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Differential Killing of *Actinobacillus actinomycetemcomitans* and *Capnocytophaga* spp. by Human Neutrophil Granule Components

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The purpose of this study was to determine whether granule fractions of human neutrophils differentially kill *Actinobacillus actinomycetemcomitans* and *Capnocytophaga* spp. Granule extracts were subjected to gel filtration, and seven fractions (designated A through G) were obtained. Under aerobic conditions at pH 7.0, representative strains of *A. actinomycetemcomitans* were killed by fraction D and variably by fraction B. In contrast, the *Capnocytophaga* spp. were killed by fractions C, D, F, and G. Fractions A (containing lactoferrin and myeloperoxidase) and E (containing lysozyme) exerted little bactericidal activity under these conditions. Anaerobiosis had little effect on the bactericidal activity of fractions D and F but inhibited that of fractions B and C. Electrophoresis, zymography, determination of amino acid composition, and N-terminal sequence analysis revealed that fraction C contained elastase, proteinase 3, and azurocidin. Fraction D contained lysozyme, elastase, and cathepsin G. Subfractions of C and D containing elastase (subfraction C4), a mixture of elastase and azurocidin (subfraction C5), and cathepsin G (subfraction D9) were found to be bactericidal. The bactericidal effects of fraction D and subfraction D9 against *A. actinomycetemcomitans* was not inhibited by heat inactivation, phenylmethylsulfonyl fluoride, or *N*-benzyloxycarbonylglycylleucylphenylalanylchloromethyl ketone. We conclude that (i) *A. actinomycetemcomitans* and *Capnocytophaga* spp. were sensitive to the bactericidal effects of different neutrophil granule components, (ii) both were sensitive to the bactericidal effects of neutral serine proteases, and (iii) the killing of *A. actinomycetemcomitans* by cathepsin G-containing fractions was independent of oxygen and neutral serine protease activity.

Severe forms of periodontal disease, such as localized juvenile and rapidly progressive adult periodontitis, have been associated with infections by the gram-negative facultative coccobacillus *Actinobacillus actinomycetemcomitans* and the gram-negative gliding capnophiles *Capnocytophaga* spp. (26, 34). Significantly, both juvenile and rapidly progressive adult periodontitis have been associated with either intrinsically or extrinsically derived neutrophil defects (12, 26, 31). These observations suggest an important role for neutrophils in controlling these organisms within the periodontium.

Neutrophils can kill *A. actinomycetemcomitans* by both oxidative and nonoxidative mechanisms (19). Nonoxidative mechanisms play a prominent role in the killing of *Capnocytophaga* spp. (30). Although dioxygen tensions of the periodontal pocket are sufficient to support respiratory burst activity of neutrophils at about 75% of levels observed under normoxic conditions (6, 15), the oxidative burst capacity of crevicular neutrophils in periodontitis is diminished (16). Further, Chediak-Higashi syndrome and specific granule deficiency, but not chronic granulomatous disease, have been associated with severe periodontal complications (4). Among other structural and functional defects, neutrophils from subjects with Chediak-Higashi syndrome and specific granule deficiency are deficient in members of the neutral serine protease (NSP) family and defensins, respectively, which are components of nonoxidative bactericidal systems (7). Therefore, nonoxidative killing mechanisms may be of great importance in protecting the host against periodontal disease.

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The human defensins are low-molecular-weight peptides that have demonstrated potent bactericidal effects against *Capnocytophaga* spp. but not *A. actinomycetemcomitans* (8, 18). Four members of the NSP family have been identified in neutrophils; cathepsin G, leukocyte elastase, p29b (and related or identical molecules AGP7, proteinase 3, p29, and ANCA-c), and the enzymatically inert azurocidin (and the related or identical molecule CAP 37) (3, 10, 20, 21, 25, 33). The bactericidal effects of the NSP against periodontal bacteria has not been fully examined; uniquely, cathepsin G kills *Capnocytophaga sputigena* ATCC 33123 in an enzyme-dependent fashion (17). The purpose of this study was to compare the sensitivity of *A. actinomycetemcomitans* and *Capnocytophaga* spp. to human neutrophil granule fractions under aerobic and anaerobic conditions and to identify some of the potential bactericidal components against oral pathogens.

