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In Vitro Sensitivity of Oral, Gram-Negative, Facultative Bacteria to the Bactericidal Activity of Human Neutrophil Defensins

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Neutrophils play a major role in defending the periodontium against infection by oral, gram-negative, facultative bacteria, such as *Actinobacillus actinomycetemcomitans*, *Eikenella corrodens*, and *Capnocytophaga* spp. We examined the sensitivity of these bacteria to a mixture of low-molecular-weight peptides and highly purified individual defensin peptides (HNP-1, HNP-2, and HNP-3) isolated from human neutrophils. Whereas the *Capnocytophaga* spp. strains were killed significantly by the mixed human neutrophil peptides, the *A. actinomycetemcomitans* and *E. corrodens* strains were resistant. Killing was attributable to the defensins. The bactericidal activities of purified defensins HNP-1 and HNP-2 were equal, and both of these activities were greater than HNP-3 activity against strains of *Capnocytophaga sputigena* and *Capnocytophaga gingivalis*. The strain of *Capnocytophaga ochracea* was more sensitive to defensin-mediated bactericidal activity than either *C. sputigena* or *C. gingivalis* was. The three human defensins were equipotent in killing *C. ochracea*. *C. ochracea* was killed under aerobic and anaerobic conditions and over a broad pH range. Killing was most effective under hypotonic conditions but also occurred at physiologic salt concentrations. We concluded that *Capnocytophaga* spp. are sensitive to oxygen-independent killing by human defensins. Additional studies will be required to identify other components that may equip human neutrophils to kill *A. actinomycetemcomitans*, *E. corrodens*, and other oral gram-negative bacteria.

Current evidence suggests that neutrophils and other phagocytes play a major role in controlling microorganisms in the periodontium, thereby protecting the host against both periodontal and systemic infections (34). Among those organisms which are apparently controlled by phagocytes are gram-negative, facultative bacteria which inhabit dental plaque, including *Actinobacillus actinomycetemcomitans*, *Eikenella corrodens*, and *Capnocytophaga* spp. All isolates of *A. actinomycetemcomitans*, some isolates of *E. corrodens*, and isolates of *Capnocytophaga* spp. from blood are not killed effectively by serum in vitro (6, 32, 39). These organisms have been isolated from lesional sites in rapidly destructive forms of periodontal infection, such as localized juvenile periodontitis and rapidly progressive adult periodontitis (7, 31, 33, 40), diseases which also have been associated with cell-derived or serum-derived defects in phagocyte function (5, 18, 35). Systemic infections by these oral gram-negative, facultative bacteria have been observed in cases of neutropenia. For example, cases of *E. corrodens* and *Capnocytophaga* spp. septicemia have been associated with granulocytopenia (8, 10). One case report has documented the association of severe periodontal disease with precipitating serum antibodies against *A. actinomycetemcomitans* and chronic neutropenia (1).

Neutrophils have been shown to be capable of killing representative strains belonging to the genus *Capnocytophaga* and the species *E. corrodens* and *A. actinomycetemcomitans* (2). At present, there is little information regarding the precise bactericidal mechanisms involved. We have reported that both oxidative and nonoxidative mechanisms appear to be operative in the killing of *A. actinomycetemcomitans* and that oxidative killing is primarily a result of activity of the myeloperoxidase-H_2O_2-Cl^- system (22).

The nonoxidative killing of microorganisms by human neutrophils is thought to be a result of the bactericidal action of (i) cationic peptides (such as the defensins) and proteins (such as the bactericidal permeability-increasing protein and members of the neutral serine protease family, including azurocidin, cathepsin G, elastase, and p29b); (ii) enzymes, such as collagenase and lysozyme; (iii) polyamines, such as spermine and spermidine; and (iv) binding proteins, including apolactoferrin and possibly others (3, 12, 15–17, 24, 27, 38). Defensins are small (molecular weights, 3,000 to 3,500), cationic, amphipathic, arginine- and cysteine-rich peptides that are composed of 29 to 34 amino acids. They are important constituents of the azurophil granules of neutrophils (26, 29), making up 5 to 7% of the total protein and 30 to 50% of the azurophil granule content of human neutrophils (12). Human defensins HNP-1, HNP-2, and HNP-3 possess net charges of +3, +3, and +2, respectively (29). A fourth defensin, HNP-4, has been isolated from human blood leukocytes and characterized recently. This antimicrobial defensin, whose cell origin is somewhat uncertain, has a net charge of +4 and is substantially less abundant than the other defensins (9, 37).

