Proteolytic Inactivation of the Leukocyte C5a Receptor by Proteinases Derived from *Porphyromonas gingivalis*

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The anaerobic bacterium *Porphyromonas gingivalis* has been implicated as a primary causative agent in adult periodontitis. Several proteinases are produced by this bacterium, and it is suggested that they contribute to virulence and to local tissue injury resulting from infection by *P. gingivalis*. Cysteine proteinases with specificities to cleave either Arg-X or Lys-X peptide bonds (i.e., gingipains) have been characterized as predominant enzymes associated with vesicles shed from the surface of this bacterium. It has recently been demonstrated that these proteinases are capable of degrading the blood complement component C5, resulting in the generation of biologically active C5a. By using an affinity-purified rabbit antibody raised against residues 9 to 29 of the C5a receptor (C5aR; CD88), we demonstrate that noncysteinyll proteinases associated with vesicles obtained from *P. gingivalis* cleave the C5aR on human neutrophils. Proteolytic attack of the C5aR by enzymes from the *P. gingivalis* vesicles was inhibited by TPCK (tolylsulfonyl phenylalanyl chloromethyl ketone), PMSF (phenylmethylsulfonyl fluoride), and dichloroisocoumarin, suggesting that serine proteinases are primarily responsible for this degradative activity. The purified vesicle proteinase Lys-gingipain but not Arg-gingipain also cleaved the N-terminal region of the C5aR on the human neutrophils. Lys-gingipain activity was essentially resistant to these inhibitors but was inhibited by TLCK (*N*- α -p-tosyl-L-lysine chloromethyl ketone) and iodoacetamide. A synthetic peptide that mimics the N-terminal region of C5aR (residues 9 to 29; PDYGHY DDKDTLDLNTVPDKT) was readily cleaved by chymotrypsin but not by trypsin, despite the presence of two potential trypsin (i.e., lysyl-X) cleavage sites. The specific sites of cleavage in the C5aR 9-29 peptide were determined by mass spectroscopy for both chymotrypsin and Lys-gingipain digests. This analysis demonstrated that the C5aR peptide is susceptible to cleavage at both potential Lys-gingipain sites (i.e., between residues 17 and 18 [K-D] and 28 and 29 [K-T]) and at two chymotrypsin sites (between residues 14 and 15 [Y-D] and 20 and 21 [L-D]), respectively. These studies suggest that *P. gingivalis* contains at least two enzymes capable of cleaving the C5aR, Lys-gingipain and a second nontryptic serine proteinase that is distinct from either Arg- or Lys-gingipain.

Severe adult periodontitis (AP) is characterized by an acute inflammatory response with excessive granulocyte infiltration into the gingival tissue which becomes a chronic disease state typified by extensive tissue degradation and eventual bone erosion and loss of teeth (34). AP affects 36% of Americans over the age of 19, and the severity of this disease increases significantly with age (19). About half of all Americans have lost their teeth to AP by the age of 65, and approximately 10 to 20% of AP patients are refractory to present-day treatment including antibiotics. It is agreed that gram-negative anaerobes are mainly responsible for AP and that the bacterium *Porphyromonas gingivalis* is a major pathogen in this form of oral disease (3, 10, 20, 36). Gingival crevicular fluid accumulates in periodontitis as gingival tissue degradation progresses at the foci of infection. Proteinases produced by this anaerobe are thought to play a critical role in the virulence of the organism (10, 18). Initially, the bacterial proteinases promote collagen and connective tissue degradation and induce granulocytic infiltration (29, 34). Granular enzymes from activated polymorphonuclear leukocytes (PMNs) also contribute to local gingival injury as they are recruited to the site of inflammation by bacterial chemotactic factors and by newly generated humoral chemotactic factors and host cell chemokines. As the tissue

injury advances, edema fluid increases and plasma proteins, including complement components, are delivered to the injury site for processing by the bacterial enzymes. This process further enhances the inflammatory state. Hence, it is bacterial proteinases that appear to play a central role in initiating and sustaining the progressively advancing tissue injury characteristic of AP.