MATERIALS AND METHODS

Preparation of neutrophil granule fractions. Purified human defensins (HNP-1, HNP-2, and HNP-3) and fresh human neutrophils, granules, and granule extracts were prepared as previously described (8, 17, 18). Crude granule extracts were separated by gel filtration with Sephadex G-100 (Pharmacia-LKB Biotechnology, Piscataway, N.J.) and pooled into seven fractions, A through G (Fig. 1A). Fractions A through E were desalted by dialysis against 10^9 volumes of distilled water at 4°C. Fractions F and G were desalted by reversed-phase chromatography (18). All samples were concentrated to dryness with a SpeedVac dryer (Savant Instruments, Inc., Farmingdale, N.Y.). Protein and peptide concentrations were determined by the dye-binding method (2) with bovine

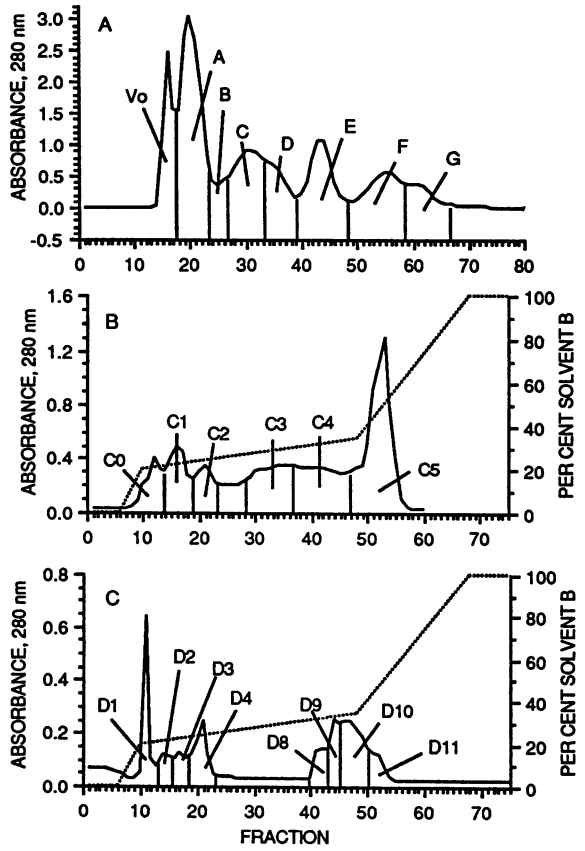

FIG. 1. (A) Chromatographic profile of the A_{280} of CETAB-solubilized crude granule extract separated on Sephadex G-100. Approximately 10^{10} cell equivalents were loaded. The identifiable component of each fraction is indicated. (B) Chromatographic separation of subfractions C4 and C5 by fast protein liquid chromatography on a Mono-S HR 5/5 column. The column was equilibrated in 0.02 M sodium acetate (pH 4.5). Proteins were eluted with 0.02 M sodium acetate–2.0 M NaCl (pH 4.5). (C) Separation of subfraction D9 with a Mono-S column equilibrated in 0.2 M NaCl–0.02 M sodium acetate (pH 4.5) and eluted with 0.02 M sodium acetate–2.0 M NaCl (pH 4.5).

serum albumin as a standard. Fractions C and D were separated into subfractions by fast protein liquid chromatography with a Mono-S HR 5/5 column (Pharmacia-LKB) (Fig. 1B and C).

Microenzyme assays. Elastase, cathepsin G, and lysozyme activities were assayed in microtiter format by using *N*-benzyloxycarbonyl-L-alanyl-2-naphthyl ester (Sigma Chemical Co., St. Louis, Mo.), *N*-benzoyl-DL-phenylalanyl-2-naphthyl ester (Sigma), and *Micrococcus leisodeikticus* (Sigma), respectively, as substrates (17, 28).

CAT-PAGE. Cationic polyacrylamide gel electrophoresis (CAT-PAGE) was performed at pH 5.2 with a 20% acrylamide resolving gel and a 3% acrylamide photopolymerized sample gel, both containing 0.5% Triton X-100 and 0.01% digitonin (Sigma) (29). Samples were dissolved in water and mixed 1:1 with 2× sample gel buffer containing 20% glycerol (29). Electrophoresis was performed in slabs measuring 0.75 by 120 by 95 mm (Tall Mighty Small; Hoefer Scientific Instruments, San Francisco, Calif.) with reversed polarity for 6 to 8 h at 10 mA of constant current per slab. Gels were stained in 0.125% Coomassie blue R-250 in 50% methanol–10% acetic acid and destained in 50% methanol–10% acetic acid.

Zymography. Slabs were preincubated for 10 min in 0.1 M sodium phosphate (pH 7.0). Thirty milligrams of substrate, including naphthol AS-D acetate (Sigma), *N*-acetyl-DL-phenylalanine-β-naphthyl ester (Sigma), or α-naphthyl acetate (Sigma), was dissolved in 3 ml of *N*,*N*-dimethylformamide (Sigma). Twenty milligrams of fast blue RR (Sigma) was dissolved in 2 ml of *N*,*N*-dimethylformamide. The slabs were submerged in 60 ml of 0.1 M phosphate buffer containing 20% *N*,*N*-dimethylformamide, and the substrates and fast blue RR were added and mixed rapidly. The gels were incubated, with rocking, until defined bands appeared. The reaction was terminated in 7% acetic acid (5).

