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Cleavage of human complement component C5 by cysteine proteinases from *Porphyromonas (Bacteroides) gingivalis*. Prior oxidation of C5 augments proteinase digestion of C5

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SUMMARY

Since severe periodontitis is characterized by an acute inflammatory response with cellular infiltration and microbial overgrowth, plasma proteins could be exposed to both proteinases and oxidants released from the granulocytes, as well as to proteinases from the microorganisms. When human complement component C5 was digested by cysteine proteinases (i.e. gingipain-R and gingipain-K) from *Porphyromonas gingivalis*, limited cleavage of the C5 molecule was observed. If C5 was first oxidized by hydroxyl radicals, these gingipains converted modified C5 to fragments that exhibited significantly greater pro-inflammatory activity than did digests of unmodified C5. After cleavage of oxidized C5 by gingipain-R, the digest exhibited measurably greater neutrophil enzyme release and chemotaxis of human polymorphonuclear leukocytes (PMNs) compared with the activities of unoxidized C5 digests. Gingipain-K generates virtually no polarization or chemotactic activity of human PMNs from C5, nor is enzyme release stimulated by these C5 digests. However, when oxidized C5 was digested by gingipain-K, human PMNs were stimulated for polarization, chemotaxis and enzyme release indicating that an active fragment had been generated. Proteolysis of oxidized C5 evokes greater neutrophil activation than does proteolysis of unoxidized protein, a fact which supports the hypothesis that oxidation and proteolysis may be coupled to enhance the destructive effects of the inflammatory process. These results, in which digests of both oxidized and unmodified complement component C5 were evaluated, support the general concept that oxidation and proteolysis may participate cooperatively in amplifying both the severity and duration of the inflammatory reaction.

INTRODUCTION

The gram-negative microorganism *Porphyromonas gingivalis* has been implicated as a pathogen associated with periodontitis, a severe inflammation of gingiva and periodontal tissues.[1] *P. gingivalis* is known to secrete potent proteinases that have been implicated as major contributors to the virulence of these organisms.[2–4] The two cysteine proteinases secreted by this microbe have specificities for arginyl-X and lysyl-X peptide bonds (i.e. gingipain-R and gingipain-K), respectively.[5,6] During periodontitis both acute and chronic stages of inflammation develop which results in severe periodontal tissue damage.[7] In addition to massive neutrophil influx, invasion by both activated eosinophils and basophils was observed in gingival crevicular fluid from patients with periodontitis.[8] Destruction of host tissue appears to be both a consequence of direct action of microbial products and agents, or of factors secreted by the massive sequestration of granulocytes.[7]

One mechanism by which proteinases such as gingipain-R or gingipain-K may evoke granulocyte influx is by cleaving human complement components C3 or C5.[9] This may release biologically active C3a or C3a-like fragments from C3, or C5a or C5a-like fragments from C5. Both C3 and C5 are two chain plasma proteins (MW $\approx$ 192 000) that fulfil a number of important immune functions. C3 products participate in phagocytic functions and C5 in assembly of the membrane attack complex of complement. The reactive activation peptides, C3a and C5a, are potent spasmogens in addition to their role in the recruitment and activation of granulocytes.[10–13]

It has been recognized that during inflammation, oxidants

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Abbreviations: BSA, bovine serum albumin; EBSS, Earle's balanced salt solution; FCS, fetal calf serum; gingipain-R and gingipain-K, cysteinyl proteases from *Porphyromonas gingivalis*; MOPS, morpholinopropane sulfonic acid; PAGE, polyacrylamide gel electrophoresis; PMN, polymorphonuclear leukocyte; SDS, sodium dodecyl sulfate.

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and proteinases do not operate as separate entities. Coupling the effects of both agents maximizes the observed destructive effects.[14] Recognizing that oxidation and proteolysis may influence the nature of the digestion products, we compared gingipain-R and gingipain-K digests of oxidized and native C5 for their ability to activate neutrophils.

It was recently demonstrated that C5 acquires C5b-like properties following oxidation by hydroxyl radicals, which permits assembly of the C5b-9 complex; however, chemotactic capacity for neutrophils (i.e. C5a-like activity) was not observed as a result of this treatment.[15–18] We have extended these findings involving the effects of C5 oxidation by showing that oxidized C5 expresses greater pro-inflammatory activity than does unmodified C5 when these molecules are cleaved by the proteinases gingipain-R or gingipain-K. Examining the combined effects of oxidation and proteolysis on C5 is warranted because oxygen radical-producing granulocyte infiltration and microbial overgrowth characterizes severe inflammatory reactions such as periodontitis. Therefore, it is likely that plasma proteins such as C5, which occurs in the exudate at sites of inflammation, will be exposed to both high concentrations of oxidants as well as proteinases secreted by both microorganisms and the granulocytes.