The nonoxidative mechanisms involved in the killing periodontal pathogens remain to be defined. Certain mech-

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anisms which may play a role in the killing of these bacteria have been investigated. *A. actinomycetemcomitans* Y4 has been shown to be lysed by both chicken egg white lysozyme and human lysozyme isolated from the urine of a subject with chronic monocytic leukemia (16). Lysis required the pretreatment of cells with EDTA and occurred optimally under hypotonic and mildly alkaline conditions. Apolactoferrin killed more than 99% of anaerobically grown *A. actinomycetemcomitans* A7154 cells at concentrations between 40 and 160 μg/ml in an isotonic buffer (pH 5.5) (17). No bacterial growth was observed in the controls.

Defensins, which are believed to play a significant role in the neutralization of pathogens by neutrophils, have antimicrobial effects against fungi, viruses, and nonoral bacteria at concentrations below 100 μg/ml (13, 14, 30, 36). In general, the defensins kill bacteria optimally under low-ionic-strength conditions and at slightly alkaline pHs (13, 19; R. I. Lehrer, A. Barton, K. A. Daher, S. S. L. Harwig, T. Ganz, and M. E. Selsted, unpublished data). We have found that a defensin peptide isolated from rabbit neutrophils (NP-1) is quite potent in killing many of the oral, gram-negative, facultative bacteria under such conditions (Miyasaki, Bodeau, Selsted, Ganz, and Lehrer, Oral Microbiol. Immunol., in press), thus confirming the susceptibility of oral bacteria to the defensin mechanism. The purposes of this study were to determine the microbicidal activity of low-molecular-weight peptides from human neutrophils and to assess the susceptibility of oral, gram-negative, facultative bacteria to the bactericidal effects of defensins HNP-1, HNP-2, and HNP-3 in vitro.

MATERIALS AND METHODS

Bacteria. Bacteria were grown to early log phase (10^9 cells per ml) and subsequently adjusted to a concentration of 10^6 cells per ml in 0.1% (vol/vol) Trypticase soy broth (BBL Microbiology, Cockeysville, Md.) containing 10 mM sodium phosphate (pH 7.0) as described elsewhere (Miyasaki et al., in press). In anaerobic studies, the organisms were grown for 24 h in an anaerobic chamber (Forma Scientific, Marietta, Ohio) containing 85% N_2, 10% H_2, and 5% CO_2. Anaerobiosis in this system was verified by using methylene blue dye indicators, by the growth of fastidious anaerobes, and by the reduction of neutrophil respiratory burst activity 40- to 100-fold (22). In anaerobic studies, bacteria were inoculated into anaerobically preequilibrated Trypticase soy broth, grown to mid-log phase, and inoculated into anaerobically preequilibrated 10 mM sodium phosphate. The bacterial cell concentration was determined turbidometrically (an optical density at 540 nm of 0.1 to 0.3 was equivalent to a concentration of 10^9 cells per ml).

Preparation of fractions F and G. Fresh human neutrophils were purified from granulocyte-enriched, leukocyte concentrates (Hemacare, Sherman Oaks, Calif.), and neutrophil granules were isolated from intact neutrophils as previously described (23). Granules were disrupted by sonication in 0.5% cetyltrimethylammonium bromide–0.2 M NaCl–0.02 M sodium acetate (pH 4.5) at 4°C. The resultant mixture was clarified by centrifugation at 39,000 × *g* for 20 min at 4°C and subjected immediately to gel filtration liquid chromatography over a column (100 by 1.6 cm) containing Sephadex G-100 (Pharmacia-LKB Biotechnologies, Piscataway, N.J.) in 0.2 M NaCl–0.02 M sodium acetate (pH 4.5) by using a flow rate of 12 ml/h. The resultant Sephadex G-100 fractions of interest, designated fractions F and G, were desalted by reversed-phase chromatography, using a PepRPC HR 5/5 column (Pharmacia-LKB Biotechnologies), equilibrated with 0.1% trifluoroacetic acid (TFA) in water, and eluted with 30% acetonitrile containing 0.1% TFA. Fractions F and G were subfractionated on the PepRPC HR 5/5 column by using a linear 0 to 30% acetonitrile gradient in the presence of 0.1% TFA. Samples were concentrated to dryness by SpeedVac vacuum centrifugation (Savant Instruments, Farmingdale, N.Y.).

Preparation of HNP-1, HNP-2, and HNP-3. Methods for purifying defensins have been described previously (13, 29). The least cationic defensin, HNP-3, was separated from HNP-1 and HNP-2 by cation-exchange, high-pressure liquid chromatography on Bio-Sil TSK IEX-535 CM (Bio-Rad Laboratories, Richmond, Calif.) and desalted as described above. HNP-1 and HNP-2 were separated by reversed-phase high-pressure liquid chromatography, using a Vydak C18 column (Separations Group, Hesperia, Calif.) and an acetonitrile gradient. Samples were distributed into 0.5- to 1.0-mg aliquots and concentrated to dryness by SpeedVac vacuum centrifugation.