Electroelution, SDS-PAGE, and transfer to PVDF membranes. Samples were inactivated by treatment with 10 μg of phenylmethylsulfonyl fluoride (Sigma) per ml in 10 mM sodium phosphate (pH 7.0), dialyzed against 10^9 volumes of distilled water, and concentrated to dryness with a SpeedVac. CAT-PAGE was performed, and Coomassie blue-stained bands were excised with a razor blade. Gel segments were presoaked for 3 h in 2.1% sodium dodecyl sulfate (SDS)–20 mM ammonium bicarbonate. The gels were electroeluted in 0.1% SDS–20 mM ammonium bicarbonate with a model 1750 electroelution tank (ISCO Inc., Lincoln, Neb.) at 10 mA for 3 h. The electroeluted protein was dialyzed against 10^9 volumes of distilled water and concentrated to dryness with a SpeedVac. Samples were resuspended in SDS-PAGE sample buffer, boiled for 3 min, and electrophoresed with a 12% acrylamide resolving gel at 100 V for 75 min in slabs measuring 0.75 by 80 by 95 mm by the method of Laemmli (11). Gels were presoaked for 5 min in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid–10% methanol (pH 11.0) (CAPS buffer). Electrophoretic transfers to polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore Corp., Bedford, Mass.) were performed for 45 min at 500 mA in CAPS buffer (13). Blots were stained for 5 min with 0.1% Coomassie blue R-250 in 50% methanol and destained in 50% methanol–10% acetic acid.

Amino acid composition and N-terminal amino acid analysis. Amino acid composition and N-terminal sequences were determined from the aqueous phase or from PVDF membrane blots, as specified in Results. The amino acid composition was analyzed with an automated amino acid analyzer (Beckman Instrument Co., Fullerton, Calif.). N-terminal amino acid analysis was performed using an automated protein sequencer (Porton Instruments, Inc., Tarzana, Calif.).

Bactericidal assay. Mid-log-phase bacteria prepared as previously described (18) were incubated with neutrophil granule fractions and subfractions at 37°C (maintained in a temperature block) in 10 mM sodium phosphate containing 1% (vol/vol) tryptic soy broth. The pH was adjusted (as specified in Results) by varying monobasic and dibasic sodium phosphate proportions. Anaerobic assays were modified such that all reagents and cells were equilibrated anaerobically and the bactericidal reaction was under an atmosphere containing 85% N_2, 10% H_2, and 5% CO_2 (18). Inhibitors, including phenylmethylsulfonyl fluoride, *N*-benzyloxycarbonylglycylleucylphenylalanylchloromethyl ketone (ZGLPCK; Enzyme System Products, Dublin, Calif.), and methoxysuccinylalanylalanylprolylvalylchloromethyl ketone (MSAAPVCK; Sigma), were dissolved in dimethyl sulfoxide, diluted in 10 mM sodium phosphate (pH 7.0), and preincubated with granule fractions for 10 min to 2 h, based upon their $k_{obsd}/[I]$ (22). Both aerobic and anaerobic reac-

TABLE 1. Killing of *A. actinomycetemcomitans* and *Capnocytophaga* spp. by Sephadex G-100 fractions prepared from neutrophil granules[a]

Organism	Killing by the indicated fraction											
	Aerobic						Anaerobic					
	A	B	C	D	E	F/G or HNP	A	B	C	D	E	F/G or HNP
A. actinomycetemcomitans												
ATCC 29523	−	+	−	±	−	−	−	−	−	++	−	ND
Y4	−	++	±	++	−	−	−	−	−	++	−	ND
NCTC 9709	−	−	−	+	−	−	−	−	−	+	−	ND
Capnocytophaga spp.												
ATCC 33123	−	−	+++	++++	−	+	−	−	±	+++	−	ND
ATCC 33124	−	−	+	+++	−	+	−	−	−	++	−	ND
ATCC 27872	−	−	++	++++	−	++	−	−	−	++	−	+++
E. coli ML-35	−	−	−	+++	−	++++	−	−	−	++	−	−

[a] Killing was determined at 100 μg of protein per ml after 2 h of 37°C and pH 7.0. Symbols represents the mean values determined in quadruplicate assay: each + represents 1 order of magnitude of reduction in CFU per milliliter. −, no killing; ±, variable or equivocal killing. ND, not determined.

tions were terminated by 1:10 dilution in 10 mM sodium phosphate (pH 7.0). The resultant suspension was spread on clarified, laked blood agar plates with a Spiral plater (Spiral Systems, Bethesda, Md.). After 48 to 72 h of incubation, the CFU were enumerated. The viability of control cultures at the initiation of the bactericidal reaction was 5×10^5 to 10×10^5 CFU/ml. Bactericidal activity was quantified as the $\log_{10}$ reduction in CFU, $\delta[\log_{10}$ (CFU/ml)] (17, 18).

