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Gingipains from *Porphyromonas gingivalis* Increase the Chemotactic and Respiratory Burst-Priming Properties of the 77-Amino-Acid Interleukin-8 Variant▽

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***Porphyromonas gingivalis*, a gram-negative anaerobe which is implicated in the etiology of active periodontitis, secretes degradative enzymes (gingipains) and sheds proinflammatory mediators (e.g., lipopolysaccharides [LPS]). LPS triggers the secretion of interleukin-8 (IL-8) from immune (72-amino-acid [aa] variant [IL-8_{72aa}]) and nonimmune (IL-8_{77aa}) cells. IL-8_{77aa} has low chemotactic and respiratory burst-inducing activity but is susceptible to cleavage by gingipains. This study shows that both R- and K-gingipain treatments of IL-8_{77aa} significantly enhance burst activation by fMLP and chemotactic activity ($P < 0.05$) but decrease burst activation and chemotactic activity of IL-8_{72aa} toward neutrophil-like HL60 cells and primary neutrophils ($P < 0.05$). Using tandem mass spectrometry, we have demonstrated that R-gingipain cleaves 5- and 11-aa peptides from the N-terminal portion of IL-8_{77aa} and the resultant peptides are biologically active, while K-gingipain removes an 8-aa N-terminal peptide yielding a 69-aa isoform of IL-8 that shows enhanced biological activity. During periodontitis, secreted gingipains may differentially affect neutrophil chemotaxis and activation in response to IL-8 according to the cellular source of the chemokine.**

Inflammatory periodontal diseases have an infectious etiology and are characterized by excess inflammation within the periodontal tissues, which can progress to alveolar bone loss and ultimately tooth loss (4). The primary etiological agent for periodontitis is the subgingival plaque biofilm, and disease progression is associated with an ecological shift in biofilm composition to a predominantly anaerobic flora (5, 27). Evidence indicates that this in turn triggers the host response, which in susceptible patients is abnormal, involving excess generation of proteolytic enzymes (9) and reactive oxygen species (ROS) (18), both of which are important determinants of disease progression and severity (5, 11). Neutrophilic inflammation is the major source of the tissue-destructive species (6), and recent studies have demonstrated that peripheral blood neutrophils from periodontitis patients are both hyperreactive to Fcγ-receptor stimulation and also demonstrate baseline hyperactivity with respect to extracellular ROS release (17, 18). The extracellular ROS production from neutrophil infiltrates into the periodontium is significant (17) but modest; however, any process which results in enhanced polymorphonuclear leukocyte recruitment to or retention (e.g., delayed apoptosis) within the periodontal tissues may contribute to ROS-mediated tissue damage.

The oral anaerobic rod *Porphyromonas gingivalis* is the organism most strongly associated with active periodontitis (1). This organism possesses a number of virulence determinants which potentially contribute to its pathogenicity, including the ability to secrete a range of degradative proteinases (1); among these, the gingipains have been extensively studied (15). Furthermore, the pathogenic bacteria within the subgingival environment shed proinflammatory mediators such as lipopolysaccharide (LPS). LPS in turn triggers the secretion of chemokines/cytokines such as interleukin-1β (IL-1β), tumor necrosis factor alpha (TNF-α), IL-6 and IL-8 (72-amino-acid [aa] variant [IL-8_{72aa}]) from resident inflammatory cells, which contribute to the initial inflammatory response (20).

IL-8 is a major chemokine with potent stimulatory effects on neutrophils, including chemotaxis, degranulation, and cytoplasmic Ca^{2+} elevation. IL-8 is a small polypeptide with a molecular mass of 8 to 10 kDa (22) that was originally isolated from monocytes (2). Subsequent studies have shown that IL-8 is also produced from a wide range of cell types, including fibroblasts, epithelial cells/keratinocytes, lymphocytes, endothelial cells, and neutrophilic polymorphonuclear leukocytes (neutrophils). In response to stimulus, IL-8 is produced as a 99-aa-long precursor polypeptide (2), which is subsequently processed into a biologically active peptide. IL-8 varies in length from 79 aa to 77-, 72-, 71-, 70-, and 69-aa variants (23). Although IL-8 is subject to variable processing at the N terminus, the IL-8_{72aa} and IL-8_{77aa} peptides have been identified as the predominant variants. The major form of IL-8_{72aa} has been extensively studied for its potent ability to prime neutrophils to stimulate the respiratory burst to a secondary stimulus, such as *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) (12, 14).