AU-PAGE. Acid-urea polyacrylamide gel electrophoresis (AU-PAGE) was performed in a Tall Mighty Small system (Hoefer Scientific Instruments, San Francisco, Calif.) by using a modification of the method of Panyim and Chalkley (25). Briefly, 12.5% acrylamide gels (thickness, 0.75 mm) were modified to incorporate 3 M urea and preelectrophoresed in 5% acetic acid at 150 V for 2 h. Samples were dissolved in deionized 6 M urea containing 7% acetic acid, and subsequent electrophoresis was performed at 200 V until the methylene green tracking dye eluted from the bottom of the gel. Gels were stained in 0.125% Coomassie blue R-250 in 40% methanol–15% formaldehyde–5% acetic acid and destained in 25% methanol containing 3.7% formaldehyde, as previously described (13). Electrophoretic transfer to nitrocellulose membranes was accomplished by using 25 mM sodium acetate buffer (pH 4.5). Blots were developed by using polyclonal rabbit anti-human neutrophil defensins and ^{125}I-labeled protein A.

Bactericidal activity. The bactericidal activity assay was performed as described elsewhere (Miyasaki et al., in press). Briefly, bactericidal activity in certain assays was quantified as the $\log_{10}$ reduction in colony-forming units, which was determined with a Spiral Plater (Spiral Systems, Bethesda, Md.), and was calculated by using the following formula: $\delta(\log_{10}\text{killing}) = \log_{10} n_0 - \log_{10} n_t$, where n_0 and n_t were the numbers of bacterial colony-forming units per milliliter at time zero and time t, respectively. The variance of δ was described, where applicable, as a maximum estimate of the standard deviation by using the following formula: $sd_\delta = \log_{10} n_t - \log_{10} (n_t - sd)$, where sd was the standard deviation of the number of colony-forming units per milliliter. All reagents were preequilibrated under anaerobic conditions for at least 2 h for assays involving anaerbiosis. The anaerobic assays were performed in an anaerobic chamber. Anaerobiosis was confirmed by using methylene blue as an indicator.

RESULTS

Initial observations with fractions F and G. The low-molecular-weight peptides eluted from Sephadex G-100 as a bifid peak (designated fractions F and G) (Fig. 1). The primary, Coomassie blue-stainable constituents of fractions F and G were defensins HNP-1, HNP-2, and HNP-3; this was confirmed by using AU-PAGE (Fig. 1, inset) and Western blot analysis (data not shown). Fraction F con-

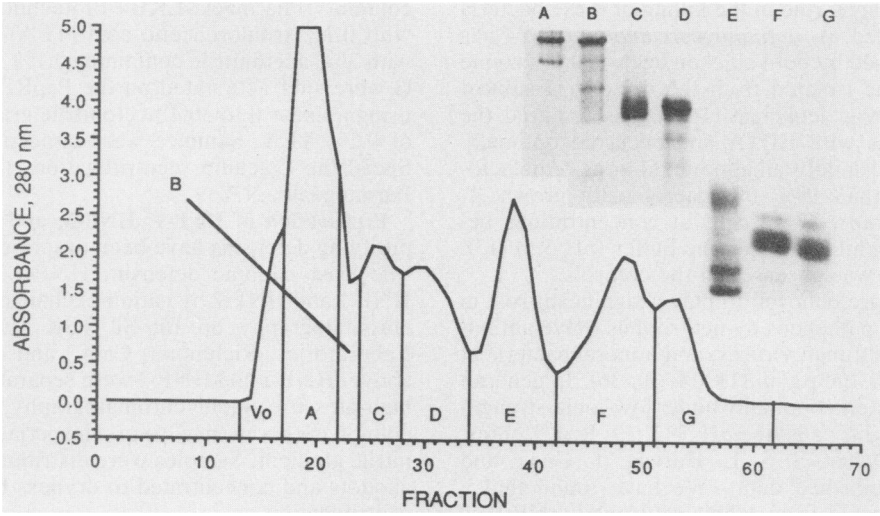

FIG. 1. Chromatogram from Sephadex G-100 column. Seven fractions, labeled A through G, were collected. The AU-PAGE gel (inset) shows the separation of fractions A through G. Peak A and valley B contained mainly myeloperoxidase and lactoferrin, peak C and D contained members of the neutral serine protease family, and fraction E contained lysozyme and low-molecular-weight components. Three isomers of defensins, HNP-1, HNP-2, and HNP-3, were visualized in both fraction F and fraction G. Fractions were loaded at a rate of 5 μg per lane. The components of each fraction have been identified elsewhere.

tained nondefensin components which were not visualized by AU-PAGE and Coomassie blue staining, as evidenced by a relatively low numbers of cysteinyl residues in its amino acid composition. Fraction F appeared to be more enriched for the defensins than fraction G (Fig. 1, inset).

