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# Biphasic Effect of Gingipains from *Porphyromonas gingivalis* on the Human Complement System<sup>1</sup>

Katarzyna Popadiak,<sup>\*†</sup> Jan Potempa,<sup>‡‡</sup> Kristian Riesbeck,<sup>§</sup> and Anna M. Blom<sup>2\*</sup>

Periodontitis is an inflammatory disease of the supporting structures of the teeth and is caused by, among other agents, *Porphyromonas gingivalis*. *P. gingivalis* is very resistant to killing by human complement, which is present in a gingival fluid at 70% of the serum concentration. We found that the incubation of human serum with purified cysteine proteases of *P. gingivalis* (gingipains) or *P. gingivalis* wild-type strains W83 and W50 resulted in a drastic decrease of the bactericidal activity of the serum. In contrast, serum treated with *P. gingivalis* mutants lacking gingipains (particularly strains without HRgpA) maintained significant bactericidal activity. To understand in detail the mechanism by which gingipains destroy the serum bactericidal activity, we investigated the effects of gingipains on the human complement system. We found that all three proteases degraded multiple complement components, with arginine-specific gingipains (HRgpA and RgpB) being more efficient than lysine-specific gingipain (Kgp). Interestingly, all three proteases at certain concentrations were able to activate the C1 complex in serum, which resulted in the deposition of C1q on inert surfaces and on bacteria themselves. It is therefore plausible that *P. gingivalis* activates complement when present at low numbers, resulting in a local inflammatory reaction and providing the bacteria with a colonization opportunity and nutrients. At later stages of infection the concentration of proteases is high enough to destroy complement factors and thus render the bacteria resistant to the bactericidal activity of complement. *The Journal of Immunology*, 2007, 178: 7242–7250.

Periodontitis is an inflammatory condition with an infective etiology that leads to the loss of tooth support. In addition to *Treponema denticola* and *Tannerella forsythensis*, *Porphyromonas gingivalis* is the part of the “red complex” of microorganisms most often associated with periodontitis (1). Although this periodontopathogen can be cultured occasionally from healthy sites, the bacteria multiplies to high numbers during active periodontal disease (2, 3) despite the fact that there is a significant Ab response (4). Furthermore, several periodontal health indicators correlate inversely with the presence of *P. gingivalis* (5), and *P. gingivalis* is able to induce periodontal disease in animal models (6). Virulence factors used by *P. gingivalis* include outer membrane vesicles, adhesins, lipopolysaccharides, hemolysins, and proteinases (7). Although *P. gingivalis* apparently secretes several different proteolytic enzymes, the cysteine proteinases of the gingipain family are responsible for >85% of the general proteolytic activity generated by this bacterium (8). These enzymes are encoded by three genes (*rgpA*, *rgpB*, and *kgp*), strictly conserved

among *P. gingivalis* strains and clinical isolates, that code for two closely related arginine-Xaa-specific proteinases (RgpA and RgpB) and one lysine-Xaa-specific proteinase (Kgp) and occur in multiple molecular forms because of proteolytic processing and glycosylation (9). In most *P. gingivalis* strains the gingipains are associated with the bacterial cell surface. Whereas RgpA and Kgp occur in a form of noncovalent complexes of unique catalytic domains with practically identical hemagglutinin/adhesion domains, RgpB is a single-chain enzyme. Working in concert, gingipains are able to cleave not only the constituents of periodontal tissues such as basement membranes and the structural proteins collagen and elastin but also degrade the host proteins used for protection such as Abs and components of the complement system (10).

Complement is a major arm of the innate immune defense system and its main function is to recognize and destroy microorganisms (11). The three pathways of human complement ensure that virtually any nonhost surface is recognized as hostile. The classical pathway is usually mediated by the binding of the C1 complex (composed of the recognition molecule C1q and two proteases, C1s and C1r) to immunoglobulins recognizing invading pathogens. Thus, complement enhances the effectiveness of the existing “natural” or specifically generated Abs in pathogen clearance. The lectin pathway is able to recognize, via the mannose-binding lectin (MBL),<sup>3</sup> “foreign” polysaccharide molecules normally present only on microbial surfaces. Finally, complement can also be activated through the alternative pathway, which is not so much an activation pathway as a failure to appropriately regulate the constant low-level spontaneous activation of C3 (constantly initiated due to the inherent instability of this protein). All three pathways lead to the opsonization of pathogen with C3b, which enhances phagocytosis. Furthermore, the anaphylatoxins C5a and C3a are

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<sup>3</sup> Abbreviations used in this paper: MBL, mannose-binding lectin; GVB<sup>2+</sup>, gelatin barbiturate (veronal) buffer; DGVB<sup>2+</sup>, dextrose-GVB<sup>2+</sup>; LB, Luria-Bertani; NHS, normal human serum; pAb, polyclonal antibody.

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Table I. Description of *P. gingivalis* strains used in this study

<i>P. gingivalis</i> strains	Characteristics	RgpA	RgpB	Kgp	Reference
W83	Wild type	Yes	Yes	Yes	32
W50	Wild type	Yes	Yes	Yes	33
W50/E8	$\Delta rgpA \Delta rgpB$ Tc <sup>r</sup> , Em <sup>r</sup>	No	No	Yes	33
W83/Kgp $\Delta$ Ig/HA	$\Delta kgp$ (602–1732) Em <sup>r</sup>	Yes	Yes	No	32
W83/RgpB+B	$rgpB \Delta rgpA$ Em <sup>r</sup> , Cm <sup>r</sup> mutant for RgpB complemented	No	Yes	Yes	34
W83/RgpB $\Delta$ 495	$\Delta rgpA \Delta rgpB^{\Delta 495-B}$ Cm <sup>r</sup> , Em <sup>r</sup>	No	No	Yes	34

released and attract phagocytes. Finally, the end result of the complement cascade is the formation of a membrane attack complex (3) and lysis. Host cells, as well as certain pathogens, protect themselves from bystander damage following complement activation through the expression of membrane-bound regulators or the recruitment of soluble endogenous complement regulators (12, 13).

Every successful human pathogen must develop the means to circumvent complement and, in the case of *P. gingivalis*, proteases appear to play an important role in the process. It has been shown that most strains of *P. gingivalis* are resistant to the bactericidal lytic activity of human serum (14, 15), and gingipains are implicated as the major factor exerting protection against complement. HRgpA cleaves purified C3 and C5, leading first to the release of active fragments such as C5a and C3b followed by degradation and loss of function by these complement factors (16). It has also been shown that Rgp proteases degrade C3 and immunoglobulins (17). Phagocytosis-resistant strains of *P. gingivalis* accumulate less C3 than noninvasive strains, and this phenomenon is affected by protease inhibitors (18). A bacterial mutant lacking HRgpA is less able to degrade C3 as compared with the wild type, which results in an increased phagocytosis by neutrophils (19). In contrast, a recent study showed that although bacterial strains lacking HRgpA and RgpB or Kgp were more efficiently opsonized by C3b and the membrane attack complex than the parental wild-type strain, they were not lysed (20).