MATERIALS AND METHODS

Materials

Frozen human plasma was obtained from the San Diego Blood Bank (San Diego, CA). Ascorbic acid, bovine serum albumin (BSA), morpholinopropane sulfonic acid (MOPS), cytochalasin B, p-nitrophenyl-β-D-glucuronide, and hydrogen peroxide were purchased from Sigma Chem. Co. (St Louis, MO). Ferrous sulfate was obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ). RPMI-1640 and fetal calf serum (FCS) were obtained from Bio-Whittaker (Walkerville, MD). Ficoll, Hypaque, and Percoll were purchased from Pharmacia Biotech (Piscataway, NJ). Earle's balanced salt solution (EBSS) was supplied by Gibco BRL (Grand Island, NY). Goat anti-mouse IgG-coated magnetic beads and magnetic separation unit were purchased from Advanced Magnetics Inc. (Cambridge, MA). Unconjugated mouse anti-human CD16 monoclonal antibodies and goat anti-(rabbit IgG) antibodies were purchased from Biosource International (Camarillo, CA). Microconcentrators were supplied by Amicon Inc. (Beverly, MA), and nitrocellulose membranes were obtained from Bio-Rad Laboratories (Hercules, CA).

Protein preparations

Gingipain-R and gingipain-K were purified from *P. gingivalis* culture supernatants following published procedures using ammonium sulfate precipitations, gel filtration, ion exchange chromatography, and arginine-Sepharose affinity chromatography.[5,6] Human complement components C3 and C5 were purified using affinity chromatography from frozen plates by following a published method.[19] The functional activities of C3 and C5, nominally 10000 CH_{50} U/mg of C3 and 130000 CH_{50} U/mg of C5, were determined using haemolytic assays.[19] C3 and C5 used in these studies were stored at 4° and used within 2 weeks of purification. C5a was purified from C5a,b, and the extinction coefficient used to quantify C5a was $E_{1\%,280\,nm} = 3{\cdot}5$ OD ml/dg cm.[20]

Oxidation of C5

C5 (0·7 mg/ml) was dissolved in 30 mM sodium phosphate buffer at pH 7·0 and oxidized by incubation with 10 mM H_2O_2/30 μM ferrous EDTA/3 mM ascorbic acid for 1 hr at 37°. The oxidized protein was dialysed against 30 mM sodium phosphate at pH 7·0 and then 20 mM Tris HCl at pH 8·0 containing 0·15 M NaCl.

Digestion of C3 and C5 by gingipain-R and gingipain-K

Gingipain-R and gingipain-K (0·25 mg/ml) were activated by incubating the enzymes with 10 mM cysteine for 10 min at 23°. Subsequently, the enzyme was added to C3 (0·9 mg/ml) or C5 (0·6 mg/ml) at molar ratios of enzyme to substrate of 1-to-25 and 1-to-100 for C3 and C5, respectively. The buffer was 20 mM Tris HCl and 0·15 M NaCl at pH 8·0. The digests were incubated at 37° for various times, and aliquots were removed for analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and for biological assays.

Neutrophil and eosinophil isolation

Neutrophils and eosinophils were isolated from peripheral blood of healthy human donors following procedures as previously described.[13] Both neutrophil and eosinophil preparations were greater than 95% pure.

Polarization assays

Aliquots (125 μl) of PMNs suspended at 2×10^6 cells/ml in EBSS with 10 mM MOPS-HCl at pH 7·35 were incubated at 37° for 30 min with varying concentrations of C3, C5 or oxidized C5 which had been digested by gingipain-R or gingipain-K. The polarization reactions were terminated by addition of an ice-cold solution of 2·5% glutaraldehyde and 0·04% eosin. After 1 hr, 200 cells from each sample were examined microscopically using 400× magnification. Cells with shapes other than the typical spherical forms were scored as polarized, and the results were expressed as the percentage of cells polarized over the total cells counted.

Polyclonal neutralizing antibody to the C5a receptor[21] was affinity purified and used for C5a-receptor inhibition experiments. Granulocytes (4×10^6 cells/ml) and an equal volume of anti-C5a receptor antibody (20 μg/ml) were incubated for 15 min at room temperature. Then an equal volume of test solution was added to the granulocyte antibody mixture and then incubated at 37° for 30 min. Polarization was assessed as described above.