For a long time, the number of proteinases elaborated by *P. gingivalis* was a subject of controversy (for a review, see reference 24), but it is now clear that multiple forms of two cysteine proteinases are solely responsible for all of the so-called trypsin-like activity of this bacterium (25). Two forms of cysteine proteinases specific for arginyl-X bonds (50 and 95 kDa) (4) and one lysyl-X bond-specific enzyme (105 kDa) (22) have been purified and characterized and are referred to as Arg-gingipain and Lys-gingipain, respectively, to underline their strict specificities. In most *P. gingivalis* strains, the majority of these proteolytic activities is associated with the cell surface and in vesicles, which are blebs of outer membranes released from the bacterium.

Arg- and Lys-gingipains have little similarity in sequence to members of other cysteine proteinase families, including the papain, calpain, or viral proteinases. Over a decade ago, it was demonstrated that proteinases from *Bacteroides gingivalis* were capable of degrading complement factors C3 and C5 as well as immunoglobulin G (IgG) and IgM. It was proposed that this

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property may contribute to the pathogenicity of this organism (30). More recent studies have shown that Arg-gingipain will cleave both C3 and C5 to generate C3a- and C5a-like fragments (35). In the case of C3, Arg-gingipain selectively cleaves the alpha chain at or near the C3a-C3b bond junction (i.e., residues 77 and 78 [Arg-Thr]), reducing the size of the digested alpha chain by approximately 10,000 Da. There was no evidence of C3a activity in the C3 digests, indicating that cleavage occurred N-terminal to the exact C3a-C3b scission site at Arg-77 and/or any C3a generated was rapidly degraded by the enzyme. In the case of C5 cleavage by Arg-gingipain, the alpha chain of C5 was not cleaved initially at the C5a-C5b bond junction but rather at an alternative internal site. The first alpha chain scission site was Arg-Gly at positions 715 and 716, a known plasmin cleavage site in C5, generating an 86- and a 30-kDa fragment of the alpha chain. As degradation continued, C5a activity was generated, which indicated that the C5a-C5b scission site in the C5 alpha chain, between residues 74 and 75 (Arg-Gly bond), was cleaved.

More recent studies using Lys-gingipain have shown degradation patterns with C3 and C5 similar to those observed with Arg-gingipain (6). As with Arg-gingipain, there was no C3a activity generated by the lysine-specific enzyme. However, C5 cleavage did result in low levels of C5a-like activity. This was explained by the fact that C5 alpha chain fragments that were cleaved carboxyl to the C-terminal Arg-74 in C5a express C5a-like activity (33). These results provided evidence that bacterial granular enzymes could generate chemotactic activity (i.e., C5a) from plasma proteins in the crevicular edema fluid. Generation of anaphylatoxins by gingival proteinases could be one mechanism for recruiting granulocytes into the inflamed tissue and for inducing neutrophil degranulation and generation of oxygen radicals at that site. A key question in understanding the progression of chronic AP, therefore, is how *P. gingivalis* protects itself from destruction by these activated neutrophils.

In the study presented here, we asked whether the *P. gingivalis* enzymes could also act on signal transduction molecules (i.e., receptors) expressed on the surface of infiltrating cells. If the bacterial proteinases can degrade these signal transducing molecules on the surface of the cell, then they have the capacity to alter or eliminate host cell responses to mediators or activating factors that would normally result in phagocytosis and bacterial killing.

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MATERIALS AND METHODS

Reagents. Aprotinin, E64, leupeptin, pepstatin, TLCK (*N* α -*p*-tosyl-L-lysine chloromethyl ketone), and TPCK (tolylsulfonyl phenylalanyl chloromethyl ketone) were obtained from Calbiochem, La Jolla, Calif. Dichloroisocoumarin, iodoacetamide, elastatinal, PMSF (phenylmethylsulfonyl fluoride), and chymotrypsin were purchased from Sigma Chemical Co., St. Louis, Mo. Trypsin was obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind. The peptide representing residues 9 to 29 of the C5a receptor (C5aR 9-29 peptide) was synthesized by standard solid-phase methodology with an Applied Biosystems (Foster City, Calif.) model 430A synthesizer as described previously (21). Fluorescein isothiocyanate-labeled goat anti-rabbit IgG was from Jackson Laboratories, West Grove, Pa. ZFKck (benzoyl phenylalanyl lysyl chloromethyl ketone) was a generous gift from Alan Krantz (Syntex, Toronto, Ontario, Canada). The calcium-sensitive fluorescent probe Indo-1 was obtained from Molecular Probes, Eugene, Ore.