In contrast to published papers on the effects of gingipains on complement, we have in the present study included a full panel of reagents and methods for comparing the different types of gingipains and we define in detail the sites of their action in the complement cascade. We are in a unique position, having access to soluble forms of HRgpA, RgpB, and Kgp as well as wild-type bacterial strains and isogenic strains deficient in these proteases. Furthermore, we have developed detailed methods of evaluation of the effects of external factors on complement function at various stages. These allowed us now to address in detail the influence of different gingipains on human complement. Interestingly, we present evidence for a biphasic effect of gingipains on complement due to the fact they are not only able to degrade and inactivate complement factors but that they also initially increase complement activation via the deposition of C1q when present at low concentrations.

# Materials and Methods

## Proteins

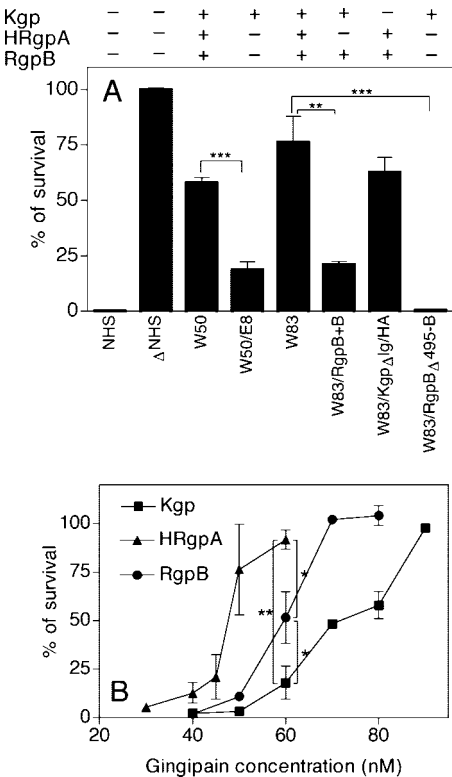
Purified complement proteins were purchased from Complement Technologies. Arginine-specific (HRgpA and RgpB) and lysine-specific (Kgp) gingipains were obtained from the *P. gingivalis* HG66 strain culture fluid as described previously (21, 22). Briefly, Kgp and HRgpA were purified using gel filtration and affinity chromatography on arginine-Sepharose, whereas RgpB was purified using a combination of gel filtration and anion-exchange chromatography on Mono Q (GE Healthcare). The purity of each enzyme was checked by SDS-PAGE. The amount of active enzyme in purified gingipains was determined by active site titration using Phe-Pro-Arg-chloromethyl ketone and benzoyloxycarbonyl-Phe-Lys-CH<sub>2</sub>OCO-(2,4,6-Me<sub>3</sub>)phenyl·HCl (Z-FK-ck) (both from Bachem) as active site ti-

trants for Rgps and Kgp, respectively (23). The same inhibitors were used to obtain inactivated gingipains with covalently modified active site cysteine residues. The final preparations of gingipains were assayed for possible contamination with LPS using *Limulus* test and found to be negative (<10 U).

As cysteine proteinases, gingipains require pretreatment with a reducing agent to become active enzymes. Therefore, stock solutions of gingipains were diluted in 0.2 M HEPES and 5 mM CaCl<sub>2</sub> (pH 8.0) supplemented with 20 mM cysteine and incubated at 37°C for 15 min. The activated gingipains were then further diluted to the appropriate final concentrations with 0.2 M Tris-HCl (pH 7.4) containing 0.1 M NaCl, 5 mM CaCl<sub>2</sub>, and 20 mM cysteine.

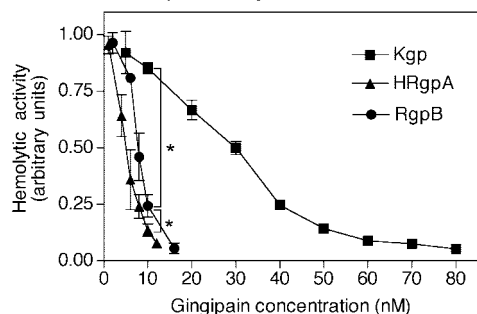
## Bacterial strains and their culture

The *P. gingivalis* strains listed in Table I were grown in enriched tryptic soy broth medium (TSB) or in blood TSB agar at 37°C in an anaerobic

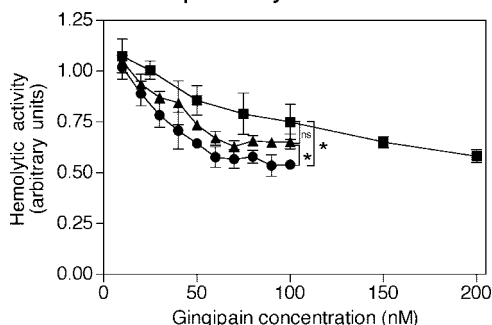


**FIGURE 1.** Gingipains destroy the bactericidal activity of NHS. *A*, *E. coli* DH5 $\alpha$  was incubated with NHS preincubated with the *P. gingivalis* strains listed in Table I and the surviving bacteria were enumerated after an overnight culture on LB agar plates. As a control, the heat-inactivated NHS ( $\Delta$ NHS) was used. *B*, *E. coli* DH5 $\alpha$  was incubated with human serum supplemented with increasing concentrations of purified gingipains and the surviving bacteria were enumerated after an overnight culture. An average of three independent experiments is presented with bars indicating SD. The statistical significance of differences between the groups was estimated with Student's *t* test; \*, *p* < 0.05; \*\*, *p* < 0.01; and \*\*\*, *p* < 0.001. At 60 mM, which is the only common concentration tested for the three gingipains (*B*), the statistical significance of differences between the gingipains was assessed and is presented in the graph.