Chemotaxis assays

Chemotaxis assays were performed by quantifying the migration of cells through a Micropore filter (Sartorius, Gottingen, Germany) using a method similar to that of Dahinden *et al.*[22] as modified by Ember *et al.*[23] The wells of modified Boyden chambers (Models P1 and 1/2 SC, Adaps Inc., Dedham, MA) were filled with solutions (700 μl) of samples containing possible chemoattractants in EBSS-MOPS/1% BSA. Micropore inserts (with pores of nominally 8 μm in diameter) were placed into the assembly, and for each well a suspension of PMNs containing $3–5 \times 10^6$ cells in 500 μl was pipetted onto the filter. Following an incubation period of 90 min at 37°, the cells that migrated through the micropore filter were counted using a Sysmex Microcellcounter Model F300 (TOA Medical

Electronics Co., Kobe, Japan). Results were expressed as a percentage of the total cells migrating. Purified human C5a was used as a positive control.

β-glucuronidase release assay

The release of β-glucuronidase from neutrophils was performed according to a procedure described by Schröder *et al.*[24] PMNs were suspended in EBSS containing 1% BSA to give a concentration of 10^7 cells/ml at 37°. Following 10 min of incubation with cytochalasin-B (5 μg/ml) at 37°, aliquots of the neutrophils (100 μl) were exposed to solutions containing proteolysed forms of C5 or oxidized C5 for 1 hr at 37°. Solutions of C5 and oxidized C5 were employed as negative controls, and human C5a was the positive control. The supernatants were incubated with an equal volume of 0·01 M p-nitrophenyl-β-D-glucuronide for 18 hr. The reactions were quenched by addition of an equal volume of 0·4 M glycine at pH 10. Colour intensity was measured at 405 nm with a Titertec Multiscan/340 enzyme-linked immunosorbent assay (ELISA) plate reader (Labsystems, Needham Heights, MA). β-glucuronidase release was expressed as the percentage of the total cellular content of enzyme following addition of 0·2% Triton X100.

Filtration of gingipain-K-digested oxidized C5

C5 was oxidized and digested using gingipain-K as described above. Then a portion (500 μl) was centrifuged at 10 000 *g* for 5 min over either a 30 000 or a 100 000 MW cut-off microconcentrator. Approximately half of the volume eluated through the filters using these conditions; this fraction was designated the filtrate. The retained material was diluted with equal volume of buffer and again centrifuged in order to deplete this fraction of smaller filterable polypeptides. The final solution, retained after repeating 15 centrifugations, was designated the retentate. As a positive control, a solution of C5a,b was treated similarly using a 30 000 MW cut-off microconcentrator.

RESULTS

Digestion of C5 by gingipain-K

The cleavage patterns of human complement components C3 and C5 by gingipain-R were reported previously;[9] however, the effects of gingipain-K on C3 and C5 have not been studied. Therefore, we initiated experiments to examine proteolysis of C3 and C5 by gingipain-K. Figure 1 shows the SDS–PAGE patterns of C3 and C5 cleaved by gingipain-K as a function of time. C3 is more refractory to digestion by this proteinase than is C5, and both the α- and β-chains of C3 were cleaved by gingipain-K. In contrast, the α-chain of C5 is the main target of the proteinase digestion. Although the proteolysis of C5 is complicated by apparent scission of several peptide bonds, a major cleavage involved splitting the α-chain into 85 000 and 30 000 MW fragments. The haemolytic activities of both C3 and C5 were lost in conjunction with proteolysis of these molecules (data not shown).

Neutrophil activation by gingipain-R and gingipain-K digests of C5

Several neutrophil-activation responses, including polarization, chemotaxis, and enzyme release, were evaluated. The human PMNs were incubated with samples of either C3, C5 or oxidized C5 digested by either gingipain-R or gingipain-K. The method employed to oxidize C5 was based on an earlier published method.[17] The major modification was to use 30 μM ferrous EDTA because this level is within the range of plasma ferrous concentration.[25] Although most of the ferrous ion in plasma is bound by transferrin, filtrates of plasma entering zones of inflammation would be exposed to a lower pH condition which may cause ferrous ion to be released from transferrin.[26] Moreover, ferrous ion may also be released from transferrin as a result of its degradation by proteinases secreted from *Bacteriodes*.[27]