IL-8_{77aa} is recognized as a less-potent variant for neutrophil activation. It exhibits reduced chemotactic properties and attenuates neutrophil adhesion to endothelial cell walls (10). Recent reports have suggested that this longer-amino-acid form is susceptible to cleavage by proteolysis into a biologically

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active form (13, 24, 25). It has also been shown that IL-8_{77aa} is susceptible to cleavage by gingipains, the principal secreted cysteine proteases of *P. gingivalis* (19). The potential for gingipains to enhance the activity of IL-8_{77aa} presents an additional mechanism of the organism's pathogenicity, whereby *P. gingivalis* promotes enhanced neutrophil recruitment, activation, and further local tissue degradation. To date, the effect of gingipain processing of IL-8 on subsequent chemotactic activity or respiratory burst priming has not been determined. Therefore, this report investigates whether gingipains from *P. gingivalis* can modify IL-8_{77aa} chemotactic and priming activities using primary neutrophils and differentiated cells (dHL60) of the neutrophil-like HL60 cell line as responder cells.

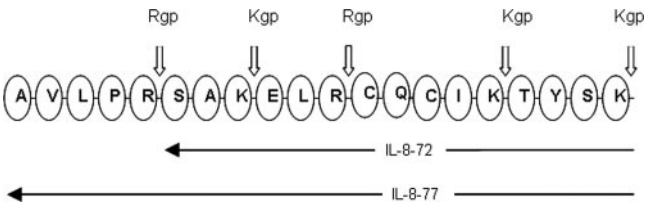

FIG. 1. N-terminal amino acid sequence of IL-8 and possible Rgp and Kgp cleavage sites. IL-8_{77aa} (IL-8-77) has a peptide sequence extended by 5 aa relative to IL-8_{72aa} (IL-8-72). Rgp has specific amino peptidase activity to R-X peptide bonds, and Kgp has specific amino peptidase activity to K-X peptide bonds.

MATERIALS AND METHODS

Materials. *P. gingivalis* W83 was kindly provided by A. Roberts (Periodontal Research Group, School of Dentistry, University of Birmingham, United Kingdom). All reagents were obtained from Sigma Chemical Company (Poole, United Kingdom) and solvents from Fisher (Loughborough, United Kingdom) unless otherwise stated. RPMI 1640, fetal bovine serum, and penicillin (1,000 U ml^{-1})/streptomycin (10,000 μg ml^{-1}) were obtained from GibcoBRL (Paisley, United Kingdom). Recombinant IL-8_{72aa}, endothelium-derived recombinant IL-8_{77aa}, and monoclonal anti-human IL-8 antibody, clone 6218, were purchased from R&D systems (Abingdon, United Kingdom).

HL60 cell cultures. The human promyelocytic cell line HL60 was purchased from the European Collection of Cell Cultures (ECACC no. 98070101) and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum and 1% penicillin-streptomycin at 37°C in a 5% CO_2–95% air humidified atmosphere. The cells were induced to differentiate in the presence of 1.0% dimethyl sulfoxide for 5 days after seeding at cell density of 2×10^5 cells/ml.

Collection and isolation of peripheral blood neutrophils. Venous blood was collected from systemically and periodontally healthy donors into 4% sodium citrate (wt/vol) in phosphate-buffered saline, with a citrate/blood ratio of 1:9, and neutrophils were isolated as described by Matthews et al. (7). Isolated cells were washed and resuspended in physiological salt solution (PSS: 115 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 10 mM glucose, 1 mM $MgSO_4$, 1.25 mM $CaCl_2$, 25 mM HEPES-Na supplemented with 0.1% bovine serum albumin at pH 7.4).

Cultivation of *P. gingivalis* strain W83. Cultures were grown statically in 200 ml of liquid medium containing 6.0g of Trypticase soy broth (Difco, Detroit, MI), 2.0 g of yeast extract, supplemented with 1 mg of hemin, 200 mg of L-cysteine, 20 mg of dithiothreitol, and 0.5 mg of menadione at 37°C in an anaerobic atmosphere of 10% H_2, 10% CO_2, and 80% N_2 for 48 h (miniMACS anaerobic workstation; Don Whitley Scientific).

Isolation of Rgp and Kgp. Gingipains were isolated according to the method described by Yun et al. (29) using a 5-ml packed-volume arginine-Sepharose column for the final affinity purification stage. Column fractions (1 ml) containing lysine-specific gingipain (Kgp; eluted with 0.75 M L-lysine) and arginine-specific gingipain (Rgp; eluted with 1 M L-arginine) were dialyzed against Tris buffer overnight at 5°C.

Enzyme activity assays. The amidolytic activities of the purified Rgp and Kgp were measured with the substrate α-*N*-benzoyl-L-arginine *p*-nitroanilide hydrochloride (L-BAPNA). One hundred microliters of each of the Rgp and Kgp fractions was incubated with L-BAPNA (final concentration of 1 mM) in 100 μl of 0.2 M Tris-HCl, 0.1 M NaCl, 5 mM $CaCl_2$, and 10 mM L-cysteine at pH 7.6 and 37°C. After 1 h of incubation, the reaction was stopped by addition of 10 μl of glacial acetic acid. The optical density was measured at 405 nm for each fraction, and the values were corrected by subtraction of negative control values (without proteinases).