RESULTS

Susceptibility to crude granule extract. *A. actinomycetemcomitans* ATCC 29523 was used to examine the bactericidal potency of the crude granule extract. A portion of the crude granule extract was desalted by gel filtration with a prepacked column containing Sephadex G-25 (Pharmacia-LKB). *A. actinomycetemcomitans* ATCC 29523 exhibited a $\log_{10}$ CFU reduction of 3.8 when exposed to the desalted crude granule extract (100 μg of protein per ml, 2 h, 37°C, pH 7.0). No killing was observed when the crude extract was not desalted. Controls such as water and buffer exerted no bactericidal effects.

Susceptibility to the G-100 fractions under aerobic conditions. The bactericidal activities of seven fractions, A through G, were measured against strains of *A. actinomycetemcomitans* that were representative of serotypes a, b, and c (ATCC 29523, Y4, and NCTC 9709, respectively) and representative strains of *Capnocytophaga* spp. by incubation with bacteria for 2 h at 100 μg of protein per ml at pH 7.0 and 37°C (Table 1). *A. actinomycetemcomitans* exhibited sensitivity primarily to fraction D, which produced a reduction in CFU of between 0.8 and 2.1 orders of magnitude in 2 h. Controls also showed some loss of viability ($\log_{10}$ CFU decrease = 0.06 to 0.63), and the net bactericidal effect attributable to fraction D was between 0.6 and 1.5 orders of magnitude. Fraction C exerted significant bactericidal effects against *A. actinomycetemcomitans* Y4 (net, 1 order of magnitude) but not against ATCC 29523 or NCTC 9709. Fraction B (valley between fractions A and C) killed *A. actinomycetemcomitans* and ATCC 29523 and Y4 but not NCTC 9709. Of the three strains tested, *A. actinomycetemcomitans* Y4 was the most susceptible to the bactericidal activity of fraction D; however, controls also exhibited the highest loss of viability. In contrast to the results with *Capnocytophaga* spp., neither fraction F nor G, nor purified human defensin HNP-1, HNP-2, or HNP-3 exerted significant bactericidal activity against strains of *A. actinomycetemcomitans* under these conditions.

Susceptibility to the G-100 fractions under anaerobic conditions. In general, anaerobiosis reduced the sensitivity to bactericidal activity in all fractions. However, anaerobiosis did not affect the killing of *A. actinomycetemcomitans* by fraction D (100 μg/ml) (Table 1), which reduced the number of CFU per milliliter by 1 to 2.8 orders of magnitude in 2 h under anaerobic conditions. Anaerobiosis suppressed killing by fractions B and C (100 μg/ml). *Capnocytophaga* spp. exhibited substantial death in the presence of fraction D under anaerobic conditions, although killing was somewhat diminished in comparison with killing under aerobic conditions.

Characterization of fraction C and D by zymography. CAT-PAGE and zymography were used to characterize the NSP content of fractions C and D (5). Coomassie blue stain patterns revealed about 7 to 9 bands in fraction D; in comparison, 8 to 10 bands were detected in fraction C (Fig. 2, gel 1). Cytochrome *c* was added as a tracer that migrated ahead of the NSP. Elastase was visualized by its interaction with both naphthol AS-D acetate and α-naphthyl acetate and appeared as four bands present at high concentrations in fraction C and lower proportions in fraction D (Fig. 2, gels 2 and 4). Proteinase 3, in fraction C, was identified as a slowly migrating band that was strongly reactive with α-naphthyl acetate (Fig. 2, gel 4). The chymotrypsin substrate *N*-acetyl-DL-phenylalanine-β-naphthyl ester intensely stained four bands in fraction D, indicative of cathepsin G isomers (Fig. 2, gel 3). Azurocidin, which lacks enzymatic activity, was not identified with these methods.

Concentration dependency of the G-100 fractions. We tested the effect of concentration on the bactericidal activity of fractions A through E. Killing by fractions B, C, and D was observed at pH 7.0. *A. actinomycetemcomitans* Y4 was more sensitive to the bactericidal effects of B and D than was *A. actinomycetemcomitans* ATCC 29523. Both strains exhibited significant death in the presence of fractions B and D at 50 μg/ml and virtually no death in the presence of the same fractions at 10 μg/ml (Fig. 3).