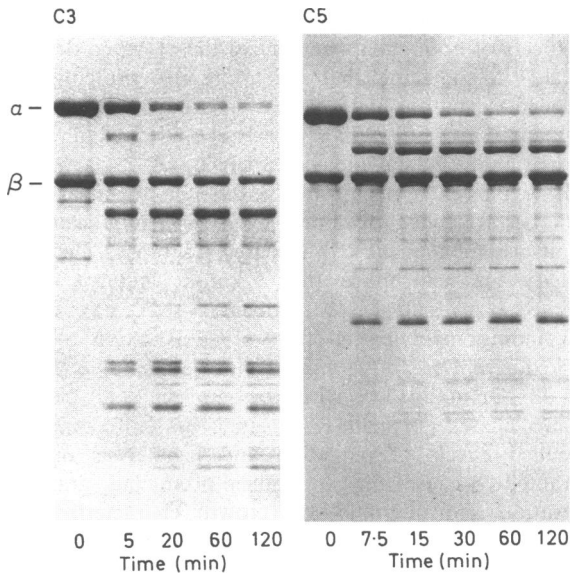

Figure 1. SDS–PAGE gel illustrating the time courses for cleavage of human complement components C3 and C5 by gingipain-K. The left panel reveals the pattern for C3 degradation at 37° using a 1-to-25 molar ratio of gingipain-K (Lys-G) to C3. The pattern for degradation of C5 at 37° using a 1-to-100 molar ratio of gingipain-K to C5 is shown in the right panel. All samples were reduced with 2% β-mercaptoethanol and boiled before electrophoresis.

Polarization of neutrophils was the first biological effect that we investigated. Gingipain-R and gingipain-K generated polarization activity from both C5 and oxidized C5 when examined as a function of time (see Fig. 2). However, only the oxidized form of C5 (at 780 nm) digested by gingipain-K contained neutrophil-polarization activity. There was no polarization activity measured above background levels in samples of cleaved or uncleaved C3, undigested C5, oxidized C5, C5 cleaved by gingipain-K, or from either of the two enzymes in the absence of C3 or C5. We found it necessary to use freshly prepared C5, because samples of C5 stored for more than a month at 4° could elicit spontaneous polarization activity. Although there was no apparent degradation of the stored protein, there might have been alterations in the folded structure of C5 that exposed the C5a region of the molecule. Oxidized C5 cleaved by gingipain-K generated neutrophil polarization activity that peaked after about 15–30 min of proteolysis, but was still detectable after 120 min of digestion. Interestingly, the neutrophil polarization activity generated by cleaving oxidized C5 with gingipain-R was greater than that

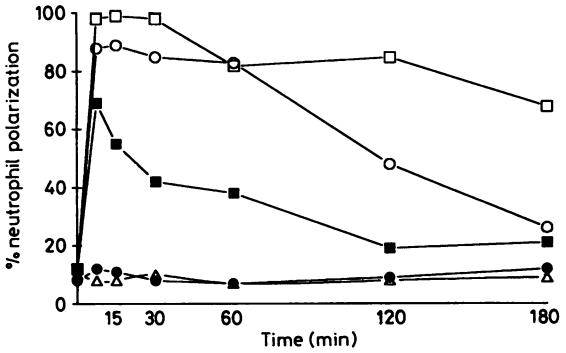

Figure 2. A time course for the generation of neutrophil polarization activity from C5 or oxidized C5 by digestion with either gingipain-R or gingipain-K. Aliquots were removed at various times during proteolysis of the C5 samples and assayed for neutrophil polarization. Polarization was measured after incubating the neutrophils with 780 nM of each sample for 30 min. Controls using gingipain-R or gingipain-K in the absence of C5 samples did not evoke polarization responses above background levels (i.e. 6%). (○) C5 + gingipain-R; (□) oxidized-C5 + gingipain-R; (●) C5 + gingipain-K; (■) oxidized C5 + gingipain-K; and (△) C3 + gingipain-K.

from unoxidized C5. While both activities were maximal between 15–30 min of incubation, the oxidized sample retained much of its activity after 2 hr, whereas the unoxidized protein had been essentially inactivated.

Since it is now known that eosinophils but not neutrophils are activated by C3a,[13] the effects of C3 digestion by gingipains-K and -R were studied using eosinophils. Whereas neither enzyme cleaved C3 into fragments that generated eosinophil polarization activity, both enzymes cleaved C5 to evoke this activity (Fig. 3). Time courses for generation of eosinophil polarization activity in gingipain-K and gingipain-R digests of either C5 or oxidized C5 is shown in Fig. 3. This time course for generation of eosinophil polarization activity is similar to that obtained for neutrophils with maximal activation