Anti-C5aR antibody. An anti-C5aR antibody was raised in rabbits to a synthetic fragment of C5aR (i.e., residues 9 to 29) based on the N-terminal sequence of the neutrophil C5aR (21). The antibody that was raised to the C5aR 9-29 peptide not only binds to the receptor on the intact PMN but also blocks C5a binding and inhibits C5a-induced cellular responses (7, 21). These results indicated that the N-terminal region of the receptor was directly involved in the

ligand-receptor interaction. Anti-C5aR antibody was affinity purified by using immobilized C5aR 9-29 peptide.

Growth of bacteria and preparation of vesicle fraction. Strain ATCC 53978 (W50) was grown in a total volume of 1.0 liter as described previously (4) and then centrifuged (6,000 $\times$ g, 30 min, 4°C) to remove cells. The culture medium obtained was subjected to ultracentrifugation (100,000 $\times$ g, 2 h, 4°C) to pellet the vesicles. The pellet was resuspended and washed with 20 mM Bis-Tris–150 mM NaCl–5 mM CaCl₂–0.02% NaN₃ (pH 6.8) supplemented with 0.3 mM 2,2'-dithiopyridine. This suspension was reultracentrifuged, and the pellet was resuspended in 5 ml of Bis-Tris buffer and regarded as the vesicle fraction.

Proteinase purification. Lys-gingipain (105 kDa) and two molecular mass forms of Arg-gingipain (50 and 95 kDa) were purified from strain H66 culture fluid by a combination of acetone precipitation, gel filtration, arginine-Sepharose affinity chromatography, and anion-exchange chromatography (22). The final gingipain preparations were dialyzed against Bis-Tris buffer and stored on ice. The quantity of active enzymes in each batch of purified proteinase as well as gingipains associated with vesicles were determined by active-site titration with ZFKck and D-Phe-Pro-Arg-CH₂Cl, respectively, for Lys-gingipain and Arg-gingipain (23a). Briefly, activated gingipains were incubated with increasing quantities of inhibitors, and then measurements of residual enzyme activity were made with the appropriate substrates (i.e., BAPNA and Z-Lys-pNA for Arg-gingipain and Lys-gingipain, respectively). On the basis of these titrations, vesicles used in this study contained approximately 0.3 μ M Lys-gingipain activity per mg of protein and 0.6 μ M Arg-gingipain activity per mg of protein. Purified Arg-gingipain and Lys-gingipain were determined to be 30% active on a molar basis, on the basis of the active-site titration results.

Neutrophil expression of C5aR. Neutrophils were isolated from peripheral blood of normal human donors by density gradient centrifugation essentially as described by Ferrante and Thong (9). Cells were washed twice in Earle's balanced salt solution buffered with 10 mM MOPS [3-(*N*-morpholino)propanesulfonic acid] at pH 7.3. Incubations with the W50 vesicles or proteolytic enzymes were carried out at 37°C in Earle's balanced salt solution. Unless otherwise indicated, Lys-gingipain and Arg-gingipain were activated with 10 mM cysteine for 10 min at room temperature before addition to the neutrophils. Where indicated, enzymes were preincubated with proteinase inhibitors for 10 min at room temperature before initiation of the incubation. At the specified times after proteinase treatment, cells were placed on ice and allowed to cool to 4°C before centrifugation and removal of supernatants. Cells were then incubated with anti-C5aR antibody, washed, and treated with fluorescein isothiocyanate-labeled goat anti-rabbit IgG antibody. Expression of C5aR was quantitated by flow cytometry by use of either FACScan or FACSort flow cytometers. Cellular viability was assessed by propidium iodide staining, and live gating was carried out to ensure analysis of only live cells. Data are expressed as mean channel fluorescence or the percent change in mean channel fluorescence. Nonimmune rabbit IgG was used as a negative control.