Inactivation of fraction D. Treatment with phenylmethylsulfonyl fluoride (20 μg/ml) or fraction D heated to 90°C for 10 min resulted in a 95.6 to 100% reduction in enzymatic

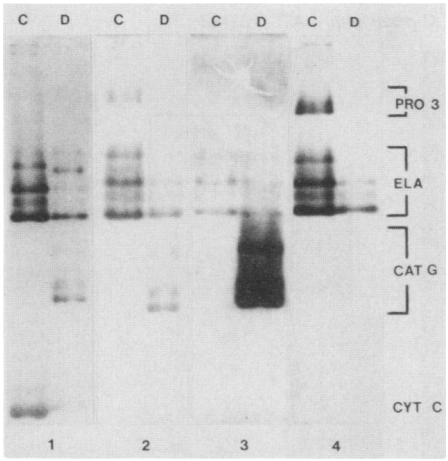

FIG. 2. CAT-PAGE and zymographic identification of components of fractions C and D. Gel 1 was stained with Coomassie brilliant blue R-250, gel 2 was stained with naphthol AS-D acetate, gel 3 was stained with *N*-acetyl-DL-phenylalanine-β-naphthyl ester, and gel 4 was stained with α-naphthyl acetate. Lanes labeled C and D contained 5 μg of G-100 fractions C and D, respectively.

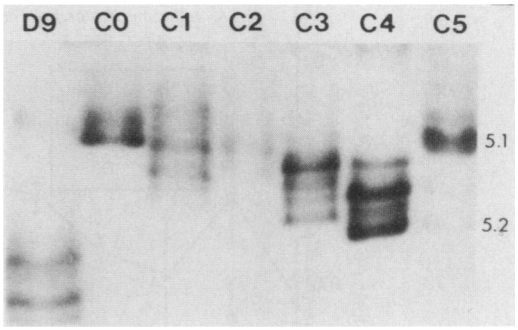

FIG. 4. CAT-PAGE analysis of Mono-S subfractions of fraction C. C0 represents the first fraction eluted by the NaCl gradient, and C5 represents the last. The bands designated C5.1 and C5.2 were excised and subjected to electroelution, SDS-PAGE, PVDF blots, and N-terminal sequence analysis. Band C5.1 was identified as azurocidin.

activity yet did not inhibit killing of *A. actinomycetemcomitans*. Specific inhibitors of cathepsin G and elastase (ZGLPCK and MSAAPVCK, respectively) also had very little effect alone or in combination.

Characterizing bactericidal subfractions of fraction C. Subfractions C0 and C5 were the least and most cationic, respectively, as assessed by elution from Mono-S columns. Predictably, subfractions C0 through C4 exhibited increasingly greater mobility when analyzed by CAT-PAGE (Fig. 4). The major component of subfraction C5 migrated more slowly than expected and comigrated with the major band in subfraction C0. Only subfractions C4 and C5 exhibited substantial bactericidal activity against *C. sputigena* ATCC 33123 (data not shown). The amino acid compositions of subfractions C4 and C5 were consistent with that of elastase, although subfraction C5 exhibited relatively higher proportions of glutamate or glutamine and arginine, consistent with CAP 37 (azurocidin) (25, 27). The N-terminal amino acid sequence of subfraction C4 was indicative of elastase (IVGGR RARPH A-PFM VS) (27). Subfraction C5 was further characterized by electroelution of the main bands from CAT-PAGE, electrophoresis in SDS-PAGE, electrophoretic transfer to PVDF, and N-terminal sequence determination. Subfraction C5 exhibited multiple bands on CAT-PAGE, including a slower-migrating major band designated C5.1 and a more rapidly migrating band designated C5.2 (Fig. 4). Band C5.1 exhibited a molecular mass of 32 kDa (by SDS-PAGE) and gave a sequence (IVGGR KARPR QFPFL ASIQN) that was identical to that reported for azurocidin (33). Band C5.2 (IVGGR–ARPH AFPFM VSLTQ) exhibited an elastaselike sequence for the first 11 positions and was identical to the major elastase glycoisomer in subfraction C4 by CAT-PAGE criteria.

Characterizing bactericidal subfractions of fractions D. The subfractions of fraction D (Fig. 1C) have been characterized