Proteolytic degradation of IL-8 by purified gingipains. Pooled fractions for each gingipain were activated as described by Mikolajczyk-Pawlinska et al. (18). Rgp and Kgp were adjusted to equimolar concentrations in Tris buffer, pH 7.6. Activated gingipains (3 mM) were mixed with 1.65 μM IL-8_{77aa} or IL-8_{72aa} and incubated at 37°C for 30 min. Enzyme activity was terminated postincubation by addition of 1 μl of a protease inhibitor cocktail containing leupeptin hemisulfate.

MS/MS analysis of Kgp- and Rgp-treated IL-8_{77aa} and IL-8_{72aa}. Kgp- or Rgp-treated IL-8_{77aa} and IL-8_{72aa} (0.14 pg/μl) were diluted in 50% methanol in water with acetic acid (1%) to enhance ionization and subjected to mass analysis after injection at 1 μl/min using a Thermo LTQ tandem mass spectrometer (MS/MS) in electrospray mode. The machine was externally mass calibrated using peptides, caffeine, and Ultramark 1621 (ABCR GmbH & Co.). The acceleration voltage was set at 20 kV, and data were collected as the average total scan of 100 scans with the scan range set from 100 to 2,000 *m/z* to search for fragmented IL-8_{77aa} and IL-8_{72aa}. Multiply charged molecular ions were subjected to collision-induced dissociation (CID) with argon gas, and the resulting MS/MS data were recorded for comparison against SWISSPROT predicted cleavage sites in IL-8.

Neutralization of IL-8_{77aa} peptide chemotactic activity. In order to investigate whether the enhanced biological activity of Kgp- or Rgp-treated IL-8 could be ascribed to the resultant formation of IL-8_{72aa} rather than to the released peptides, neutralizing anti-human IL-8 antibody (5 μg/ml), which recognizes the whole molecule, was added to Kgp- or Rgp-treated IL-8_{77aa} (8.25 nM) for 30 min at room temperature prior to addition of neutralized IL-8 to the lower chambers for chemotaxis experiments.

Chemotaxis assay. dHL60 cell/neutrophil chemotaxis was measured by Boyden's technique using (2- or 5-μm pore size, respectively) polyvinylpyrrolidone-free polycarbonate filters in a 96 multiwell chamber (Neuroprobe, Inc.). dHL60 cells/neutrophils (1×10^5) were washed and resuspended in PSS. Pre- and post-gingipain-treated IL-8 (8.25 nM) samples, gingipain-treated and antibody-neutralized IL-8_{77aa}, and untreated gingipains were added to the lower wells of the chamber, the filter was fixed in place, and the upper wells were loaded with 1×10^5 dHL60 cells/neutrophils in 100 μl of PSS at 37°C for 90 min. After chemotaxis, cell-containing buffer from the upper chamber was removed and the top of the filter was washed with PSS. The microplate/filter assembly was centrifuged at $400 \times g$ for 10 min. The filter was carefully removed, and cell counts in the lower chamber were taken by flow cytometry (Coulter Epics XL). Results were expressed as specific cell migration after subtraction of background migration. *Escherichia coli* LPS (serotype 0111:B4; 1 μg/ml) was used as a positive control in all assays, and Rgp and Kgp alone in the presence of protease inhibitor cocktail acted as a negative control.

Chemiluminescent assay for respiratory burst activity. Chemiluminescence assays were performed using lucigenin to detect total superoxide production by neutrophils or dHL60 cells. Assays were performed (37°C) using a Berthold microplate luminometer (LB96v). Neutrophils (5×10^5 cells) were added to each well containing 100 μM lucigenin in PSS and incubated for 30 min at 37°C. Light emission in relative light units (RLU) was recorded during the 30-min prestimulation period to establish a steady baseline. Cells were then incubated with gingipain-treated or untreated IL-8 isoforms for 10 min prior to stimulation with 1 μM fMLP. The RLU peak values were analyzed for each treatment, and time to peak for each stimulus was recorded.

Data analysis. Statistical analysis was performed by one-way analysis of variance followed by Tukey's comparison test analysis. $P < 0.05$ was considered significant.