Neutrophil myeloperoxidase release. Neutrophils were incubated with *P. gingivalis* enzymes as described above. Cells were then centrifuged, washed, and resuspended in Earle's balanced salt solution containing 5 μ g of cytochalasin B per ml. The neutrophils were incubated with cytochalasin B for 15 min at 37°C before the addition of either C5a or phorbol myristate acetate. After 30 min, supernatants were removed and assayed for myeloperoxidase content as described by Lundberg and Arfors (17). Total myeloperoxidase content was determined by sonicating cells for 10 s in 0.5% hexadecyltrimethylammonium bromide. Data are expressed as mean percent release of total myeloperoxidase stores $\pm$ standard error of the mean. Datum points were determined in triplicate, and the experiment was carried out three times.

Measurement of intracellular calcium. Measurement of neutrophil calcium flux was carried out with Indo-1 as originally described by Grynkiewicz et al. (13). Neutrophils were subjected to proteolysis by either Lys-gingipain or vesicles as described above. The cells were washed twice and loaded with the acetoxymethyl ester of Indo-1 at 5 μ g/ml for 30 min at room temperature. After washing, the cells were used to determine responsiveness to either intact C5a or a synthetic peptide (ISFKDMLGR) based on the active C-terminal region of C5a. The activity of this peptide has been characterized previously (8), and it has been found that this and similar peptides retain their biologic activity either in the presence of antibodies to the N-terminal region of the receptor or when the N terminus is proteolytically cleaved (7, 21, 28). Calcium influx in response to either C5a or the synthetic peptide was determined cytochemically with a FACStar Plus (Becton Dickinson) by monitoring the emission ratio of 520 nm/405 nm.

Mass spectrometry. The mass spectrometry experiments were performed with an API III Perkin-Elmer SCIEX triple-quadrupole mass spectrometer. Electrospray samples were introduced into the analyzer at a rate of 4.0 μ l/min. The positive ions generated by the ion evaporation process entered the analyzer through an interface plate and a 100- μ m orifice, while the declustering potential was maintained at 100 V to control the collisional energy of the entering ions. The sample was scanned from *m/z* 500 to 1500.

RESULTS

Sensitivity of the C5aR to trypsin and chymotrypsin. The anti-C5aR 9-29 antibody binds to an extracellular region of the

FIG. 3. Mass spectral analysis of the chymotrypsin digest of C5aR 9-29 peptide. The C5aR 9-29 peptide was digested with chymotrypsin (1:50, wt/wt) for 30 min at 37°C. The digest was then analyzed by ion spray mass spectrometry. The fragments KAPDYGHY, DDKDTL, and DLNTVPDKT were identified, indicating that the enzyme had cleaved primarily the Y-D (positions 14 and 15) and L-D (positions 20 and 21) bonds in the receptor peptide.

indicating a serine proteinase (Fig. 6B). Activity in the vesicles was also inhibited by high concentrations of iodoacetamide and TLCK but was less sensitive to these inhibitors than was purified Lys-gingipain. These results strongly suggest that, although Lys-gingipain can cleave the C5aR on neutrophils, the primary C5aR-cleaving activity associated with crude vesicles is distinct from this enzyme.

Inhibition of C5a-induced neutrophil enzyme release by *P. gingivalis* proteinases. The N-terminal region (amino acids 9 to 29) of the C5aR has been shown previously to be important for ligand interactions and/or activation of the receptor (7, 21). Cleavage of this region of the receptor by proteinase treatment was therefore presumed to inactivate the receptor. To confirm this hypothesis, we examined the effects of proteolysis on C5a-induced PMN activation. C5a-induced enzyme release (i.e., of myeloperoxidase) was found to be reduced or eliminated by Lys-gingipain and by proteinases from the isolated vesicles (Fig. 7). No spontaneous release was observed from proteinase treatment alone. Treatment with the *P. gingivalis* proteinases had no effect on phorbol myristate acetate-induced enzyme release, demonstrating that inhibition was specific to receptor-mediated activation.

Inhibition of C5a-mediated calcium flux by *P. gingivalis* proteinases. Previous studies have demonstrated that, although the N-terminal region of the receptor is required for cellular activation by intact C5a, activation by synthetic analogs of C5a occurs independently of this N-terminal region (7, 28).