## A Classical pathway



## B Alternative pathway



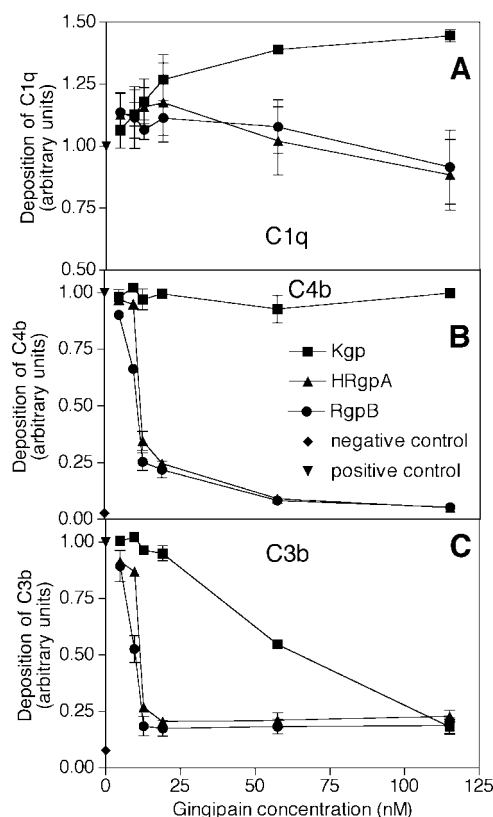
**FIGURE 2.** Gingipains destroy the hemolytic activity of human serum. Sheep erythrocytes sensitized with Abs (A, classical pathway) or rabbit erythrocytes (B, alternative pathway) were incubated with 1.25 or 10% NHS, respectively. Serum was supplemented with various concentrations of gingipains. After 1 h (classical pathway) or 1.5 h (alternative pathway) incubation at 37°C, the degree of lysis was estimated by the measurement of released hemoglobin (absorbance at 405 nm). The lysis obtained in the absence of gingipains was set as 1. An average of three independent experiments is presented with bars indicating SD values. The statistical significance of differences between the groups of gingipains at 10 nM (classical) and 100 nM (alternative) was estimated with Student's *t* test, \*,  $p < 0.05$ ; ns, not significant.

chamber (Concept 400; Biotrace) with an atmosphere of 90% N<sub>2</sub>, 5% CO<sub>2</sub>, and 5% H<sub>2</sub>. For growth selection of mutants on a solid medium, antibiotics were used at 5 µg/ml erythromycin and 1 µg/ml tetracycline. The concentration of antibiotics was doubled for selective *P. gingivalis* mutant growth in a liquid culture. *Escherichia coli* strain DH5α (Invitrogen life Technologies) was grown on a standard Luria-Bertani (LB) agar plates or in LB broth. *Prevotella nigrescens* (ATCC no. 25261) was grown on BBL Columbia II agar containing 8.5% horse blood, 0.04% L-cysteine HCl, 2.5% hemin, and 1% vitamin K1.

## Bactericidal assay

Strain *E. coli* DH5α was cultured in LB broth until the exponential growth phase. Cells were harvested, washed once in gelatin barbiturate (veronal) buffer (GVB<sup>2+</sup>) (5 mM veronal buffer (pH 7.3), 140 mM NaCl, 0.1% gelatin, 1 mM MgCl<sub>2</sub>, and 0.15 mM CaCl<sub>2</sub>) and adjusted to an OD at 600 nm of 0.6. Normal human serum (NHS) was prepared from blood taken from six healthy volunteers and pooled. NHS was diluted in GVB<sup>2+</sup> to a concentration of 2% and incubated with various concentrations of gingipains for 15 min at room temperature. Thereafter, 10<sup>4</sup> bacteria cells were added and incubated with serum supplemented with gingipains for 20 min at 37°C. After incubation, aliquots were removed, diluted serially, and spread onto LB agar plates. Heat-inactivated serum (56°C for 30 min) was used as a negative control. Plates were incubated for 12 h at 37°C after which colonies were counted and the percentages of the surviving bacteria were calculated.

*P. gingivalis* from 5-day-old agar plate cultures were harvested and washed once in GVB<sup>2+</sup> and adjusted to an OD at 600 nm of 0.6. Thereafter, 2 × 10<sup>6</sup> (W50 strain and its mutants) or 8 × 10<sup>5</sup> bacterial cells (W83 and its corresponding mutants listed in Table I) were mixed with 2% serum diluted in GVB<sup>2+</sup> and incubated for 15 min at room temperature. Thereafter, 10<sup>4</sup> *E. coli* cells were added and incubation was continued for 20 min



**FIGURE 3.** Gingipains inhibit the classical pathway. Human IgM was immobilized on microtiter plates and allowed to activate 1% NHS containing various concentrations of gingipains. After 45 min of incubation the plates were washed and the deposited C1q (A), C4b (B), and C3b (C) were detected with specific Abs. Positive controls show how much of each complement factor was deposited in the absence of any gingipain, whereas the negative control shows how much signal was obtained in the absence of serum. The absorbance obtained in the absence of gingipains was set as 1. An average of three independent experiments is presented with bars indicating SD. The enhancement of C1q deposition seen in A was statistically significant (at least  $p < 0.05$ ) at four (13, 19, 58, and 115 nM), two (5 and 19 nM) and one (13 nM) concentrations for Kgp, RgpB, and HRgpA, respectively.

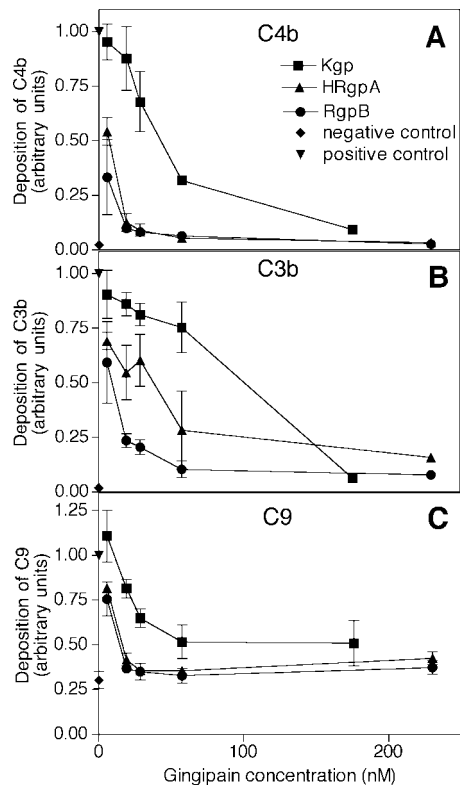
at 37°C. As described above, the aliquots were removed, diluted serially, and spread onto LB agar plates. Plates were incubated for 12 h at 37°C after which colonies were counted and the percentages of the surviving bacteria were calculated.

## Hemolytic assay

To assess the activity of the classical pathway, sheep erythrocytes were washed three times with DGVB<sup>2+</sup> buffer (2.5 mM veronal buffer (pH 7.3), 70 mM NaCl, 140 mM glucose, 0.1% gelatin, 1 mM MgCl<sub>2</sub>, and 0.15 mM CaCl<sub>2</sub>). The cells were incubated with a complement-fixing Ab (amboceptor (Dade Behring) diluted 1/3000 in DGVB<sup>2+</sup> buffer) at a concentration of 10<sup>9</sup> cells/ml for 20 min at 37°C. After two washes with DGVB<sup>2+</sup>, 0.5 × 10<sup>9</sup> cells/ml were incubated for 1 h at 37°C with 1.25% NHS diluted in DGVB<sup>2+</sup> buffer. Before incubation with erythrocytes, NHS was preincubated with various concentrations of gingipains for 15 min at room temperature. The buffer used for the activation of gingipains did not interfere with the hemolytic assay or erythrocytes. The samples were centrifuged and the amount of the lysed erythrocytes was determined by spectrophotometric measurement of the amount of released hemoglobin (405 nm).