RESULTS

Kgp and Rgp specifically cleave the N terminus of IL-8_{77aa}. Rgp has two theoretical, amino peptidase cleavage sites for IL-8_{77aa}, and Kgp has three amino peptidase cleavage sites in IL-8_{77aa} (Fig. 1). At the N terminus of IL-8_{72aa}, Rgp has one theoretical cleavage site, while Kgp has three sites. To investigate the effects of gingipain activity on IL-8_{77aa}, released N-terminal peptides corresponding in mass to Rgp- and Kgp-cleaved IL-8_{77aa} isoforms were investigated by MS/MS with

TABLE 1. Release of amino-terminal peptides by gingipain treatment of IL-8_{77aa} and IL-8_{72aa} as determined by MS/MS[a]

IL-8 isoform and gingipain treatment	*m/z*	aa position		Sequence
		Start	End	
IL-8_{77aa}				
Rgp	555.7020	1	5	AVLPR
	703.8212	6	11	SAKELR
Kgp	842.0352	1	8	AVLPRSAK
IL-8_{72aa}				
Rgp	703.4097	1	6	SAKELR
Kgp	305.1819	1	3	SAK
	1135.4	4	11	ELRCQCIK
	498.5	12	15	TYSK

[a] The table documents *m/z* ratios, amino acid positions, and sequences of peptides released post-Kgp treatment of IL-8_{77aa}.

CID. Rgp treatments preferentially cleaved the N terminus of IL-8_{77aa}, releasing peptides of *m/z* 555.7 and 703.82 corresponding to residues 1 to 5 and 6 to 11 of the N-terminal region with the sequences reported in Table 1, to produce 72- and 66-aa-long IL-8 peptides. Rgp treatment also released a peptide of *m/z* 703.41 and sequence SAKELR from IL-8_{72aa} to yield an IL-8_{66aa} polypeptide. However, Kgp treatment of IL-8_{77aa} released an 8-aa-long polypeptide of *m/z* 842.03 (AVLP RSAK) from the N terminus, resulting in a 69-aa-long IL-8 polypeptide. Kgp treatment of IL-8_{72aa} released peptides of *m/z* 305.18, 1135.4, and 498.5, resulting in 69-, 61-, and 57-aa-long peptides, respectively.

Kgp and Rgp increase the chemotactic properties of biologically inactive IL-8_{77aa}. In order to determine the effect of Rgp and Kgp treatment on chemotactic properties of IL-8 isoforms, dHL60 cells/primary neutrophils were allowed to undergo chemotaxis toward pre- or post-gingipain-treated IL-8_{72aa} and IL-8_{77aa} isoforms. The effect of gingipain treatment on IL-8-dependent chemotaxis was corrected for chemotaxis toward either inactivated Rgp or inactivated Kgp, where migration was always lower toward gingipains than to LPS or IL-8 isoforms. IL-8_{72aa} demonstrated higher chemotactic activity than the native IL-8_{77aa} isoform. Both Rgp (Fig. 2A) and Kgp (Fig. 2B) treatments significantly decreased the chemotactic activity of IL-8_{72aa} ($P < 0.001$) toward HL60 cells. In contrast, Kgp treatment significantly increased IL-8_{77aa} chemotactic properties ($P < 0.05$). In order to compare the behavior of dHL60 cells with peripheral blood neutrophils, chemotaxis of primary neutrophils toward IL-8 isoforms was measured. Confirming the observations in dHL60 cells, both Kgp (Fig. 2C) and Rgp (Fig. 2D) treatments significantly increased the chemotactic prop-