Sensitivity to fractions F and G. Both fraction F and fraction G were bactericidal. Fractions F and G produced 1.5 and 1.3 $\log_{10}$ orders of killing, respectively, under aerobic conditions after 2 h of incubation when they were tested against *Capnocytophaga ochracea* ATCC 27872 at concentrations of 100 μg/ml. The initial viability of the bacterial suspension was 3.9×10^5 to 4.0×10^5 CFU/ml. Fraction F exhibited bactericidal activity against *C. ochracea* ATCC 27872 under both aerobic and anaerobic conditions (Fig. 2). At a concentration of 100 μg/ml, more than 3 $\log_{10}$ orders of killing was observed under anaerobic conditions and more than 2 $\log_{10}$ orders of killing was observed under aerobic conditions after 4 h of incubation. Fraction F did not kill *A. actinomycetemcomitans* ATCC 29523, Y4, or NCTC 9709.

Role of defensins in the bactericidal activity of fraction F. Using a PepRPC H/R 5/5 column, we separated fraction F into eight subfractions (designated subfractions I through VIII) (Fig. 3). The pooled subfractions were dried by vacuum centrifugation and analyzed for bactericidal activity. Only subfractions IV and V exhibited microbicidal activity. Only subfractions IV and V contained the defensin peptides, as determined by AU-PAGE. The AU-PAGE pattern for subfraction IV is shown in Fig. 3 (inset). Thus, almost all of the bactericidal activity of fraction F could be attributed to defensins. Similar results were obtained with fraction G; only the subfractions which contained defensins exhibited bactericidal activity (data not shown).

Sensitivity to purified human defensins. Purified human defensins HNP-1, HNP-2, and HNP-3 exhibited bactericidal effects against *Capnocytophaga* spp. but not against *A. actinomycetemcomitans* or *E. corrodens* in dose-response studies (Fig. 4 and Table 1). The *Capnocytophaga* spp. exhibited approximately 1 to 2 $\log_{10}$ orders of killing after 2 h when they were exposed to 100 to 500 μg of defensin per ml. Rabbit defensin NP-1 was used as a positive control (Table 1). Both HNP-1 and HNP-2 were capable of killing *C. ochracea* ATCC 27872 under anaerobic conditions (Fig. 2). Neither *A. actinomycetemcomitans* nor *E. corrodens* was killed significantly. Purified HNP-1 and HNP-2 killed *C. ochracea* ATCC 27872 at pH values ranging between 6.0 and 8.0 (Fig. 5). Mildly acidic and alkaline conditions (pH 6.0 and 7.6) resulted in slightly greater bacterial killing. Also, fraction F was capable of killing *C. ochracea* ATCC 27872 at pH values between 6.0 and 8.0.

Effect of salts on killing by HNP-1. The effect of salts on the bactericidal activity of HNP-1 against *C. ochracea* ATCC 27872 was investigated by using NaBr, NaCl, and KCl under aerobic conditions for 4 h at 37°C. In general, increasing the

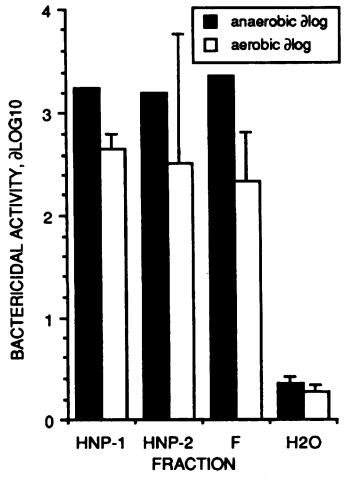

FIG. 2. Killing of *C. ochracea* ATCC 27872 under aerobic and anaerobic conditions by partially purified defensins, fraction F, and purified defensins HNP-1 and HNP-2 (all at a concentration of 100 μg/ml, 37°C, 4 h). The control was defensin solvent (water). The initial levels of viability were 1.7×10^5 to 2.4×10^5 CFU/ml. The vertical bars and vertical lines indicate the means and standard deviations of quadruplicate assays, respectively.