To confirm functional inactivation of the receptor by *P. gingivalis* proteinases and to determine whether responsiveness to C5a peptide analogs is retained, changes in intracellular calcium were measured. As shown in Fig. 8, neutrophils responded to both C5a and the analog (ISFKDMLGR) with a rapid increase in intracellular calcium. Treatment of neutrophils with Lys-gingipain abolished the cellular responsiveness to intact C5a, while the response to the analog C5a peptide was essentially unchanged. Digestion by the proteinases in *P. gingivalis* vesicles resulted in cellular unresponsiveness to either C5a or the synthetic peptide. These results suggest that degradation by Lys-gingipain may be limited to the N-terminal region of the receptor or to other regions not essential for ligand interaction. Vesicle proteinase cleavage apparently is not limited to the N-terminal region of the C5aR but must also include other ligand-binding sites and/or signal transduction elements of the receptor.

DISCUSSION

Recent studies have shown that Lys-gingipain and Arg-gingipain, proteinases derived from *P. gingivalis*, can cleave the complement proteins C3 and C5, generating fragments with anaphylatoxin-like activities (6, 35). These studies provided evidence that bacterial proteinases could generate chemotactic activity (i.e., C5a) from plasma proteins in the crevicular edema fluid. The implication of these results is that infection

FIG. 4. Flow cytometry analysis of human PMNs after exposure to *P. gingivalis* vesicle enzymes (B), Arg-gingipain (C), or Lys-gingipain (D). Cells were incubated in the presence of *P. gingivalis* vesicles (strain W50; 100 μ g of protein per ml) or either cysteine-activated Arg-gingipain (100 μ g/ml) or Lys-gingipain (50 μ g/ml) for 60 min at 37°C. Expression of the C5aR was monitored by use of the anti-C5aR 9-29 peptide antibody as described in the legend to Fig. 2. Shaded histograms depict receptor expression on enzyme-treated cells; open histograms represent receptor expression on untreated cells. The shaded histogram in panel A represents binding of control rabbit IgG. Only the Arg-gingipain failed to degrade the C5aR on PMNs.

by *P. gingivalis* can lead to proteolytic events that generate fragments from plasma components capable of recruiting granulocytes and monocytes to the site of tissue injury and thus promote or amplify the inflammatory response.

In the study presented here, we asked whether the *P. gingivalis* enzymes could also act upon receptors (i.e., signal transduction molecules) expressed on the surface of infiltrating PMNs. Initial experiments were designed to establish that this region of the C5aR was susceptible to proteolysis. Since the C5aR region of residues 9 to 29, to which the antibody binds, contained both potential trypsin and chymotrypsin cleavage sites, it was expected that either trypsin or chymotrypsin could degrade the receptor and destroy the antibody binding site. In fact, we found that the C5aR on PMNs was relatively resistant to trypsin. This result might be explained because one potential trypsin cleavage site in the N-terminal C5aR region (TDDK-DTLD) is surrounded by anionic aspartyl side chains and the other site at positions 28 and 29 (K-T) may be protected by proximity to, or insertion in, the membrane. The finding that Lys-gingipain was active at these sites suggests that the selectivity of Lys-gingipain for the Lys-X bonds in the C5aR 9-29 region must be distinctly different from that of trypsin. The inability of *P. gingivalis* vesicles to cleave the C5aR 9-29 peptide and yet still eliminate antibody binding to the receptor may be explained by one of at least three possibilities. First, the active proteinase may recognize and cleave the intact receptor on the cell at or near the N-terminal binding site but not cleave the peptide in solution. Second, the proteinase may cleave the C5aR at a region other than the N terminus, which may lead to a conformational change in the receptor which prevents antibody recognition of the N-terminal region. Finally, the proteinase may cleave the N-terminal region of the receptor C-terminal to the region of residues 9 to 29. This possibility seems least likely, since it is thought that the first transmem-

brane segment of the receptor enters the cell membrane shortly beyond residue 29.