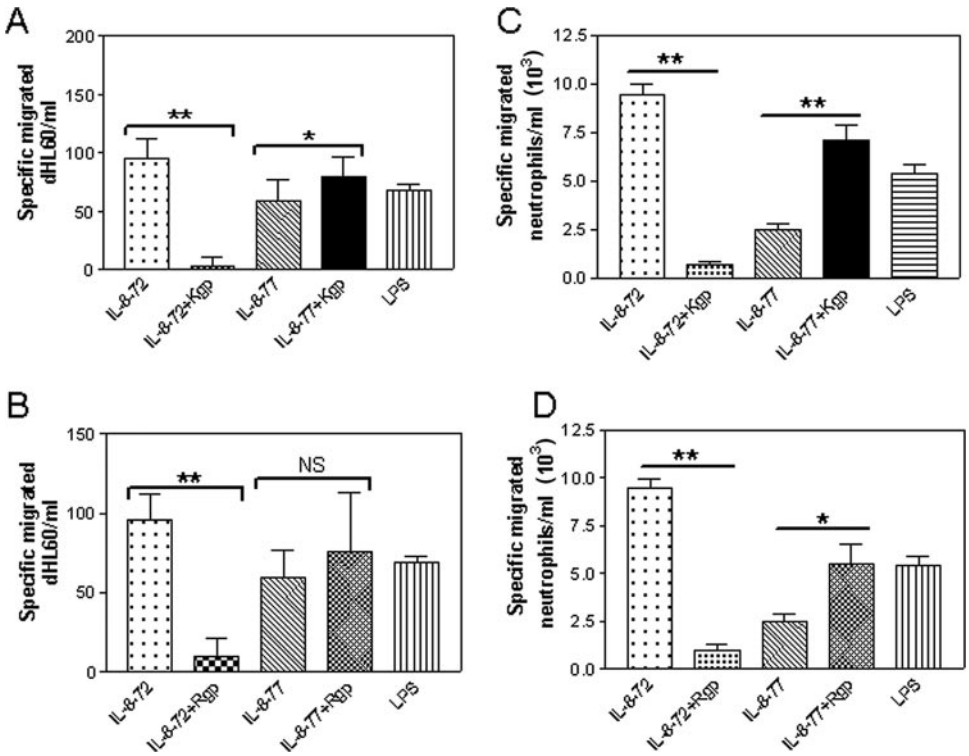

FIG. 2. Gingipain treatment increases chemotactic activity of IL-8_{77aa} toward dHL60 cells and neutrophils. IL-8 isoforms of 72- and 77-aa-long peptides (IL-8-72 and IL-8-77, respectively) were treated with 10 mM cysteine activated Kgp or Rgp for 30 min. Chemotaxis (corrected for background) of 1×10^5 dHL60 cells through a 2-μm filter toward Kgp (A)- and Rgp (B)-treated or untreated IL-8 isoforms was observed for 90 min in a multiwell chemotaxis chamber. Specific chemotaxis of 1×10^5 primary neutrophils toward Kgp (C)- and Rgp (D)-treated IL-8 isoforms through a 5-μm filter was observed for 90 min in a multiwell chemotaxis chamber. Cell counts in the lower wells were taken by flow cytometry and are expressed as mean cell number migrated ± standard error of the mean, where $n = 3$ independent experiments performed in triplicate. *, $P < 0.01$; **, $P < 0.001$; and NS, not significant by Tukey's multiple comparison test.