To assess the activity of the alternative pathway, rabbit erythrocytes were washed three times with Mg<sup>2+</sup> EGTA buffer (2.5 mM veronal buffer (pH 7.3) containing 70 mM NaCl, 140 mM glucose, 0.1% gelatin, 7 mM MgCl<sub>2</sub>, and 10 mM EGTA). Erythrocytes at a concentration of 0.5 × 10<sup>9</sup> cells/ml were then incubated for 1.5 h at 37°C with 10% NHS diluted in Mg<sup>2+</sup> EGTA buffer. The NHS used was pretreated with various concentrations of gingipains for 15 min at room temperature. The samples were





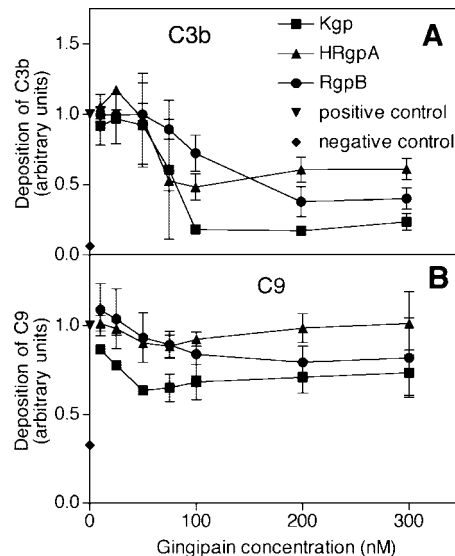
**FIGURE 4.** Gingipains inhibit the lectin pathway. Mannan was immobilized on microtiter plates and allowed to activate 5% NHS containing various concentrations of gingipains. After 45 min of incubation the plates were washed and the deposited C4b (A), C3b (B) and C9 (C) were detected with specific Abs. Positive controls show how much of each complement factor was deposited in the absence of any gingipain, whereas the negative control shows how much signal was obtained in the absence of serum. The absorbance obtained in the absence of gingipains was set as 1. An average of three independent experiments is presented with bars indicating SD. Difference between HRgpA and RgpB was statistically significant (at least  $p < 0.05$ ) only at 20 and 30 nM in the assay measuring deposition of C3b; no differences were significant for the depositions of C4b and C9 at any concentration tested. Differences between Kgp and HRgpA/RgpB were significant (at least  $p < 0.05$ ) at 6, 20, 30, and 60 nM concentrations for all three panels.

centrifuged and the amount of the lysed erythrocytes was determined spectrophotometrically. We determined experimentally that gingipains do not cause direct lysis of erythrocytes and that they do not affect the sensitivity of the hemolytic assays (i.e., the erythrocytes pretreated with gingipains were equally sensitive to hemolysis by serum as untreated ones).

#### Complement activation assays

Microtiter plates (Maxisorp; Nunc) were incubated overnight at 4°C with 50  $\mu$ l of a solution containing 2  $\mu$ g/ml human IgM (I-8260; Sigma-Aldrich), 100  $\mu$ g/ml mannan (M-7504; Sigma-Aldrich), or 20  $\mu$ g/ml zymosan (Z-4250; Sigma-Aldrich) in 75 mM sodium carbonate (pH 9.6).

Between each step of the procedure the plates were washed four times with 50 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20 (pH 7.5). The wells were blocked with 1% BSA (Sigma-Aldrich) in PBS for 2 h at room temperature. NHS was diluted in DGVB<sup>2+</sup> buffer and used at a concentration of 1 or 5% for the classical and the lectin/alternative pathways, respectively. These concentrations were chosen on the basis of initial titrations. NHS was mixed with various concentrations of gingipains and incubated in the wells of microtiter plates for 45 min at 37°C with shaking in the case of the alternative and the lectin pathways. For the classical pathway, NHS was incubated with gingipains for 15 min at room temperature and the gingipains were inhibited to avoid degradation of the IgM deposited on the plates. The inhibitors used were 3.1  $\mu$ M Z-FK-ck for Kgp or 16.4  $\mu$ M antipain (Bachem) for HRgpA and RgpB. Immediately after the addition of inhibitors, NHS was incubated in microtiter plates for 45



**FIGURE 5.** Gingipains inhibit the alternative pathway. Zymosan was immobilized on microtiter plates and allowed to activate 5% NHS containing various concentrations of gingipains. After 45 min of incubation the plates were washed and the deposited C3b (A) and C9 (B) were detected with specific Abs. Positive controls show how much of each complement factor was deposited in the absence of any gingipain, whereas the negative control shows how much signal was obtained in the absence of serum. The absorbance obtained in the absence of gingipains was set as 1. An average of three independent experiments is presented with bars indicating SD.

min at 37°C with shaking. Complement activation was assessed by detecting the deposited complement factors using rabbit anti-C1q, anti-C4b, anti-C3d polyclonal Abs (pAbs; DakoCytomation), and goat anti-C9 pAb (Complement Technologies) diluted in the blocking buffer. Bound Abs were detected with HRP-labeled anti-rabbit or anti-goat secondary pAb (DakoCytomation). Bound HRP-labeled pAbs were revealed using 1,2-phenylenediamine dihydrochloride (OPD) tablets (DakoCytomation) and the absorbance was measured at 490 nm.

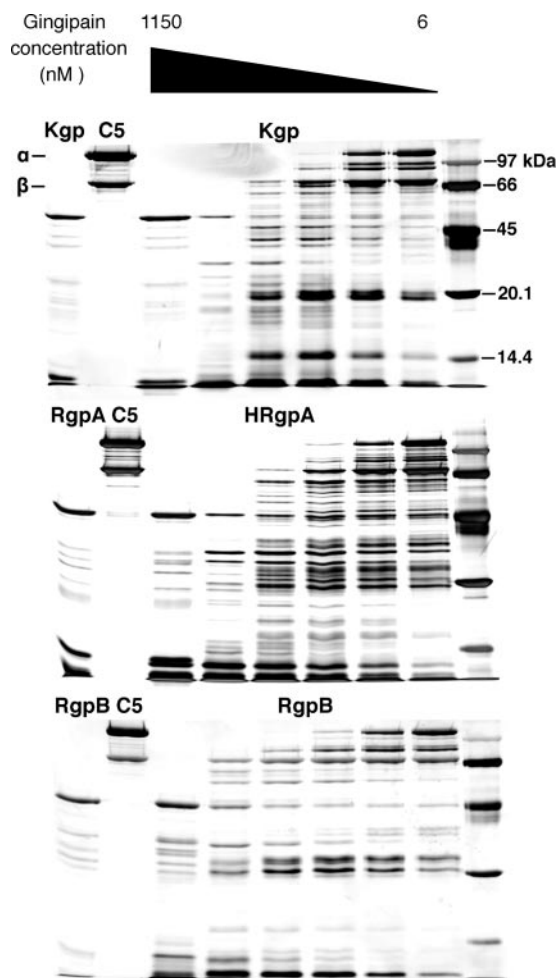
To assess the deposition of C1q/C1 from normal NHS as well as purified C1 and C1q on microtiter plates without any complement activator, plates were blocked with 1% BSA in PBS for 2 h at room temperature. NHS at a concentration of 5% or purified C1q and C1 at concentration of 4  $\mu$ g/ml were diluted in DGVB<sup>2+</sup> buffer and mixed with various concentration of gingipains. Plates were incubated for 45 min at 37°C with shaking and the deposited C1q was detected with specific Abs.

