 11

$F.\ nucleatum$ 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 $P.\ gingivalis$, $F.\ nuclea-
tum$, 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 experi-

ments in a triplicate assay.

$\ ^b$ $P = 0.183$ (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, $A.\ actinomycetemcomi-
tans$, $F.\ nucleatum$, 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 stimula-

tion by $A.\ actinomycetemcomitans$ and $F.\ nucleatum$. This suggests that all three bacteria induce an epithelial-cell re-

sponse 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.\ gingo-

givalis$ (2, 4, 11, 12, 20, 25, 43), the most likely cause is the degradation of ICAM-1 and IL-8 proteins by proteases. Con-
sistent 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 de-

grade 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 po-
tent activity in degrading IL-8 in purified form (25) and may account for the degradation from crude supernatant, as demonstra-

ted by our study (Table 3); however, bacterial cell-associa-

ted 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 se-

creted 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 estab-

lish 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 accumu-

lated 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.\ gingo-

givalis$ 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. Se-

quential 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.
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.

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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