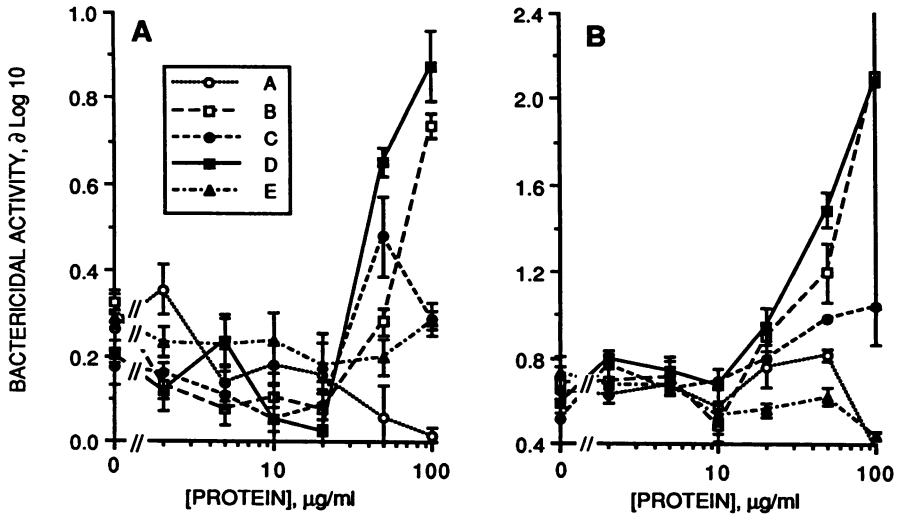

FIG. 3. Effect of concentration on bactericidal activity against *A. actinomycetemcomitans* ATCC 29523 (A) and *A. actinomycetemcomitans* Y4 (B) of G-100 fractions A through E. Fractions B and D exerted the greatest bactericidal activity. Points represent the mean $\delta[\log_{10}(\text{CFU/ml})]$ of quadruplicate assay, and vertical lines represent the maximum standard deviations.

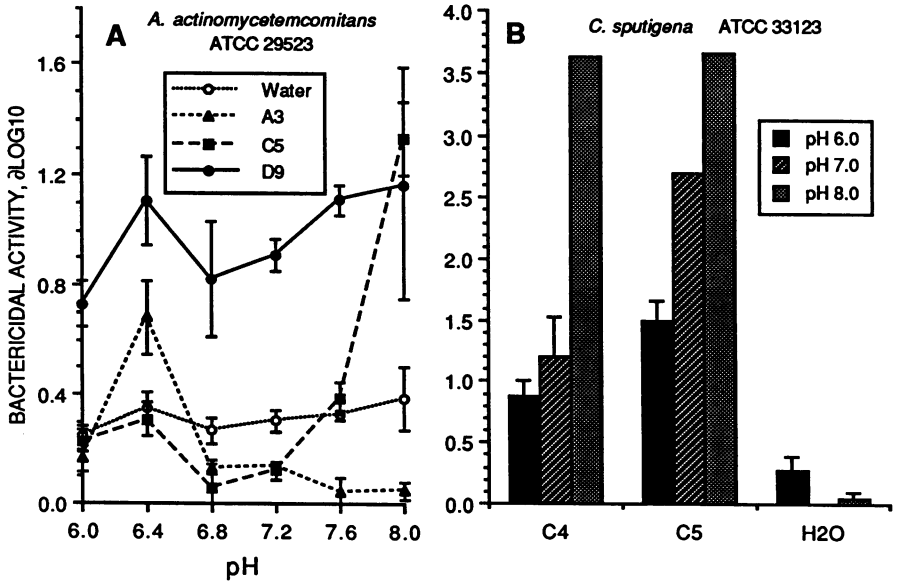

FIG. 5. (A) Killing of *A. actinomycetemcomitans* ATCC 29523 by subfractions C5 and D9. Subfraction A3 was a relatively cationic subfraction of G-100 fraction A and served as a control. Enhanced killing by subfraction C5 was observed at alkaline pH. Samples were tested at 100 μg/ml for 2 h at 37°C. Points represent the mean $\delta\log_{10}$ of quadruplicate assays, and vertical lines represent the maximum standard deviations. (B) Killing of *C. sputigena* ATCC 33123 by subfractions C4 and C5 at pH 6.0, 7.0, and 8.0. Samples were tested at 100 μg/ml for 2 h at 37°C. Vertical bars represent the mean $\delta\log_{10}$ of quadruplicate assays, and vertical lines represent the maximum standard deviations.

elsewhere (17). Briefly, subfraction D1 contained lysozyme. Subfractions D3 and D4 contained elastolytic activity and exhibited amino acid compositions and N-terminal sequences consistent with elastase. Subfractions D8 through D11 contained chymotryptic activity and exhibited amino acid compositions consistent with cathepsin G. Subfraction D9 exhibited an amino acid composition and N-terminal sequence (IIGGR ESRPH SRPYM AYLQI) consistent with that of cathepsin G inferred from cDNA sequences (17, 23). The bactericidal activity of fraction D was confined to subfractions D8 through D11 at pH 7.0 (17).

Killing by subfractions C4, C5, and D9. Although fraction C (100 μg/ml) was not bactericidal against *A. actinomycetemcomitans* at pH 7.0, subfraction C5 exerted intense killing of *A. actinomycetemcomitans* ATCC 29523 at pH 7.6 and above (Fig. 5A). In contrast, killing of *A. actinomycetemcomitans* by subfraction D9 was observed at all pHs. Anaerobic conditions impaired the ability of subfraction C5 to kill *A. actinomycetemcomitans* ATCC 29523. Under aerobic conditions (pH 8.0) in the presence of subfraction C5 (100 μg/ml), *A. actinomycetemcomitans* ATCC 29523 exhibited a $\delta[\log_{10}(\text{CFU/ml})]$ of 3.38 ± 0.00 after 2 h. Under anaerobic conditions, a $\delta[\log_{10}(\text{CFU/ml})]$ of 0.45 ± 0.19 was observed. Subfractions C4 and C5 exerted equivocal (slight or variable) killing against other strains of *A. actinomycetemcomitans* at pH 8.0 (data not shown). In contrast, subfractions C4 and C5 exhibited brisk killing against *C. sputigena* ATCC 33123 which was substantially enhanced by alkaline conditions (Fig. 5B).