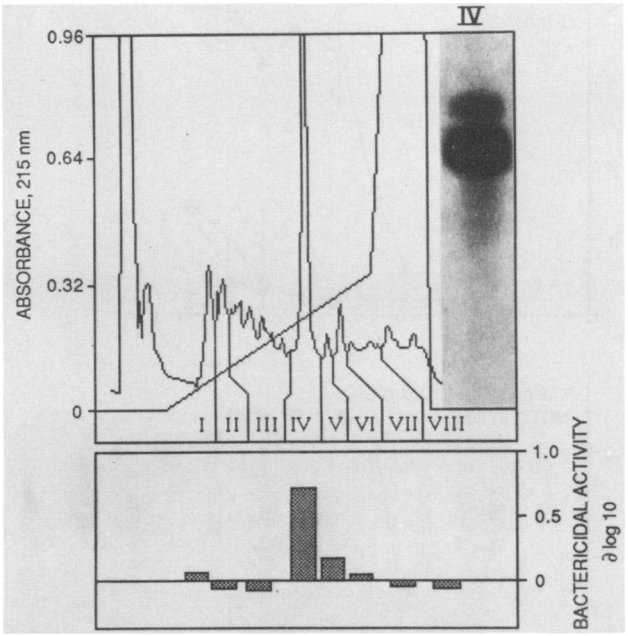

FIG. 3. Reversed-phase separation of fraction F on a PepRPC column. Fraction F (50 μg) was applied to a PepRPC HR 5/5 column and equilibrated in 0.1% TFA. Fractionation was achieved by using a 0 to 30% linear gradient of acetonitrile containing 0.1% TFA. Eight subfractions were collected (subfractions I through VIII) and concentrated to dryness in a SpeedVac apparatus. The subfractions were resuspended in equal volumes (14 μl) of sterile water and tested for microbicidal activity. The bactericidal screening was performed as described in Materials and Methods by using a 2-h incubation against *C. ochracea* ATCC 27872. The initial level of viability was 2.1×10^5 CFU/ml. (Inset) AU-PAGE analysis of fraction IV.

concentration of NaBr and NaCl to values above 20 mM resulted in a reduction in bactericidal activity; there was a 2 $\log_{10}$ order reduction in killing at 140 mM. Nevertheless, because the assay system produced more than 3 $\log_{10}$ orders of killing in the absence of salt supplementation, 90% killing was observed at concentrations of NaBr and NaCl as high as 200 mM. KCl also reduced the level of killing at concentrations above 20 mM, but was toxic itself at concentrations above 100 mM. Salts exerted similar effects on the bactericidal activity of fraction F against *C. ochracea* ATCC 27872. The initial levels of viability of the bacterial suspensions ranged between 1.5×10^5 and 3.9×10^5 CFU/ml.

Bactericidal kinetics. The bactericidal activity of the defensins exhibited simple exponential kinetics, which could be described as follows: $\log_{10}(n_t/n_0) = -kt$, where $k = k(c)$ depended on the defensin concentration (c), t was the time (in seconds), n_t was the colony count (colony-forming units per milliliter) at time t, and n_o was the initial colony count (21). Table 2 shows the killing constants (k) when 50 μg of defensin per ml was incubated with the three *Capnocytophaga* spp. strains and *A. actinomycetemcomitans* ATCC 29523. Of the three strains of *Capnocytophaga* spp., strain ATCC 27872 was killed most rapidly and exhibited the highest k. Organisms resistant to the human defensins, including *A. actinomycetemcomitans* and *E. corrodens*, exhibited k values at an NP-1 concentration of 100 μg/ml that ranged from 7×10^{-5} to 60×10^{-5} s^{-1}.

DISCUSSION

The mechanisms by which neutrophils protect the periodontium against destructive infection by certain bacterial pathogens are only partially understood. We have reported that intact neutrophils can phagocytose and kill oral bacteria, such as *A. actinomycetemcomitans*, by both oxygen-dependent and oxygen-independent mechanisms (22). The oxygen-dependent microbicidal mechanisms of neutrophils have been subjected to intense investigation during the past 20 years, and the nonoxidative antimicrobial mechanisms have been delineated more recently. These antimicrobial effectors include defensins (13, 37), cathepsin G (24), azurocidin (3), p29b (3), bactericidal permeability-increasing protein (20), (apo)lactoferrin (17), and lysozyme (16).

Congenital neutrophil disorders provide certain insights into the relative roles of oxidative and nonoxidative bactericidal mechanisms in controlling periodontopathogens. Disorders such as specific granule deficiency and Chediak-Higashi syndrome predispose individuals to severe periodontal infection (5). Significantly, the neutrophils from subjects with specific granule deficiency lack defensins, and the neutrophils from subjects with Chediak-Higashi syndrome are deficient in members of the neutral serine protease family (11). Therefore, it is plausible that periodontal infection in these individuals is a result of diminished nonoxidative bactericidal capacity of the phagocytes.