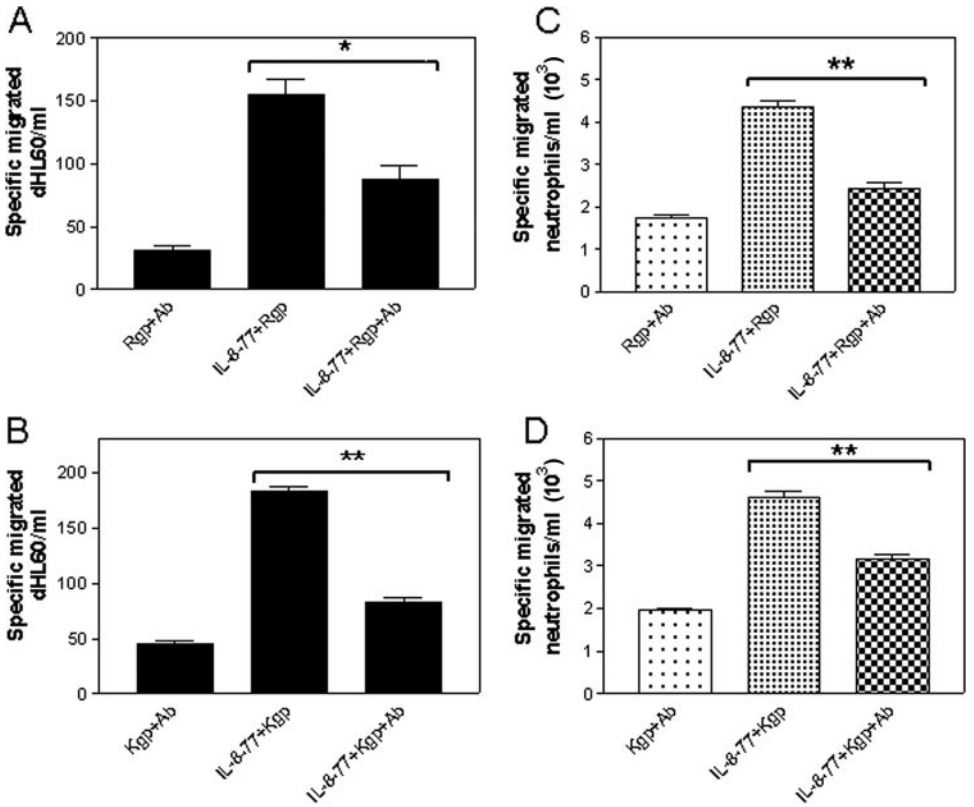

FIG. 3. Major IL-8 peptides released from gingipain treatment of IL-8_{77aa} are responsible for enhanced chemotactic activity of dHL60 cells and primary neutrophils. IL-8 isoforms of 72- and 77-aa-long peptides (IL-8-72 and IL-8-77, respectively) were treated with 10 mM cysteine-activated Kgp or Rgp for 30 min. After neutralization of gingipains, the resultant peptides were subsequently incubated with neutralizing anti-IL-8 antibody (Ab). Chemotaxis (corrected for background) of 1×10^5 dHL60 cells through a 2-μm filter toward Kgp-treated IL-8_{77aa} and Kgp-treated IL-8_{77aa} plus IL-8-neutralized isoforms (A) or Rgp-treated IL-8_{77aa} and Rgp-treated IL-8_{77aa} plus IL-8-neutralized isoforms (B) was observed for 90 min in a multiwell chemotaxis chamber. Specific chemotaxis of 1×10^5 primary neutrophils toward Kgp-treated-IL-8_{77aa} and Kgp-treated IL-8_{77aa} plus IL-8-neutralized isoforms (C) and Rgp-treated-IL-8_{77aa} and Rgp-treated IL-8_{77aa} plus IL-8-neutralized isoforms (D) through a 5-μm filter was observed for 90 min in a multiwell chemotaxis chamber. Cell counts in the lower wells were taken by flow cytometry and are expressed as mean cell number migrated ± standard error of the mean, where $n = 3$ independent experiments performed in triplicate. **, $P < 0.001$ by Tukey's multiple-comparison test.

erties of IL-8_{77aa} ($P < 0.001$ and $P < 0.01$, respectively) toward primary neutrophils.

N-terminally shortened peptide fragments of IL-8_{77aa} account for the increased chemotactic activity. To investigate whether N-terminally shortened IL-8_{77aa} peptides accounted for the observed chemotactic activity, gingipain-treated IL-8_{77aa} was neutralized with anti-human IL-8 antibody prior to chemotaxis assay. Both antibody-neutralized Rgp-treated IL-8_{77aa} (Fig. 3A) and Kgp-treated IL-8_{77aa} (Fig. 3B) showed significantly decreased chemotactic activity toward dHL60 cells ($P < 0.05$ and $P < 0.01$, respectively) compared with gingipain-treated IL-8_{77aa} in the absence of neutralizing antibody. Similarly, when the experiment was repeated using neutralized Kgp- or Rgp-treated IL-8_{77aa} as a chemotaxin for primary neutrophils, again, the enhanced biological activity of gingipain-cleaved IL-8_{77aa} was not evident (Fig. 3C and D).

Kgp and Rgp increase the priming effect of IL-8_{77aa} for the respiratory burst in response to fMLP. The priming effect of IL-8 on the fMLP-induced respiratory burst was measured by lucigenin-dependent chemiluminescence using isolated peripheral blood neutrophils. IL-8_{72aa} primed neutrophils for enhanced superoxide production after fMLP stimulation, whereas neither IL-8_{77aa} nor isolated gingipains had any priming effect (Fig. 4). However, both Rgp (Fig. 4A)- and Kgp (Fig. 4B)-treated IL-8_{77aa} primed neutrophils for fMLP-induced superoxide production, demonstrating significantly increased superoxide generation compared with native IL-8_{77aa} ($P < 0.05$). In contrast, gingipain treatment decreased the priming activity of IL-8_{72aa} ($P < 0.05$) for fMLP-stimulated superoxide production.

DISCUSSION

Infection of host tissue by pathogenic bacteria and/or stimulation by microbial components/virulence factors triggers the production of proinflammatory peptides that have the ability to activate and recruit neutrophilic polymorphonuclear leukocytes along a concentration gradient. Patients with periodontitis show increased numbers of neutrophils within periodontal tissues and pockets (21), and recent work has demonstrated baseline hyperactivity of peripheral blood neutrophils, with respect to extracellular ROS release (18) and proteolytic enzyme release (9), in periodontitis patients relative to matched healthy controls. Such mechanisms when coactive may explain a significant amount of the oxidative stress reported in peri-

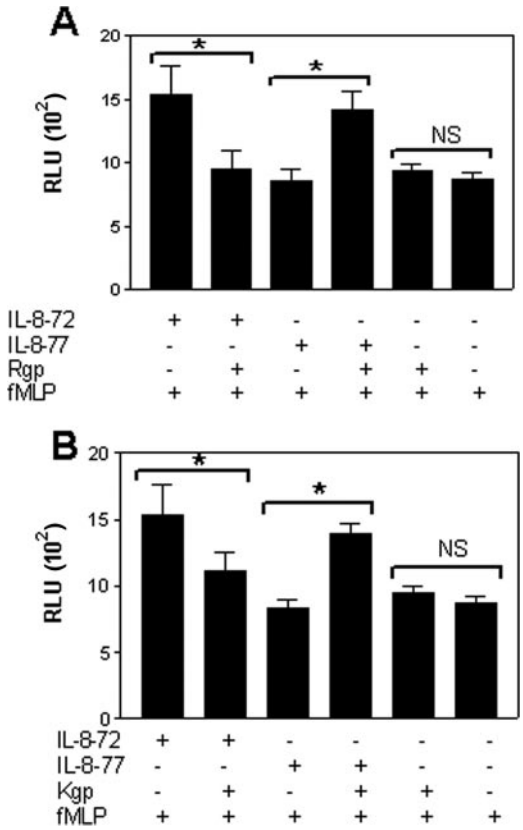

FIG. 4. Gingipain treatment enhances the priming effect of IL-8_{77aa} on neutrophils. Neutrophils (5×10^5) were primed with Rgp-treated or untreated 72- and 77-aa IL-8 isoforms (IL-8-72 and IL-8-77, respectively) (A) or Kgp-treated or untreated IL-8 isoforms (B) for 10 min prior to stimulation with 1 μM fMLP. Mean peak (RLU ± standard error of the mean; $n = 9$) chemiluminescence generated by neutrophils was recorded. Significant differences were calculated (*, $P < 0.05$) with Tukey's multiple-comparison test. NS, not significant.