#### Degradation assay

C1q, C4, C3, and C5 were incubated with different concentrations of the three gingipains (1150, 115, 46, 23, 11.5, and 5.75 nM). Incubations were conducted in 0.2 M Tris-HCl (pH 7.4) containing 0.1 M NaCl, 5 mM CaCl<sub>2</sub>, and 20 mM cysteine for 30 min at 37°C. The proteins were separated by SDS-PAGE electrophoresis using standard Laemmli procedure and 12 or 15% gels. Before electrophoresis the samples were boiled for 5 min at 95°C in a sample loading buffer containing 25 mM DTT and 4% SDS. After separation, the gels were stained with silver salts or Coomassie brilliant blue to visualize the separated proteins.

#### Deposition of C1q on bacteria

*Prevotella nigrescens* ATCC 25261 from a 2-day-old agar plate culture were harvested, washed twice in DGVB<sup>2+</sup> buffer, and adjusted to an OD at 600 nm of 1. NHS was diluted in DGVB<sup>2+</sup> to a concentration of 5%, mixed with 6  $\times$  10<sup>5</sup> cells and incubated with various concentrations of gingipain for 30 min at 37°C. Thereafter the cells were washed twice in binding buffer (10 mM HEPES, 140 mM NaCl, 5 mM KCl, 1 mM MgCl<sub>2</sub>, and 2 mM CaCl<sub>2</sub> (pH 7.2)). C1q deposition was assessed by incubation of human C1q FITC-conjugated polyclonal Abs (diluted in binding buffer 1:100; DakoCytomation) for 1 h. The cells were washed twice in a binding buffer and finally resuspended in the flow cytometry buffer (50 mM HEPES, 100 mM NaCl, 30 mM Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, and 1% BSA (pH 7.4)). Flow cytometry analysis was performed using a FACSCalibur apparatus (BD Biosciences).



**FIGURE 6.** Degradation of C5 by gingipains. Purified C5 was incubated for 30 min with increasing concentrations of gingipains and separated by SDS-PAGE on a 12% gel. One representative experiment of at least three performed is shown.

*P. gingivalis* (strain E8, RgpBΔ495-B, KgpΔIg/HA) from a 5-day-old agar plate were harvested, washed once in DGVB<sup>2+</sup>, and adjusted to an OD at 600 nm of 1. NHS was diluted in DGVB<sup>2+</sup> to a concentration of 5%, mixed with  $6 \times 10^5$  cells, and incubated with gingipains (300 nM Kgp, 37.5 nM HRgpA, and 75 nM RgpB) for 30 min at 37°C. The cells were washed twice in the binding buffer and a C1q deposition was assessed as for *P. nigrescens*.

#### Statistical analysis

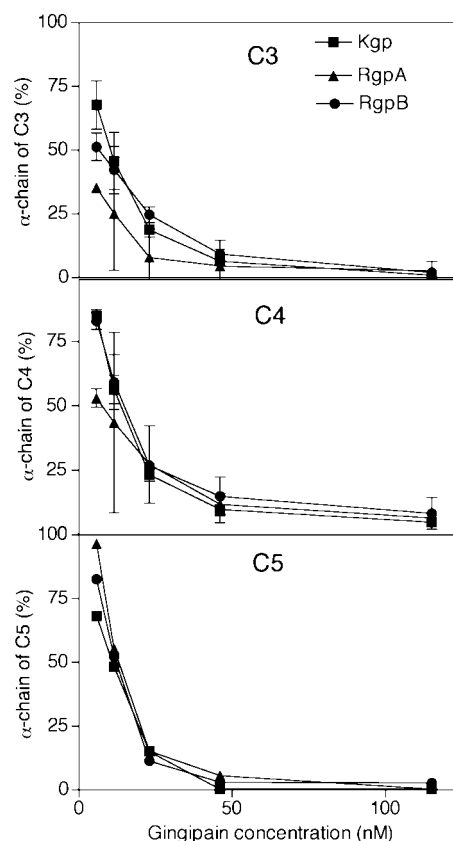
Student's *t* test was used to calculate the *p* values to estimate whether the observed differences between experimental results were statistically significant.

## Results

### Gingipains destroy the bactericidal activity of human serum

*P. gingivalis* is extremely resistant to killing by NHS and we found that a 1-h incubation of wild type strains W83 and W50 with even 80% pooled NHS collected from healthy laboratory workers did not significantly affect the survival of *P. gingivalis* (not shown).

Furthermore, the bactericidal activity of human serum was destroyed by preincubation of the serum with both *P. gingivalis* wild-type strains, W83 and W50 (Fig. 1A). Serum incubated with these two strains lost ~60% of its bactericidal activity. Serum incubated in the presence of mutants lacking gingipains lost bactericidal activity to various degrees. RgpA was the most efficient in destroying the bactericidal activity of serum, because mutants lacking RgpA were



**FIGURE 7.** Densitometric analysis of C3, C4, and C5 degradation by gingipains. Gels such as those shown in Fig. 6 were analyzed by densitometry to quantify and compare the efficiencies of gingipains in the degradation of these complement factors. The densities of protein bands corresponding to the  $\alpha$ -chains of C3, C4, and C5 were determined. An average of three independent experiments is presented with bars indicating SD. No statistically significant differences between the gingipains were found.