In this study, we investigated the bactericidal effects of low-molecular-weight peptides (fractions F and G) and purified from human neutrophils defensins against oral, gram-negative, facultative bacteria. The low-molecular-weight fractions exerted substantial bactericidal activity against *Capnocytophaga* spp. In other studies, we examined killing by higher-molecular-weight fractions A through E. Fraction D, enriched for cathepsin G, was also found to be bactericidal against both *Capnocytophaga* spp. and *A. actinomycetemcomitans*. In perspective then, the defensins are only one of the components involved in the nonoxidative killing of oral microorganisms.

All of the microbicidal activity of low-molecular-weight fractions F and G could be attributed to the defensins. Anaerobic conditions did not significantly alter the bactericidal activity of either fraction F or purified HNP-1 or HNP-2 against *Capnocytophaga* spp., indicating that the bactericidal activity of the defensins against *Capnocytophaga* spp. is both nonoxidative and oxygen independent. This is the first demonstration of defensin-mediated antimicrobial activity under anaerobic conditions. In contrast, the fungicidal activity of human defensins against *Candida albicans* is nonoxidative yet oxygen dependent, because of a requirement for active metabolism by target cell mitochondria (19).

The human defensins were less potent and more selective against the periodontopathogens than rabbit defensin NP-1. Killing of the oral pathogens by the human defensins was slower and required a higher concentration of peptides than killing by NP-1 did (Miyasaki et al., in press). HNP-1 and HNP-2 exerted significant bactericidal activity against *Capnocytophaga* spp. but not against *A. actinomycetemcomitans* or *E. corrodens* in vitro.

Most of our assays were performed at neutral pH; however, the pH of phagolysosomes is known to vary from 5.6 to 7.8 (4, 28). We found that fraction F, as well as purified HNP-1 and HNP-2, is bactericidal against *C. ochracea* ATCC 27872 over a broad pH range. Although the activity of defensins against *Candida albicans* is also expressed over a broad pH range, many bacteria are susceptible to the bacte-