Enzyme-independent killing by subfraction D9. We reported previously that subfraction D9 (relatively pure cathepsin G) required enzymatic activity to kill *C. sputigena* ATCC 33123 (17). As such, we were curious as to whether subfraction D9 also killed *A. actinomycetemcomitans* in an enzyme-dependent manner. We tested subfraction D9 against *A. actinomycetemcomitans* FDC Y4, which showed the greatest sensitivity to fraction D. In contrast to the results with *C. sputigena* ATCC 33123, enzymatic activity was not required for killing *A. actinomycetemcomitans*; neither phenylmethylsulfonyl fluoride nor the specific inhibitor of cathepsin G, ZGLPCK, exerted any inhibitory effect against killing (Fig. 6).

DISCUSSION

The precise mechanisms involved in the control of different periodontal bacteria can vary from genus to genus, from species to species, and from strain to strain. To begin to understand the oxygen-independent mechanisms whereby neutrophils kill periodontopathic bacteria, we prepared a crude granule extract from human neutrophils. We found that this extract could kill *A. actinomycetemcomitans* ATCC 29523. Desalting was required for killing, indicating that low-molecular-weight solutes interfered with the killing of *A. actinomycetemcomitans*. Also, the fact that killing occurred after desalting indicated that low-molecular-weight peptides (such as the defensins) were unnecessary for the nonoxidative killing of *A. actinomycetemcomitans* by neutrophil granule components.

We fractionated the crude granule extract by gel filtration with Sephadex G-100. We pooled seven peak or valley fractions (fractions A through G) based upon UV absorbance. The seven fractions obtained were tested for bactericidal activity against three strains of *A. actinomycetemcomitans* (representative of the three known serotypes) under both aerobic and anaerobic conditions. We tested killing in the presence and absence of dioxygen because nonoxidative mechanisms (those that do not involve the reduction of dioxygen) may be oxygen dependent (require aerobic conditions to kill target microorganisms) (14, 32). This is an

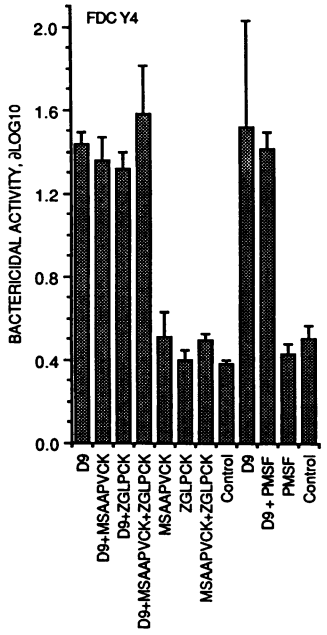

FIG. 6. Effect of ZGLPCK (5 μg/ml), MSAAVPCK (5 μg/ml), and PMSF (10 μg/ml) on the killing of *A. actinomycetemcomitans* Y4 by subfraction D9 (100 μg/ml) after 2 h at 37°C. Between 95.6 and 100% enzyme inhibition was obtained with these methods. Vertical bars represent the mean $\delta\log_{10}$ of quadruplicate assays, and vertical lines represent the standard deviations.

important consideration if the mechanism is to function within a hypoxic gingival crevice (15).

We did not observe killing of either *A. actinomycetemcomitans* or *Capnocytophaga* spp. by fraction A, which contained lactoferrin and myeloperoxidase. Lactoferrin has been shown to be bactericidal against *A. actinomycetemcomitans*, and one explanation for the lack of killing by fraction A is that the iron was not eliminated with chelating agents (9). Additionally, much of the protein in fraction A was not lactoferrin. Fraction B was bactericidal against *A. actinomycetemcomitans* under aerobic conditions but not under anaerobic conditions. Further, fraction B did not kill *Capnocytophaga* spp. to any appreciable extent. Although fraction B contained lactoferrin and myeloperoxidase, this fraction has been described as containing the Vab protein and CAP 37 (25). CAP 37 exhibits complete N-terminal amino acid sequence homology with azurocidin (3). Although it is possible that CAP 37-azurocidin may be responsible for the bactericidal activity of fraction B, the exact mechanism of killing by fraction B remains to be resolved.