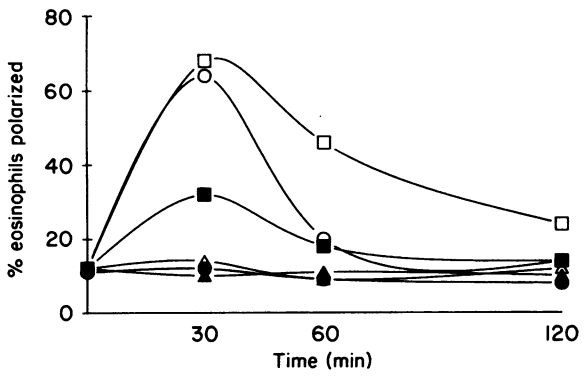

Figure 3. A time course for the generation of eosinophil polarization activity derived from C3, C5, or oxidized C5 after digestion by gingipain-R or gingipain-K. At various times in the proteolysis of C3 and C5, aliquots were removed and assayed for the ability to polarize eosinophils. Polarization was measured after 30 min of incubation of eosinophils with these samples. (○) C5 + gingipain-R; (□) oxidized-C5 + gingipain-R; (●) C5 + gingipain-K; (■) oxidized-C5 + gingipain-K; (△) C3 + gingipain-R; (△) C3 + gingipain-K.

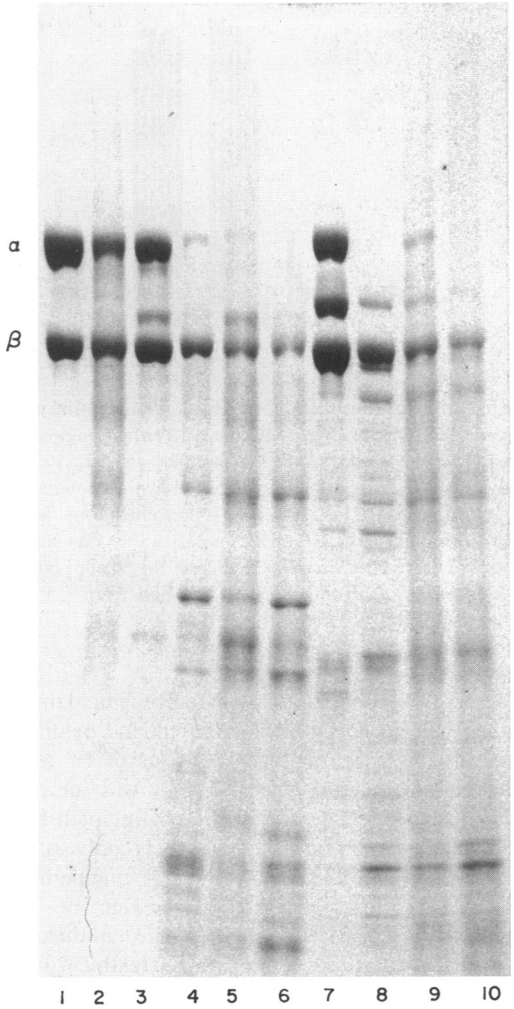

Figure 4. SDS–PAGE gel patterns of C5 and oxidized C5 cleaved by gingipain-R or gingipain-K. The samples were reduced with 0·5% 2-mercaptoethanol and boiled before electrophoresis. The order of the samples is: (lane 1) C5; (lane 2) oxidized C5; (lane 3) C5 + gingipain-R, 15 min digest; (lane 4) C5 + gingipain-R, 120 min digest; (lane 5) oxidized C5 + gingipain-R, 15 min digest; (lane 6) oxidized C5 + gingipain-R, 120 min digest; (lane 7) C5 + gingipain-K, 15 min digest; (lane 8) C5 + gingipain-K, 120 min digest; (lane 9) oxidized C5 + gingipain-K, 15 min digest; and (lane 10) oxidized C5 + Lys-gingipain, 120 min digest.

occurring after 30 min of proteolysis. Since granulocyte activity was mediated only by C5, but not C3, we continued our investigations using C5.

The SDS–PAGE gel patterns are shown for C5 and oxidized C5, along with the proteins cleaved by gingipain-R or gingipain-K for selected periods of time (Fig. 4). Reduced C5 exhibited two bands after SDS–PAGE reflecting the α- and β-chains; however, these bands were diminished in intensity for oxidized C5, while indications of fragmentation and possible cross-linking were seen, causing the sample of oxidized C5 to smear throughout the gel lane. It can be seen that both gingipain-R and gingipain-K degraded the C5 α-chain into a variety of fragments, but degradation of oxidized C5 was more substantial.