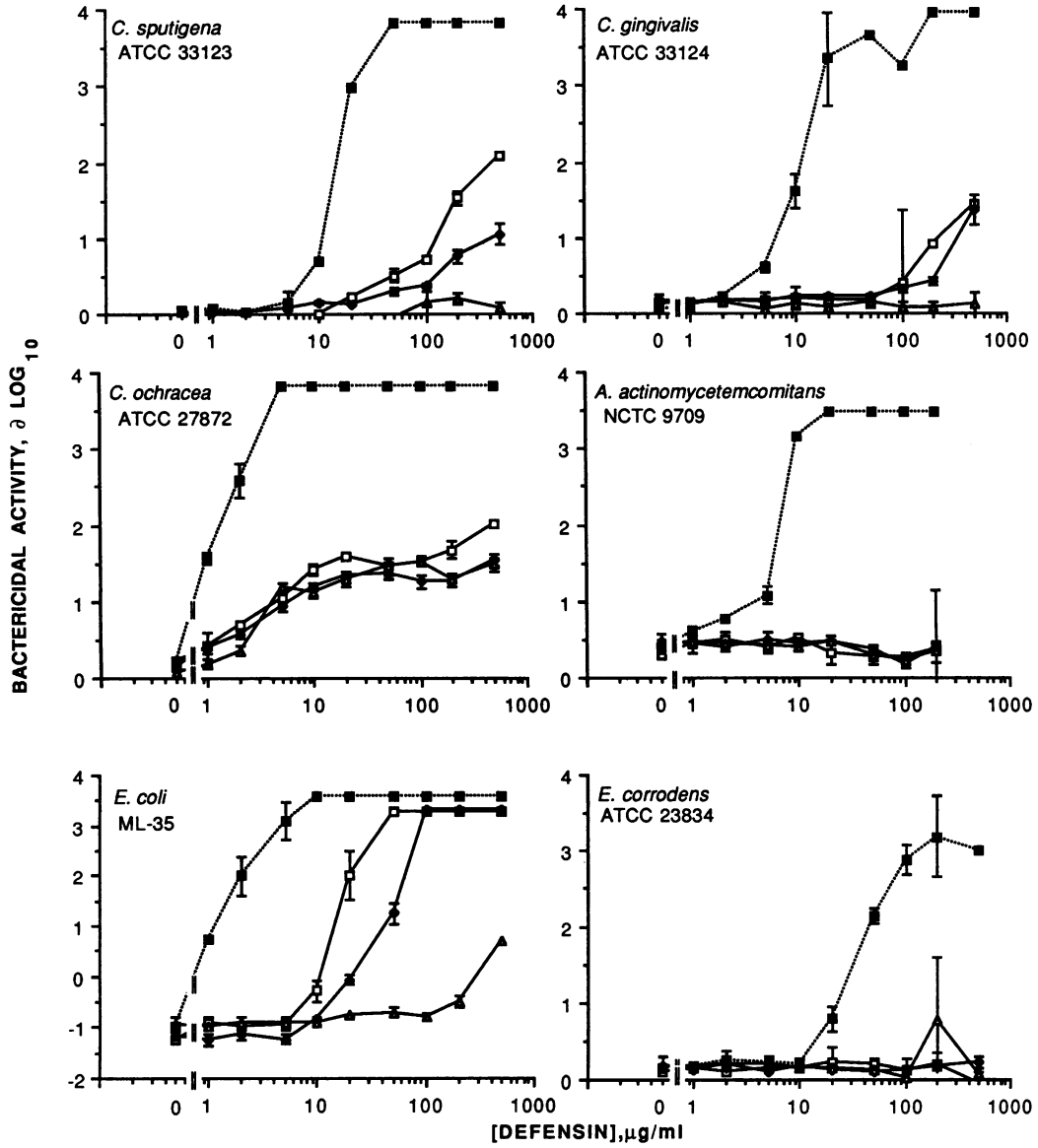

FIG. 4. Effect of defensin concentration on bacterial killing. Killing is expressed as $\delta \log_{10}$. Bacteria were exposed to defensins NP-1 (■), HNP-1 (□), HNP-2 (◆), and HNP-3 (△). The defensin concentration varied from 1 to 500 μg/ml, and incubation was for 2 h at 37°C. The initial levels of viability were 0.5×10^5 to 4.4×10^5 CFU/ml. The points and vertical bars indicate the means and sd_δ values of quadruplicate counts, respectively.

ricidal effects of the defensins exclusively between pH 6.5 and 7.8 (14). Although all three of the human neutrophil defensins possess one amino acid residue which ionizes in these pH ranges (glutamic acid at position 13), the ionization status of this residue does not appear to be sufficient to influence the potency of HNP-1 and HNP-2 against *C. ochracea* ATCC 27872. Moreover, the sensitivities of other bacteria appear to increase with mild alkaline conditions which favor decreased net cationic charge (14), and this observation is most consistent with the hypothesis that the bacterial surface compensates by increasing its net anionic charge.

Some bactericidal activity was blocked by increased tonicity of the suspending milieu; nevertheless, killing was observed even at physiologic salt concentrations. It remains to be determined whether the decrease in killing activity was a result of tonicity, ionic strength, or counterionic strength. The exact ionic composition of phagolysosomes is unknown. Regardless, we concluded that *Capnocytophaga* spp. may be killed by human defensins whether the phagolysosomal tonicity reflects the crevicular fluid salt concentration or the intragingival tissue fluid salt concentration.

In 2-h dose-response assays, HNP-1 and HNP-2 were equally potent, and both were more potent than HNP-3 when they were tested for bactericidal activity against *Capnocytophaga sputigena* ATCC 33123 and *Capnocytophaga gingivalis* ATCC 33124. All of the human defensins appeared to be equipotent for killing *C. ochracea* ATCC 27872. Neither *E. corrodens* (strain ATCC 23834) nor *A. actinomycetemcomitans* (strains ATCC 29523, Y4, NCTC 9709, and GA3-1) exhibited sensitivity to the human defensins at concentrations as high as 500 μg/ml. Catalase-deficient *A.*

TABLE 1. Sensitivity of oral, gram-negative, facultative bacteria to the bactericidal effects of defensins

Species	Strain	Sensitivity to[a]:			
		NP-1	HNP-1	HNP-2	HNP-3
A. actinomycetemcomitans	Y4	2	NS[b]	NS	NS
	GA3-1	20	NS	NS	NS
	653	20	NS	300	NS
	NCTC 9709	6	NS	NS	NS
	ATCC 29523	15	NS	NS	NS
C. sputigena	ATCC 33123	11	200	500	NS
C. gingivalis	ATCC 33124	8	200	500	NS
C. ochracea	ATCC 27872	1	10	10	10
E. corrodens	ATCC 23834	30	NS	NS	NS
Escherichia coli	ML-35	1	10	20	300

[a] Sensitivity is expressed as the 90% lethal dose (1 δ $\log_{10}$) (in microgram per milliliter) after 2 h at 37°C in 10 mM phosphate (pH 7.0).