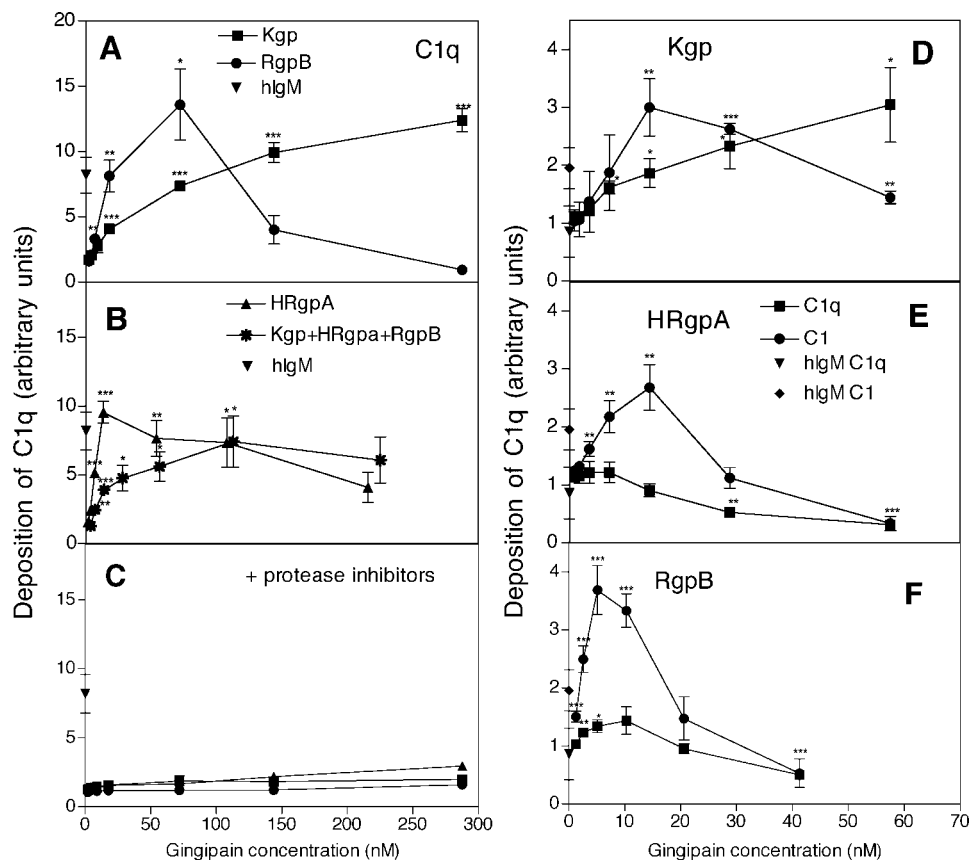
the ones having the least effect on a complement-mediated killing by serum. Serum pretreated with mutants expressing only Kgp (W50/E8 and W83/RgpBΔ495) was still very efficient in killing *E. coli*.

To quantitatively assess how three different cysteine proteinase i.e., gingipains isolated from *P. gingivalis*, affect the bactericidal activity of human serum, we used a model system. *E. coli* DH5 $\alpha$  was incubated with human serum pretreated with various concentrations of the three gingipains. We found that all three enzymes destroyed the bactericidal activity of human serum in a dose-response-dependent manner and rescued *E. coli* (Fig. 1B). However, the three gingipains differed in efficiency, with HRgpA being the strongest inactivator of the bactericidal activity of human serum and Kgp being the weakest one. The differences between the three gingipains were statistically assessed. Taken together, the results obtained with both the bacterial mutants and the purified proteins show that of the three gingipains tested, HRgpA has most impact on the bactericidal activity of human serum while Kgp displays the weakest activity.

### Gingipains destroy complement system in human serum

To understand how the gingipains destroy the bactericidal activity of NHS, i.e., complement, the three purified gingipains were incubated at various concentrations with human serum and hemolytic assays were used to assess the activity of the classical and alternative pathways of complement in the pretreated sera. We found that HRgpA and RgpB were more efficient in degradation and inactivation of complement than the Kgp in the case of both

**FIGURE 8.** Gingipains activate C1 and cause deposition of C1 on nonactivating surfaces. *A* and *B*, Microtiter plates were blocked with BSA and incubated with 5% NHS containing various concentrations of gingipains. The gingipains were tested either separately or all three together at the same time. Deposited C1q was detected with a specific Ab. The signal obtained for C1q binding to IgM is shown as a positive control. *C*, The same experiment as shown in *A* and *B* but with the addition of specific inhibitors of gingipains. *D–F*, Blocked microtiter plates were incubated with purified C1q and C1 in the presence of various concentrations of the three gingipains. The plates were washed and the deposited C1q was detected with Ab. Absorbance obtained in the absence of gingipains was set as 1. An average of three independent experiments is presented with bars indicating SD. Statistical significance of differences between experimental conditions without (set as 1) and with gingipains was estimated with Student's *t* test, \*, *p* < 0.05; \*\*, *p* < 0.01; and \*\*\*, *p* < 0.001.



pathways (Fig. 2). All three gingipains were able to fully inhibit the classical pathway when present at nanomolar concentrations, whereas the alternative pathway was only inhibited by 50% at the highest concentrations used (100 nM). It should be noted that 10% serum was used for the alternative pathway hemolytic assay vs 1.25% for the classical pathway. These concentrations were chosen on the basis of the initial titration and represent conditions in which each assay is the most sensitive to changes. The alternative pathway is known to require high concentrations of serum to function properly. We also tested the activity of gingipains in the buffers used for hemolytic assays and found that Kgp had a 20% lower activity in Mg-EGTA than in GVB<sup>2+</sup> buffer whereas HRgpA and RgpB were 40 and 70%, respectively, more active in Mg-EGTA than in GVB<sup>2+</sup> buffer (data not shown). This indicates that the presence of a chelator in the alternative pathway hemolytic assay cannot be responsible for the lower activity of gingipains toward the alternative pathway as compared with the classical one. Altogether, the classical pathway appears to be more sensitive to destruction by gingipains than the alternative one and Kgp is the weakest of the tested gingipains, the most pronounced for the classical pathway.

#### *Gingipains interfere with all three pathways by degrading several complement factors*

Each complement pathway is composed of several factors activated in a consecutive manner. To assess which complement factors were mainly affected by gingipains, we used a microtiter plate-based assay in which complement activation was initiated by various ligands depending on the pathway analyzed. The deposition of successive complement factors was detected with specific Abs.

In case of the classical pathway, complement activation was initiated by IgM deposited on plates. We found that the deposition of C1q from 1% serum was enhanced in the presence of lower

concentrations of HRgpA and RgpB as well as all concentrations of Kgp (Fig. 3A). C4 was sensitive to degradation by HRgpA and RgpB and the deposition of C4b was almost abolished at 25 nM by either of these two gingipains. In contrast, Kgp did not affect C4 in the conditions tested (Fig. 3B). C3 was sensitive to all three gingipains, although Kgp was a significantly weaker inhibitor than HRgpA and RgpB (Fig. 3C).

For the assessment of the lectin pathway we used plates covered with mannan. In this case, all three gingipains inhibited the deposition of C4b, C3b, and C9 from 5% serum with Kgp being significantly weaker than HRgpA and RgpB (Fig. 4).