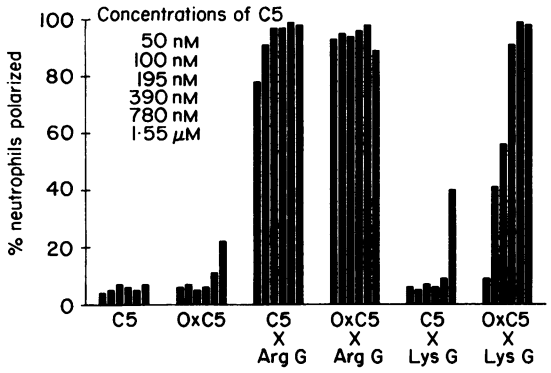

Figure 5. A comparative study of neutrophil polarization induced by C5 or oxidized C5 (OxC5) after treatment with either gingipain-R (Arg-G) or gingipain-K (Lys-G). The C5 samples (50 nM, 100 nM, 195 nM, 390 nM, 780 nM, and 1·55 μM) were digested by the proteinase for 15 min at 37°, and tested at these various concentrations for neutrophil polarization activity. In all cases, polarization was measured after exposing the neutrophils to the C5 digests for 30 min. Note that more activity is generated from oxidized C5 than from C5. C5a evoked maximal neutrophil polarization at 0·5 nM.

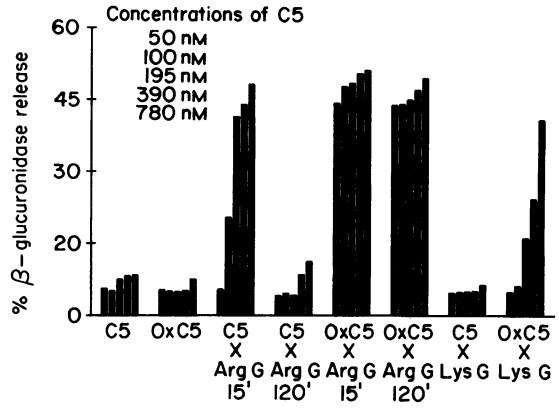

Figure 7. The release of β-glucuronidase from neutrophils by gingipain-R (Arg-G) or gingipain-K (Lys-G) digests of C5 or oxidized C5 (OxC5) was measured. Digests of C5 or oxidized C5 by gingipain-R at 15 min and 120 min were assayed. The one time period (15 min) of digestion by gingipain-K was used in evaluating activities derived from C5 or oxidized C5. For each group, the original concentrations of C5 or oxidized C5 were 50 nM, 100 nM, 195 nM, 390 nM, and 780 nM. Note that more activity is generated from oxidized C5 than from C5 after cleavage by gingipain K.

The polarization activities evoked by gingipains-R and -K digests of various concentrations of C5 and oxidized C5 were compared (Fig. 5). Gingipain-R digests of C5 and oxidized C5-elicited polarization of neutrophils with nearly maximal activity observed at 50 nM C5 levels. Gingipain-K digests of C5 generated weak polarization activity only at the highest concentration tested (1·55 μM); however, gingipain-K cleavage of oxidized C5 was considerably more extensive. The optimal concentration of oxidized C5 required for stimulation of neutrophil polarization after gingipain-K treatment was between 390–780 nM.

Results of neutrophil chemotaxis induced by C5 digests (Fig. 6) were consistent with the polarization assays. Gingipain-R

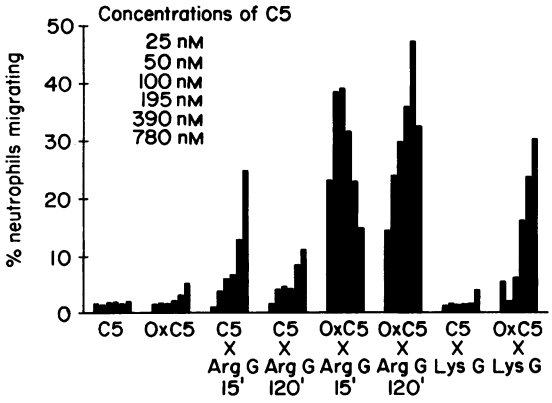

Figure 6. A comparison of neutrophil chemotactic activity generated from C5 or oxidized C5 (OxC5) by either gingipain-R (Arg-G) or gingipain-K (Lys-G). C5 or oxidized C5 was digested by gingipain-R for either 15 min or 120 min and then assayed. Samples of C5 and oxidized C5 digestion by gingipain-K were assayed for only the 15-min time period. Samples of C5 or oxidized C5 digests at concentrations of 25 nM, 50 nM, 100 nM, 195 nM, 390 nM and 780 nM were assayed. There was no measurable chemotactic activity generated by the proteinases alone.

cleavage generated higher levels of chemotactic activity from oxidized C5, and over a longer digestion period, than was generated from unoxidized C5. After 120 min of incubation of C5 with gingipain-R, using the stated conditions, virtually all of the chemotactic activity was lost. However, activity was still present after 2 hr of gingipain-R exposure to oxidized C5. Chemotactic activity was not observed for either native C5, oxidized C5, C5 cleaved by gingipain-K, or for the enzymes themselves, but it was present in both gingipains K and R digests of oxidized C5.