A number of proteolytic enzymes from *P. gingivalis*, including Arg- and Lys-gingipain, have been identified and characterized. We have found that both *P. gingivalis* vesicles and Lys-gingipain, but not Arg-gingipain, cleave the N-terminal region of the C5aR. The lack of activity by Arg-gingipain is explained by the lack of arginyl residues in this region. Despite the activity of Lys-gingipain on the C5aR, our results suggest that the primary C5aR-cleaving activity associated with the vesicles is distinct from this enzyme. This is suggested by the requirement of cysteinyl preactivation for Lys-gingipain, but not for the vesicles as well as differing profiles of sensitivity to various established proteinase inhibitors. These studies suggest that a serine proteinase in intact vesicles with sensitivity to dichloroisocoumarin, PMSF, and TPCK may play the most important role in degrading the C5aR. Besides gingipains (Lys-gingipain and Arg-gingipain), at least two more proteolytic activities were found associated with *P. gingivalis* vesicles. One enzyme is fairly well characterized (1, 12) and is localized on the surface of the bacterial cell outer membrane (11). It is a serine proteinase with glycyloprolyl peptidase activity. The second enzyme was characterized as a cysteine proteinase cleaving peptide bonds within active site loops of α_1 -antichymotrypsin and α_1 -proteinase inhibitor at sites carboxyl to aliphatic residues (unpublished data). In addition, a serine proteinase of broad specificity has been purified from culture supernatants of *P. gingivalis*. In comparison with gingipains, this enzyme cleaves insulin beta chain rather inefficiently at Gln, Cys, Glu, and Ala residues but not after Arg or Lys (14). Among these activities, the more likely candidate for being responsible for cleaving the C5aR is the glycyloprolyl peptidase because the inhibitor susceptibility of this enzyme matches closely the effects of the different inhibitors reported here (12). Attempts

FIG. 5. Mass spectral analysis of the Lys-gingipain digest of C5aR 9-29 peptide. The C5aR 9-29 peptide was treated with Lys-gingipain (1:50, wt/wt) for 60 min at 37°C. The digest mixture was then separated by reverse-phase HPLC on a C-18 column, and fractions representing the two most prominent peaks detected by A_{220} were collected and analyzed separately by ion spray mass spectrometry. Fragments KAPDYGHYDDK (B) and DTLDLNTPVDK (A) were identified in the HPLC-eluted fractions, confirming that both the Lys-Asp (positions 17 and 18) and Lys-Thr (positions 28 and 29) bonds were cleaved by the *P. gingivalis* proteinase.

to purify and fully characterize the enzyme identified in this study are currently under way.

We have also demonstrated that treatment of PMNs with *P. gingivalis* proteases resulted in functional inactivation of the receptor both in terms of calcium mobilization and myeloperoxidase release. Previous studies have suggested that the N-terminal region of the C5aR is directly involved in the ligand-receptor interaction (7, 21). Other groups have used limited proteolysis to characterize potential binding sites between C5a and the C5aR. By using proteinases derived from spider venom, Siciliano et al. (28) also concluded that the N-terminal region of the receptor is required for C5a binding and activation but not for activation by peptide analogs of C5a. Our

FIG. 6. Sensitivity of gingival enzyme cleavage of C5aR to selected proteinase inhibitors. Lys-gingipain (A) or *P. gingivalis* vesicles (B) were treated with the indicated inhibitors for 10 min at room temperature before incubation with PMNs. C5aR expression following Lys-gingipain digestion was then determined by flow cytometry as described in Materials and Methods. Inhibitor concentrations are as follows: EDTA, 5 mM; leupeptin, 50 μ M; 3,4-dichloroisocoumarin, 100 μ M; elastatinal, 100 μ M; TPCK, 50 μ M; E-64, 50 μ M; aprotinin, 1.5 μ M; pepstatin, 10 μ M; PMSF, 5 mM; ZFKck, 50 μ M. The patterns of inhibitor efficacy suggest that C5aR cleavage by vesicles results from serine proteinase activity distinct from Lys-gingipain. Data are expressed as the mean percent suppression of Lys-gingipain activity $\pm$ standard error of the mean. *N* values ranged from 4 to 10.

demonstration that cleavage of this region by bacterial proteinases results in a loss of functional responses to the natural ligand further supports this previous conclusion. Inactivation of calcium responses by Lys-gingipain to C5a but not synthetic analogs suggests that cleavage occurs at the N terminus of the receptor but not at essential internal binding domains. Inactivation of calcium responses to peptide analogs by exposure to *P. gingivalis* vesicles, on the other hand, suggests that either internal binding domains or signal transduction elements are cleaved or degraded by this proteolytic activity. Since proteolysis of cell surface receptors can affect other receptors or pathways of activation, such as PMN stimulation via cleavage of the thrombin receptor (31, 32), it was important to show that proteolysis alone does not cause the cells to be activated. Treatment with either the isolated vesicles or purified Lys-gingipain did not lead to activation of the PMNs as measured by myeloperoxidase release.