[b] NS, No significant bactericidal activity was observed at concentrations up to 500 μg/ml.

actinomycetemcomitans strain 653 exhibited slight sensitivity to HNP-1 and HNP-2 at concentrations above 200 μg/ml. The kinetics of the bactericidal reaction of the human peptides against the *Capnocytophaga* spp. were documented. Diminished killing by the human defensins compared with the rabbit peptide resulted from less rapid kinetics, as opposed to alterations in the shape of the kinetic curve. The exponential killing constant, k, was less than 22 $\times 10^{-5}$ s^{-1} at defensin concentrations as high as 500 μg/ml.

We concluded that *Capnocytophaga* spp. are killed by low-molecular-weight peptides from human neutrophils

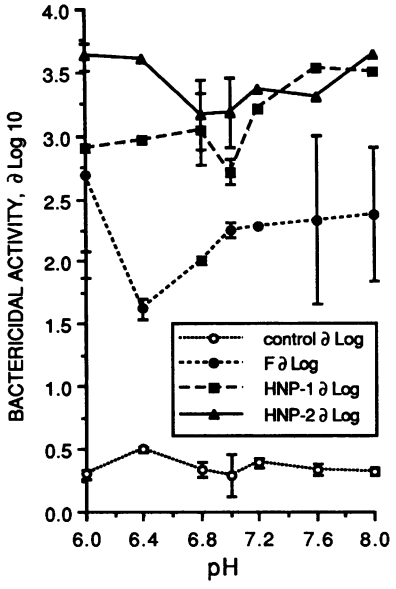

FIG. 5. Effect of pH on the bactericidal activities of HNP-1, HNP-2, and fraction F against *C. ochracea* ATCC 27872. A 10 mM sodium phosphate buffer system was used to control the pH. Bactericidal reaction mixtures were incubated aerobically for 4 h at 37°C. The initial levels of viability for the controls ranged from 1.6 $\times 10^5$ to 5.4 $\times 10^5$ CFU/ml. The initial levels of viability for test suspensions containing HNP-1 and HNP-2 were 2.7 $\times 10^5$ and 4.6 $\times 10^5$ CFU/ml, respectively, at time zero. The initial level of viability for the test suspension containing fraction F was 2.5 $\times 10^5$ CFU/ml. The points and vertical lines indicate the means and standard deviations of quadruplicate assays, respectively.

TABLE 2. Exponential killing constants for human defensin-sensitive *Capnocytophaga* spp. strains ATCC 33123, ATCC 33124, and ATCC 27872 and human defensin-resistant *A. actinomycetemcomitans* strain ATCC 29523

Strain	$k(10^{-5}, s^{-1})$[a]			
	NP-1	HNP-1	HNP-2	HNP-3
Capnocytophaga spp.				
ATCC 33123	62	11	18	10
ATCC 33124	58	10	12	3
ATCC 27872	133	21	20	18
A. actinomycetemcomitans ATCC 29523	15	3	6	7

[a] The exponential killing constants (k) were calculated at an NP-1 concentration of 50 μg/ml and at HNP-1, HNP-2, and HNP-3 concentrations of 500 μg/ml.

(fractions F and G) and that this activity is attributable to the defensins. The defensins kill *Capnocytophaga* spp. under aerobic and anaerobic conditions and over a broad range of both pH and tonicity. Defensins HNP-1 and HNP-2 are more potent than HNP-3 against *C. sputigena* ATCC 33123 and *C. gingivalis* ATCC 33124. All three defensins are equally potent against a more susceptible strain, *C. ochracea* ATCC 27872. In contrast, under the same conditions, *A. actinomycetemcomitans* and *E. corrodens* are not killed. A more cationic member of the defensin family, NP-1, which is present in rabbit granulocytes and certain macrophages, exhibits greater potency and a wider range against this panel of oral pathogens, which is consistent with its behavior in our previous studies with bacteria (Lehrer et al., unpublished data; Miyasaki et al., in press) and fungi (19).

In these studies we did not address either the bacteriostatic effects of the defensins or the effects of the defensins in combination with other neutrophil components; therefore, our data do not exclude the possibility that the defensins play a role in the antimicrobial activity against *A. actinomycetemcomitans* and *E. corrodens*. Our results suggest that the mechanisms involved in the killing of different periodontal bacteria by neutrophils can vary from genus to genus and species to species. Further definition of the specific mechanisms by which neutrophils control specific periodontopathogens could enhance our ability to predict, prevent, and treat specific periodontal infections. Additional experimentation will be required to assess the bacteriostatic capacity of the human neutrophil defensins and their ability to synergize with other components or metabolic products of neutrophils against oral pathogens.

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