Experiments investigating release of the enzyme β-glucuronidase from neutrophils (Fig. 7) were concordant with those of chemotaxis and polarization. Neither C5 alone, gingipain-R nor gingipain-K exhibited enzyme-release activity from neutrophils, but activity was measured in gingipain-R digests of both C5, oxidized C5, as well as in digests of oxidized C5 cleaved by gingipain-K. Similar to the polarization and chemotaxis results, extending digestion of C5 by gingipain-R beyond 1 hr resulted in loss of activity for enzyme release, while this activity was sustained for oxidized C5 incubated with the enzyme for 2 hr.

Specific cleavage of C5 by C5 convertase yields the bioactive peptide C5a (MW = 11 000).[20] In order to determine whether or not a small polypeptide containing the C5a domain was similarly released by gingipain-K, size filtration experiments were performed. Samples of C5a,b and the gingipain-K digest of oxidized C5 were centrifuged through microconcentrators containing 30 000 MW and 100 000 MW cut-off filters. Both the eluated and retained fractions were assayed for polarization activity, and these results were compared with the activity in the original digests. As expected, based on the 11 000 MW of C5a, greater than 95% of the specific polarization activity was found in the 30 000 MW filtrate (Fig. 8a). In contrast, only a negligible portion of polarization activity was found in the 30 000 MW filtrate of the gingipain-K digest of oxidized C5. However, about 70% of the specific polarization activity from oxidized

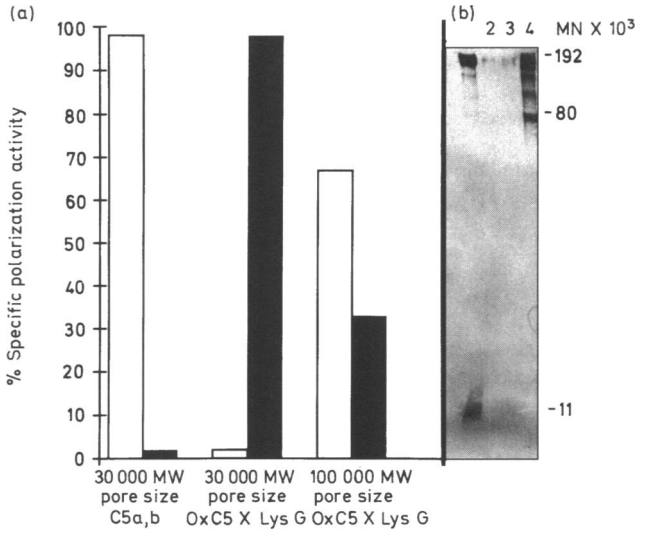

Figure 8. Demonstration that the C5a fragment is not released in gingipain-K digests of oxidized C5. (a) C5a,b or gingipain-K (Lys-G) digests of oxidized C5 (OxC5) were centrifuged through microconcentrators containing 30 000 MW or 100 000 MW cut-off filters. The neutrophil polarization activities of these filtrates (white bars) or retentates (black bars) were compared with the activities present in either C5a,b or gingipain-K digests of oxidized C5 prior to centrifugation. These comparisons were quantified as percentages of recovery and expressed as specific polarization activities. (b) A Western blot of (1) C5; (2) C5a; (3) C5b; (4) oxidized C5 × gingipain-K was developed using rabbit anti-C5a antibodies to illustrate that no low molecular weight fragments of C5 were generated by gingipain-K degradation.

C5 treated with gingipain-K was recovered in the 100 000 MW cut-off filtrate, indicating that the active fragment(s) from oxidized C5 were larger than 30 000 MW.

These studies indicate that, unlike cleavage of C5 by C5 convertase,[20] the C5a fragment is not released when oxidized C5 is cleaved by gingipain-K. This is confirmed by a Western blot which showed that the fragments of oxidized C5 in the gingipain-K digest containing the C5a domain were approximately 80 000 MW or larger (Fig. 8b).

Most published reports have attributed the pro-inflammatory activities originating from C5 to C5a. To confirm that the activity from oxidized C5 treated by gingipain-K involves the C5a domain, we undertook polarization studies in the presence or absense of a specific antibody to the C5a receptor. The outcome confirmed our expectations because anti-C5a receptor antibody blocked granulocyte polarization activity in the gingipain-K digest of oxidized C5 (data not shown). Therefore, the cellular activity does appear to be C5a mediated.