The principles and methods utilized in this study have been

FIG. 7. Effects of *P. gingivalis* proteinase treatment on C5a-induced PMN myeloperoxidase release. Isolated PMNs were incubated with either *P. gingivalis* vesicles or purified Lys-gingipain (50 μ g/ml) for 60 min at 37°C. The cells were then washed and treated for 15 min at 37°C with cytochalasin B (5 μ g/ml) and stimulated for 30 min with C5a. Myeloperoxidase was then measured in supernatants by reduction of H_2O_2 in the presence of *o*-dianisidine, and the A_{460} was recorded. Exposure to either Lys-gingipain or the mixed proteinases of the vesicles significantly reduced the cellular response to C5a stimulation. Each determination was carried out in triplicate, and the experiment was carried out three times.

successfully employed in previous studies characterizing proteolytic regulation of neutrophil adhesion molecules and their receptors. It is now well established that a number of neutrophil adhesion molecules and receptors, including CD16, CD43, CD44, L-selectin, and tumor necrosis factor receptor, are cleaved by autologous enzymes upon neutrophil activation (2, 15, 16, 23, 26). Relatively few studies have defined the effects of bacterial proteinases on cellular receptors, particularly with respect to the development of an inflammatory response. Curtis et al. have recently demonstrated that proteases from *P. gingivalis* lead to platelet activation but concluded that this did not proceed through activity of the thrombin receptor (5). The findings that bacterial proteinases can modify host responses either through generation of inflammatory mediators (i.e., C5a) or through actions on cellular receptors introduce a relatively new concept. The net effects of proteolytic generation of C5a activity at a local tissue site with corresponding inactivation of the C5aR would lead to enhanced cellular recruitment but an attenuated antibacterial host response. It is currently unclear which of these two activities may dominate in an in vivo setting and whether these activities contribute to the virulence of the organism. In this regard, we have found the same proteolytic activity on the C5aR in vesicles obtained from the nonvirulent strain ATCC 33277, although apparently at a slightly lower level (data not shown).

FIG. 8. Effects of *P. gingivalis* proteinase treatment on neutrophil responsiveness to C5a and a C5a analog decapeptide. Isolated PMNs were incubated at 37°C for 60 min in the absence of enzyme (A and B), with Lys-gingipain (C and D), or with *P. gingivalis* vesicles (E and F) as described in Materials and Methods. Cells were then loaded with Indo-1, washed, and stimulated with either C5a (A, C, and E) or a synthetic analog of C5a (ISFKDMLGR) (B, D, and F). Intracellular calcium levels were monitored by flow cytometry as the change in the emission ratio of 405 nm/519 nm. Treatment with either Lys-gingipain (C) or *P. gingivalis* vesicles (E) ablated the calcium response to C5a. Treatment with vesicles (F) but not Lys-gingipain (D) blocked the increase in intracellular calcium in response to the decapeptide.

Proteolysis by bacterial enzymes is presumably not limited to C5aR on PMNs and may affect other receptors and cell types in the tissue microenvironment. The results reported here indicate that an entire class of receptors, known as the rhodopsin family of G protein-associated receptors (27), may be particularly susceptible to limited digestion by bacterial proteinases. Proteolysis of exposed cellular receptor molecules can lead to an inability of blood and/or tissue cells to respond to bacteria and prevent or limit injury, and this destruction of effector signalling could subsequently limit normal tissue repair processes. Bacterial proteinases serve a number of functions, many of which may contribute to the virulence of the organism. Cleavage of cellular receptors clearly would represent only one of a number of activities of these proteinases. It is clear that the role of bacterial proteinases in altering tissue inflammatory responses, by perhaps eliminating normal defense mechanisms in the host, must be examined more carefully at the molecular (i.e., effector-receptor) level.

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