odontitis-affected tissues (6). Therefore, the influence of periodontal bacteria and their virulence factors on IL-8-mediated neutrophil chemotaxis and activation is important to elucidate. In contrast to biologically active IL-8_{72aa}, the IL-8_{77aa} peptide produced by epithelial cells, fibroblasts, and endothelial cells is resistant to a wide range of host proteinases; it has a low chemotactic activity (10) and less respiratory burst-priming activity. In our experiments, we have used LPS as a positive control; LPS is a well-known chemotactic bacterial component which requires serum components such as LPS-binding protein for receptor activation (28). However, as serum may also contain other chemotactic factors, it was excluded from our experiments, and the chemotactic activity of LPS in serum-free conditions here was greater than that of IL-8_{77aa} but less than that of IL-8_{72aa} toward primary neutrophils.

In this study, we have investigated a possible mechanism by which *P. gingivalis* could manipulate IL-8 cytokine-mediated neutrophil chemotaxis using a dHL60 cell model and also primary human neutrophils. Gingipains increased the priming activity induced by IL-8_{77aa} on the fMLP-induced oxidative burst in primary neutrophils, data that confirm previous studies measuring elastase release from neutrophils, where IL-8_{77aa}-induced release was shown to be increased after gingipain treatment (19).

Our results demonstrate a significant increase in the chemotactic properties of IL-8_{77aa} and a higher priming capability of IL-8_{77aa} after incubation with L-cysteine-activated gingipains under the conditions described. Compared with primary neutrophils, dHL60 cells have low CXC2 receptor expression (26), and this may explain the lower rate of dHL60 cell migration toward IL-8. The corresponding increase in data variation may account for the lack of significant increase in migration of dHL60 cells toward Rgp-treated IL-8_{77aa} compared to the increased migration of primary neutrophils. Chemotactic properties and priming abilities of truncated, gingipain-treated forms of IL-8_{77aa} were found to be two- to threefold higher than those of untreated IL-8_{77aa}. Using a neutralizing antibody against the complete sequence of IL-8, we confirmed that the increased biological activity of IL-8_{77aa} following gingipain treatment was due to the release of mature IL-8, rather than due to the release of small peptides identified by MS, as the neutralizing antibody (which does not recognize small peptide fragments) completely inhibited the increase in activity. Given that reported concentrations of reduced glutathione/cysteine in gingival fluids are 1,000-fold higher than those of serum (7), this may represent a physiologically relevant mechanism whereby gingipains contribute to neutrophil recruitment and activation at *P. gingivalis*-infected sites.

The extended amino terminus of IL-8_{77aa} folds back to interact with the essential Glu^4-Leu^5-Arg^6 (ELR) sequence; this may protect the ELR sequence from interaction with the receptor and may explain the low chemotactic activity of IL-8_{77aa} compared to IL-8_{72aa}. The N-terminal amino acid sequence of IL-8_{77aa} is AVLPRSAKELRCQCIKTYSK- (21). Rgp has theoretical, specific amino peptidase cleavage activity at Arg^5-Ser^6 and Arg^{11}-Cys^{12} (15), yielding peptides with lengths of 72 and 66 aa. After Rgp treatment, we observed cleavage products of IL-8_{77aa} by MS/MS of the 5 and 11 aa corresponding to the putative N-terminal cleavage sites. There was no evidence of low-molecular-weight peptides corresponding to cleavage at the putative C-terminal sites. Even though some reports suggest that ELR sequences in IL-8 are necessary for high-affinity binding to IL-8 receptor, recent studies have shown that IL-8_{66aa} has similar activity to IL-8_{72aa} (8). Our observations do not fully support the latter work: while an increase in activity of IL-8_{77aa} is observed after Rgp treatment, which results in products with lengths of 72 and 66 aa, a decrease in IL-8_{72aa} activity was observed after Rgp treatment, indicating that IL-8_{66aa} is not biologically active and that the observed increase in activity after treatment of IL-8_{77aa} with Rgp may be due to release of IL-8_{72aa} alone. Active IL-8 requires a properly folded protein structure with a highly conserved ELR sequence near the N terminus that is critical for its activity (16). Kgp, with its specificity for the Lys-X peptide bond, is predicted to cleave the IL-8_{77aa} amino-terminal sequence at Lys^8-Glu^9, and this product was observed in our studies by MS/MS. The resultant 69-aa-long form of IL-8 shows enhanced biological activity compared with IL-8_{77aa} in our studies of chemotactic activity and respiratory burst priming. In contrast, the more biologically active form of IL-8_{72aa} showed reduced chemotactic activity after treatment with both Rgp and Kgp. Analysis of released peptides by MS/MS confirmed further cleavage of