DISCUSSION

Oxidation and proteolysis are two characteristic events commonly associated with tissue damage and the inflammatory process. It is increasingly apparent than these two processes function concurrently and that they may inflict their damaging effects synergistically. There is now evidence that oxidation and proteolysis operate interdependently in contributing to the intensity of inflammatory reactions.[14] In order to gain further understanding of microbial-induced inflammation, we designed our experiments to test the hypothesis that oxidation may enhance the ability of bacterial proteinases to elaborate pro-inflammatory activity from plasma proteins such as complement component C5. Thus we studied effects of the cysteine proteinases gingipain-R and gingipain-K from *Porphyromonas gingivalis* on both oxidized and unoxidized C5.

Oxidation of proteins by hydroxyl radicals is known to cause peptide bond cleavage at prolyl residues, destruction of aromatic side chains, and introduction of covalent cross-links such as bityrosine formation.[28–32] Patterns of oxidized C5 observed by SDS–PAGE analysis suggest both partial fragmentation and cross-linking of the protein (Fig. 4). The smearing of the oxidized C5 sample on the gel is probably caused by a combination of oxidative peptide bond cleavage along with intermolecular and intramolecular cross-linking.

Our laboratory has previously published that gingipain-R can cleave both C3 and C5, and that this proteolysis results in the loss of haemolytic activity from both proteins. Although no neutrophil activation was detected in C3 digests, gingipain-R hydrolysed C5 to generate polarization and chemotactic activities in human PMNs.[9] We confirmed those results, and extended them by showing that prior oxidation of C5 followed by gingipain-R digestion enhances the generation of pro-inflammatory activities (Figs 2, 3, 5, 6 and 7). Enhancement of C5-derived pro-inflammatory activities by prior oxidation of the protein indicates that oxidative products from granulocytes may make plasma proteins more susceptible to degradation.

Although a role for eosinophils in pathogenesis of periodontitis has not been established, this cell type is detected in the gingival crevicular fluid during adult periodontitis.[8] Eosinophils, unlike neutrophils, are known to be activated by C3a.[13] We therefore investigated the effects of C3 digestion by gingipains-K and -R. Although neither gingipain enzyme was able to generate C3a-like eosinophil polarization activity from C3 digests, digestion of C5 and oxidized C5 by gingipain-R and oxidized C5 by gingipain-K evoked eosinophil polarization responses (Fig. 3).

In order to determine whether larger fragments containing C5a were released from oxidized C5 after gingipain-K digestion, size filtration experiments and Western blots were performed (Fig. 8). The active form(s) of oxidized C5 digested by gingipain-K, in contrast to C5a, did not pass through 30 000 MW cut-off membranes, but did penetrate the 100 000 MW cut-off membranes. Moreover, Western blots developed using rabbit anti-C5a IgG, indicated that the smallest fragment of oxidized C5 yielded by gingipain-K digestion is approximately 80 000 MW. These data suggest that the C5a domain remains associated with a large fragment of the C5 molecule after gingipain-K digestion. However, it was demonstrated that the polarization activity was caused by the C5a portion of the molecule because anti-C5a receptor antibodies blocked this polarization activity. These results extend earlier findings where it was shown that limited tryptic digestion of C5 results in exposure of the C5a domain giving rise to chemotactic activity without actually releasing the C5a fragment from the molecule.[33] Therefore, it is not necessary that the C5 α-chain be degraded so that intact C5a is liberated in order to obtain C5a-activity.

In summary, we investigated the effects of two well-characterized microbial proteinases believed to be prominent in promoting the acute inflammatory response in periodontal

disease. These studies provide a specific *in vitro* example showing how oxidation and proteolysis may participate synergistically in augmenting the intensity of an inflammatory process.

These findings along with those of others demonstrate that oxidation of C5 affects two important changes in the molecule. First, it converts C5 to a form that has C5b-like properties, this enables C5 to serve as an acceptor for C6 leading to assembly of the membrane attack complex;[15–18] secondly, it generates a molecule that exhibits greater pro-inflammatory potential after exposure to certain proteinases, e.g. gingipain-R and gingipain-K. Investigations dealing with *in vivo* synergism of oxidation and proteolysis in inflammation are merited because the potential enhancement of the inflammatory response by this mechanism has implications for a variety of disease processes. A comparison of the effects of other proteinases, especially granular proteinases from neutrophils and eosinophils, on C5 and oxidized C5, merits future study. It is probable that oxidized C5 exhibits an augmented potential to generate pro-inflammatory activity when degraded by a host of proteinases, such as those secreted by granulocytes and other inflammatory cells.

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