The alternative pathway was activated by immobilized zymosan and we found that all three proteinases were able to inhibit this pathway with a similar efficiency as determined by measurement of the deposition of C3b (Fig. 5A). Higher concentrations of the proteinases were required to inhibit the alternative pathway in 5% serum as compared with the classical and the lectin pathways. The deposition of C9 was also affected by all proteinases but to a lower extent (Fig. 5B).

Taken together, all three pathways were sensitive to gingipains, but to various degrees. Interestingly, the C1 complex appears to be quite resistant to degradation and its deposition was even more enhanced at lower protease concentrations. Furthermore, Kgp is a rather weak inhibitor of the classical pathway while its effect on the alternative pathway is similar to that of the other two gingipains.

#### *Gingipains attack preferentially $\alpha$ -chains of C3, C4, and C5 molecules*

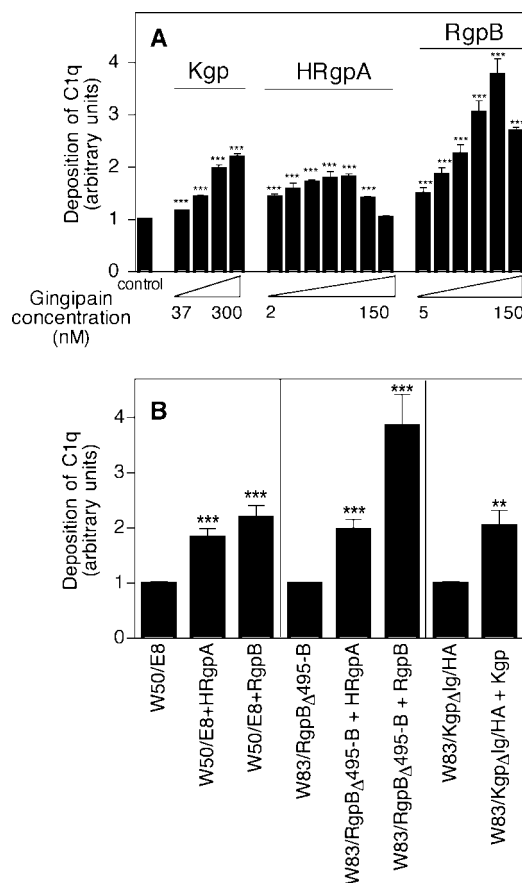
To assess the sites cleaved by gingipains in complement factors, purified C3, C4, and C5 were incubated with the three gingipains at various molar ratios. The proteins were then separated by SDS-PAGE and visualized using silver staining. Both C3 and C5 are

composed of covalently linked  $\alpha$ - and  $\beta$ -chains, whereas C4 contains  $\alpha$ -,  $\beta$ -, and  $\gamma$ -chains. When the degradation of purified C5 was analyzed it became apparent that all three proteinases first attack the  $\alpha$ -chain (Fig. 6), which is in agreement with previous reports suggesting that gingipains are able to release C3a and C5a when present at low concentrations. At higher molar ratios the gingipains degraded the C5  $\alpha$ -chain into smaller fragments and then continued the degradation of a remaining part of the molecule. C3 and C4, which are structurally related to C5, were cleaved in a similar manner (not shown). When degradation of the  $\alpha$ -chains was quantified by densitometry, we found that all three gingipains were equally efficient in degradation of C3, C4, and C5 in this purified system (Fig. 7). This suggests that Kgp prioritizes other serum proteins than complement factors in comparison to HRgpA and RgpB. These experiments confirmed a previous observation (16) that  $\alpha$ -chains of C3 and C5 are preferentially degraded by gingipains and extended it to C4.

#### *Gingipains cause activation and deposition of C1 in the absence of any activator*

When assessing the effect of gingipains on activation of the classical pathway, we observed that the deposition of C1q on IgM was not inhibited by the gingipains but was enhanced at lower concentrations of the gingipains. When human serum was incubated with gingipains in the absence of any immobilized C1 activator, we observed that all three gingipains caused a massive deposition of C1q on the empty microtiter plates blocked with BSA (Fig. 8, A and B). In case of HRgpA and RgpB, this effect was apparent at lower concentrations of the enzymes and vanished with increasing concentrations. In case of Kgp the deposition of C1q increased with the concentration of added enzyme over the whole tested range (Fig. 8A). In the absence of gingipains, the deposition of C1q from serum was negligible as expected. Furthermore, we observed a significant deposition of C1q on empty plates when the three proteases were present simultaneously (Fig. 8B). Importantly, C1q deposition was absent when not only gingipains were added but also their specific inhibitors (Fig. 8C).

To determine whether gingipains affected the recognition part of the C1 complex, C1q, or the associated proteinases C1r and C1s, we incubated purified C1q and C1 complex with gingipains followed by the measurement of a C1q deposition on the empty surface. We found that the added C1q bound only slightly over the background readout when treated with HRgpA or RgpB (Fig. 8, E and F). When the whole C1 complex was added together with these two gingipains there was a strong deposition of C1q on the empty plates (Fig. 8, E and F), comparable with the intact serum (Fig. 8, A and B). The increase in deposition vanished yet again at high gingipain concentrations for HRgpA and RgpB (Fig. 8, E and F). In case of Kgp, the latter effect was not reached at the tested concentrations and the amount of the deposited C1q was highest at the highest protease concentration tested. Interestingly, both C1q and the C1 complex deposited well on the plates in the presence of Kgp (Fig. 8D). To test whether the deposited C1 was active, we incubated human serum in the presence of Kgp in the plates blocked with BSA and observed the deposition of C4b that was otherwise lacking in the absence of Kgp (not shown). We then tested whether the gingipains will cause a deposition of C1q also on bacterial surfaces. To this end, *P. nigrescens* was incubated with NHS containing gingipains at different concentrations and the deposition of C1q was measured using flow cytometry. We found that the addition of any gingipain to NHS caused an increase in the deposition of C1q on the surface of *Prevotella* that mimicked the results obtained using microtiter plates (Fig. 9A). Furthermore, we incubated the *P. gingivalis* strain lacking Kgp but expressing



**FIGURE 9.** Gingipains cause the deposition of C1q on bacterial surfaces. A, *P. nigrescens* ATCC 25261 was incubated with 5% NHS and different concentrations of gingipains. The deposition of C1q was detected with specific FITC-labeled Abs and flow cytometry analysis. B, Mutants of *P. gingivalis* lacking Kgp (W83/KgpΔIg/HA) or HRgpA and RgpB (W50/E8 and W83/RgpBΔ495-B) were incubated with 5% NHS and missing gingipains. C1q deposition was assessed as in A. The deposition of C1q in the absence of gingipains was set as 1. An average of three independent experiments is presented with bars indicating SD. The statistical significance of differences between the groups in absence of gingipains and after incubation with gingipains was estimated with Student's *t* test; \*, *p* < 0.05; \*\*, *p* < 0.01; and \*\*\*, *p* < 0.001.