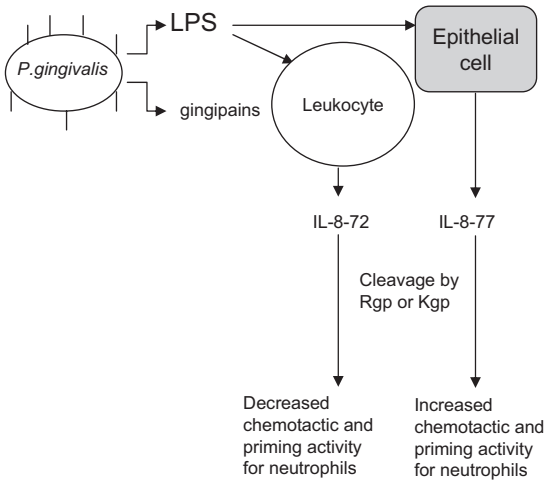

FIG. 5. Schematic representation of gingipain-modulated IL-8 response. *P. gingivalis* stimulates production of host proinflammatory mediators, including IL-8. IL-8_{77aa} (IL-8-77) secreted by host epithelial cells can be cleaved into more active, truncated forms. Collectively with IL-8_{72aa} (IL-8-72), these truncated forms may recruit more neutrophils to the site of infections and also prime their activation, which may contribute to the increased hyperactivity of neutrophils in periodontitis. Prolonged exposure to gingipains may trigger further degradation of IL-8_{77aa} which may reduce chemotaxis and neutrophil priming, thus prolonging the inflammatory lesion.

IL-8_{72aa}, releasing three peptides corresponding to the 15 N-terminal aa of IL-8_{72aa}. The presence of 5 aa at the N terminus of IL-8_{77aa} compared to IL-8_{72aa} appears to modulate cleavage of the peptide by gingipains. The difference in IL-8_{77aa} susceptibility to gingipain treatment compared with that of IL-8_{72aa} may relate to either differences in three-dimensional structures at the N terminus or specific charge differences which contribute to change in altered accessibility and thus cleavage by gingipains. Previous studies have shown that prolonged incubation with Rgp or Kgp could result total IL-8_{77aa} degradation (19); physiologically, a concentration gradient will exist, where released gingipain will be highest in closest proximity to the site of bacterial colonization and via diffusion the concentration of gingipains will be lower further away from the site. Thus, while initial enzyme release may activate local IL-8_{77aa} in the early stages of infection, the IL-8_{77aa} variant may be completely degraded in the immediate locality over time. However, further from the site of infection, diffusing gingipain may cause activation of IL-8_{77aa}. The importance of this observation should be addressed in vivo following specific inhibition of gingipain activity or expression.

Previous studies have shown that the capacity of gingipains to manipulate the host cytokine network is partly due to degradation of other cytokines such as IL-1β, IL-6, and TNF-α (3). Therefore, it has been suggested that the ability to inactivate cytokines by *P. gingivalis* in the early stages of pathogenesis is advantageous for the organism. Rgp has recently been shown to digest secretory leukocyte protease inhibitor released from neutrophils, thus reducing the protective effect against bacterial proinflammatory molecules by which disease in periodontal tissues may be accelerated (21). In contrast, degradation of proinflammatory cytokines could diminish neutrophil chemotaxis toward infected periodontal sites by lowering inflammatory cytokine secretion. However, in periodontal patients, neutrophil recruitment to the gingival crevice is maintained despite the presence of gingipains.

It is probable that an alternative mechanism exists to promote neutrophil chemotaxis and activity at periodontitis sites which may involve the secretion of the longer form of IL-8_{77aa} by nonimmune cells. This in vitro study provides a possible mechanism for *P. gingivalis*-manipulated neutrophil chemotaxis into periodontal pockets via activation of IL-8_{77aa}, as illustrated by Fig. 5. In conclusion, products from *P. gingivalis* may regulate host neutrophil accumulation at infected periodontal sites by initially stimulating the production of IL-8_{77aa} by nonimmune cells (e.g., epithelium) and promoting gingipain-dependent modification of IL-8_{77aa} into a more biologically active chemokine which promotes neutrophil chemotaxis and priming. Thereafter, after prolonged degradation by gingipains, the modified IL-8_{77aa} may reduce chemotaxis and neutrophil priming, thus prolonging the inflammatory lesion. Such a model (Fig. 5) is worthy of further investigation.

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