Fraction C contained proteinase 3 (as determined with CAT-PAGE zymography), elastase (as determined with Cat-PAGE zymography and N-terminal sequence determinations), and azurocidin (as determined from N-terminal sequences). Azurocidin was more cationic than proteinase 3 and elastase based upon its elution from Mono-S columns; however, it migrated more slowly than elastase (essentially comigrating with proteinase 3) in CAT-PAGE. SDS-PAGE analysis (data not shown) revealed that this molecule was larger than the predominant isomer of elastase (greater than 32 kDa), consistent with the behavior and sequence of CAP 37 (that is, it is most likely a large glycoisomer of azurocidin). Whereas fraction C exhibited minimal bactericidal activity against *A. actinomycetemcomitans*, it was potent against *Capnocytophaga* spp. under aerobic conditions; no bactericidal activity was apparent under anaerobiosis. The ability of fraction B to kill *A. actinomycetemcomitans* but not *Capnocytophaga* spp. and the ability of fraction C to kill *Capnocytophaga* spp. but not *A. actinomycetemcomitans* suggest that neutrophils utilize different mechanisms to kill different bacteria. Differential killing may be dependent upon environmental factors such as pH. Hence, despite the inability of fraction C to kill *A. actinomycetemcomitans*, the azurocidin-enriched subfraction C5 was bactericidal above pH 7.6. Azurocidin has been reported to exert optimal killing at mildly acidic pH (3); therefore, it is plausible that azurocidin in the presence of another neutral serine protease (but not azurocidin alone) may play a role in the early phagolysosomal killing of *A. actinomycetemcomitans* at alkaline pH. Further studies will be required to ascertain the microbicidal activities of the individual components of fraction C and the interactions among them. Studies are ongoing to determine the bactericidal activity of purified azurocidin against periodontal bacteria.

Fraction D exhibited the greatest level and spectrum of bactericidal activities of the Sephadex G-100 fractions. Fraction D killed both *A. actinomycetemcomitans* and *Capnocytophaga* spp. CAT-PAGE zymography demonstrated that fraction D consisted primarily of cathepsin G and elastase. Importantly, only fraction D killed *A. actinomycetemcomitans* and *Capnocytophaga* spp. under anaerobic conditions.

Separation of fraction D by Mono-S cation exchange revealed that bactericidal activity was associated with subfractions containing chymotrypsinlike activity. Subfraction D9, which was enriched in cathepsin G (as assessed by amino acid composition and N-terminal sequence determination), was bactericidal against both *A. actinomycetemcomitans* and *Capnocytophaga* spp. The killing of *A. actinomycetemcomitans* by fraction D and subfraction D9 was not inhibited by serine protease inhibitors or heat inactivation. This finding is consistent with other studies, which have demonstrated that the ability of cathepsin G to kill nonoral bacteria does not depend upon an intact enzyme active site (21). In fact, killing by cathepsin G has been attributed to two small peptide fragments (1). In contrast to the enzyme-independent killing of *A. actinomycetemcomitans*, the killing of *C. sputigena* by the cathepsin G-containing fraction D is dependent upon enzymatic activity (17).

Fraction E contained lysozyme and other peptides. The bactericidal activity of fraction E against *A. actinomycetemcomitans* and *Capnocytophaga* spp. was negligible either aerobically or anaerobically. The defensin-containing fractions F and G did not exert any bactericidal activity against *A. actinomycetemcomitans* but killed *Capnocytophaga* spp. We have reported elsewhere that the killing of *Capnocytophaga* spp. by fractions F and G is defensin mediated and that *A. actinomycetemcomitans* is resistant to defensin-mediated killing at defensin concentrations as high as 500 μg/ml (18).

We conclude that neutrophils use different molecules to kill different periodontal bacteria. Certain neutrophil granule fractions (i.e., fractions B and C and the defensins) killed either *A. actinomycetemcomitans* or *Capnocytophaga* spp. but not both. These findings support the concept that granule components may exert selective bactericidal activity (24). It is plausible that the composition of the periodontal micro-

flora may be determined by pleomorphic expression or delivery of certain neutrophil granule components. The identity of all the bactericidal components in these fractions remains to be determined, but fraction C contained a mixture of proteinase 3/p29b, azurocidin, and elastase, all of which are known to be bactericidal against nonoral microorganisms. In this study, we focused much attention on the cathepsin G-containing fraction D, since it exhibited the highest killing potential against periodontal bacteria. In contrast, cathepsin G-containing neutrophil granule fractions apparently have very little ability to kill *Proteus mirabilis* (24). Fraction D was capable of killing both *A. actinomycetemcomitans* and *Capnocytophaga* spp. under aerobic or anaerobic conditions. The subfraction D9, which was highly enriched with cathepsin G, used an enzyme-independent mechanism to kill *A. actinomycetemcomitans*. Since we previously observed that the killing of *C. sputigena* (but not *Escherichia coli*) by cathepsin G was enzyme dependent (17), we conclude that certain molecules, i.e., cathepsin G, may use two different mechanisms to kill oral bacteria. Studies are presently underway to determine how cathepsin G kills *A. actinomycetemcomitans* and *Capnocytophaga* spp.

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