HRgpA and RgpB with purified Kgp and observed an increase in the deposition of C1q (Fig. 9B). Similarly, *P. gingivalis* strains lacking HRgpA and RgpB but expressing Kgp were opsonized more efficiently with C1q after the addition of HRgpA and RgpB.

Taken together, our results show that the gingipains are able to cause the deposition of an active C1 complex on normally nonactivating surfaces such as BSA-coated plastic or bacteria.

## Discussion

All successful human bacterial pathogens must develop strategies to circumvent the complement system. Complement-mediated killing is relevant for *P. gingivalis*, because complement components are present in a gingival fluid at 70% of the serum concentration (24) and the fact that *P. gingivalis* activates both the classical and alternative pathways (14). It has also been demonstrated in vivo that there is high level of complement activation in a gingival fluid of patients with periodontitis (21, 25). Mounted Ab responses against *P. gingivalis* are of importance because there is a relationship between Ab titers, opsonic activity, and the accumulation of C3 (18). However, it appears that *P. gingivalis* is able to override these defense mechanisms. One strategy of the defense depends on



the production of large amount of gingipains, which are cysteine proteases able to degrade, among other proteins, immunoglobulins and complement factors. Gingipains were found at the level of 100 nM in the gingival crevicular fluid collected from the inflamed sites of adult periodontitis patients (22). Furthermore, it is likely that, in situ in dental plaque heavily populated with *P. gingivalis*, the gingipain concentration is considerably higher than in the crevicular fluid because the majority of strains retain significant amounts of gingipains on the cell surface as well as release them in the form associated with vesicles (9). Most importantly, gingipain concentration in gingival crevicular fluid correlates with periodontal attachment loss in chronic periodontitis patients (23). Host response to gingipains relies on specific Abs necessary for the efficient opsonophagocytosis of *P. gingivalis* (26).

In the present study we investigated the effects of three gingipains on the complement system. Importantly, gingipain levels used in these in vitro experiments were well within the range of enzyme concentrations found in vivo. Interestingly, low concentrations of gingipains appear to activate complement factors C3, C4, and C5 because they preferentially aim at the  $\alpha$ -chains of these proteins (16, 27). This may lead to the release of the anaphylatoxins C3a and C5a as well as the activated forms C4b, C3b, and C5b. At higher concentrations the proteases simply degrade these three complement factors into small fragments so that they can no longer propagate a complement cascade. For C3 this has also been observed in vivo in a guinea pig model (28). We also observed that the three gingipains are able to activate the C1 complex and cause its deposition on a surface that normally does not serve as an activator. One interesting question is whether this phenomenon is characteristic for C1 or whether it occurs also for related proteins such as MBL. Our assay was not sensitive enough to detect the binding of MBL from human serum to mannan. However, we could detect the deposition of MBL when using a purified MBL preparation. When tested in a similar manner as C1 for its ability to deposit on empty plates in the presence of gingipains, neither purified MBL used alone nor purified MBL added to human serum showed such an ability (not shown). This shows that the phenomenon we observed for C1 is not a general property of collectins but is specific for the C1 complex. Considering that the C1 complex is more susceptible to this event than C1q alone, it is possible that gingipains both digest and activate the C1q part of the complex but also act on C1r and C1s. Furthermore, the C1 inhibitor may be inactivated by gingipains (29), which could contribute to an increased activity of the C1 complex. C1 was deposited from a serum in the presence of gingipains not only on blocked microtiter plates but also on the surfaces of both *P. gingivalis* and *P. nigrescens*, which are found together at sites of infection. A picture of an intricate strategy emerges; the bacteria at low concentrations appear to generate C5a and C3a, chemotactic factors for neutrophils, and to activate the C1 complex, directing its deposition on their own and surrounding surfaces. This may lead to a certain level of inflammation that provides access to nutrients for the bacteria and allows colonization. At higher concentrations of bacteria and gingipains, the complement system becomes incapacitated by multiple cleavages of several participating proteins. It is also plausible that proinflammatory cytokines are readily induced by initial infection with *P. gingivalis* and can similarly have the potential to induce the supply of nutrients present in inflammatory exudates. Thus, the postulated role for the gingipains at low concentrations could be redundant.

Many successful human bacterial pathogens capture human complement inhibitors such as factor H to down-regulate complement attack. We could not detect an interaction between factor H and *P. gingivalis* (not shown), which makes the gingipain-mediated

complement destruction an even more important virulence factor.

*P. gingivalis* is very resistant to complement and survives at very high serum concentrations. Therefore, it is difficult to establish a quantitative bactericidal assay using *P. gingivalis* strains without sensitization of the bacteria with polyclonal rabbit Abs that enhance complement activation. Hence, we used *E. coli* as a model to investigate whether the bactericidal activity of serum is affected by gingipains. Although very sensitive to human serum, *E. coli* was able to fully survive when nanomolar concentrations of gingipains were added to 2% NHS. This clearly shows that purified gingipains are very efficient at destroying the bactericidal activity of NHS. Furthermore, we found that NHS preincubated with *P. gingivalis* wild-type strains also lost its bactericidal activity toward *E. coli*. A recent publication has shown that *P. gingivalis* lacking the gingipains HRgpA and RgpB or Kgp are indeed opsonized with larger amounts of C3b but that this does not lead to the lysis of bacteria (20). The authors suggest that lysis is prevented by the presence of anionic polysaccharides on a bacterial surface. The results of this study are in contrast with other published results that showed that a *P. gingivalis* mutant lacking all three gingipains was extremely sensitive to lysis by complement (30). In direct contrast, a mutant lacking HRgpA and RgpB was reported to survive in NHS equally well as the wild type (20) or to survive at 28% as compared with 87% for the wild type (30). It is unclear at the moment what the reason is for these discrepancies. However, enhanced opsonization with C3b in gingipain-deficient mutants was consistently observed in all of the studies. A decrease of opsonization with C3b leads to impaired phagocytosis, and in case of most pathogens opsonization/phagocytosis is more important than lysis for a bacterial killing. This implies that gingipain-dependent complement inactivation is indeed an important virulence factor for *P. gingivalis*. It has been shown that a mutant lacking HRgpA is opsonized and phagocytized more efficiently than the wild type (19), which ultimately leads to the fact that the mutant is less invasive in a mouse model of infection (31). Our experiments showing that gingipains will also aid the survival of bystander bacteria implies that gingipains create favorable conditions for other species of bacteria that together can create a common ecosystem that would be beneficial habitat for all participating species.

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## Disclosures

The authors have no financial conflict of interest.

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