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Defensins Mediate the Microbicidal Activity of Human Neutrophil Granule Extract against *Acinetobacter calcoaceticus*

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An acid extract of human neutrophil granules was fractionated on a Sephadex G-100 column and tested for microbicidal activity against *Acinetobacter calcoaceticus* HO-1 as described previously (M. C. Modrzakowski and C. M. Paranavitana, Infect. Immun. 32:668–674, 1981). The low-molecular-weight protein fraction, peak D, accounted for about 30% of the protein and nearly all of the activity of the crude extract against strain HO-1. Peak D protein proved to be a mixture of the three human defensin peptides HNP1, HNP2, and HNP3. Purified defensins reproduced the microbicidal activity of peak D. The data suggest that defensins could play a major role in the killing of *A. calcoaceticus* by human neutrophils.

Polymorphonuclear leukocytes (PMN) utilize both oxygen-dependent and oxygen-independent microbicidal mechanisms (1, 12, 17). During phagocytosis, ingested microorganisms are exposed not only to reactive oxygen intermediates but also to high concentrations of various microbicidal proteins released from PMN granules into phagolysosomes (3, 20). Studies of acid extracts of PMN granules and fractionation of such extracts by gel permeation chromatography indicate that both the absolute and relative microbicidal activity of the various fractions depend on the organism tested (7, 8, 10, 11). Modrzakowski and Paranavitana (8) noted that the lowest-molecular-weight (MW) fraction (peak D) of an acid extract of PMN granules was particularly active against a strain of the gram-negative bacterium *Acinetobacter calcoaceticus*. However, the chemical nature of the active component of peak D remained unknown. The identification of antimicrobial defensins as one of the principal components of human PMN azurophil granules (2) prompted us to investigate the possibility that these peptides account for the microbicidal activities of both peak D fraction and crude granule extract against *A. calcoaceticus*, a human pathogen of increasing importance (16).

MATERIALS AND METHODS

Purification of PMN. Human PMN were obtained from a commercial supplier (Hemacare, Van Nuys, Calif.) in single-donor leukophoresis packs containing 2×10^{10} to 4×10^{10} cells, with >90% viable PMN as determined by trypan exclusion and Giemsa stain differential count. Contaminating platelets and erythrocytes were subsequently removed by differential centrifugation and hypotonic lysis as described previously (2). PMN and granule preparations were processed at 0°C throughout.

Preparation of PMN granules and granule protein extraction. After suspension in 0.34 M sucrose (adjusted to pH 7.4 by titration with 7.5% $NaHCO_3$) to 50 ml, cells were homogenized four times (2 to 2.5 min each) in a Potter-Elvehjem homogenizer and subjected to low-speed centrifugation (200 × *g* for 10 min) to remove nuclei and cellular debris. The final pellet was examined by phase-contrast microscopy to ensure >90% cell disruption. The four supernatants containing PMN granules were pooled, and the granules were sedimented at 27,000 × *g* for 20 min.

Granule proteins were extracted and the extract was fractionated essentially as described by Modrzakowski et al. (7, 8). Briefly, the granule sediment was extracted three times in a total of 60 ml of 0.2 M sodium acetate buffer with 0.01 M $CaCl_2$ (pH 4). Each extraction was done overnight at 4°C with gentle stirring, and the residue was separated by centrifugation at 27,000 × *g* for 20 min. The pooled acetate extracts were concentrated by ultrafiltration (YC-05 filter; Amicon Corp., Lexington, Mass.) and placed on a Sephadex G-100 column (2.5 by 150 cm). Approximately 44 mg of crude granule protein extract was eluted with 0.2 M sodium acetate buffer (pH 4) and collected in 10-ml fractions. The fractions were pooled into four peaks (A, B, C, and D) and three valleys (AB, BC, and CD) defined by their A_{280} pattern, concentrated by ultrafiltration (Amicon YC-05 filter), and dialyzed against phosphate-buffered saline (PBS) with Spectrapore 6 tubing, MW 3,000 cutoff (Spectrum Industries, Los Angeles, Calif.).

Antibacterial assays. *A. calcoaceticus* HO-1, ATCC 14987 was used for all bactericidal assays. The organism was maintained on tryptic soy agar (Difco Laboratories, Detroit, Mich.) plates. Organisms from a single colony were inoculated into 50 ml of tryptic soy broth and cultured for 24 h at 37°C. A 1-ml portion of this culture was inoculated into 50 ml of tryptic soy broth for an additional 18 h at 37°C. The concentration of CFU was quantitated by A_{620} referenced to previously determined standards.

The bactericidal assays of the seven granule protein fractions, crude granule extract, and purified defensins (HNP1/HNP2/HNP3 in mass ratio 1:1:0.5) (2) were performed by a modification of the method of Rest et al. (10). Assay mixtures containing approximately 10^5 CFU and the test substance in a total of 0.1 ml of 0.5% tryptone–0.5× phosphate-buffered saline (pH 7.0) were incubated for 2 h at 37°C in a shaking water bath. To determine the number of remaining CFU, 0.1-ml portions of sequential dilutions were plated on tryptic soy agar plates and incubated at 37°C until visible colonies formed. Bactericidal assays evaluating the dependence of defensin activity on dose, duration of incubation, and the influence of varying ionic strengths, pH, and

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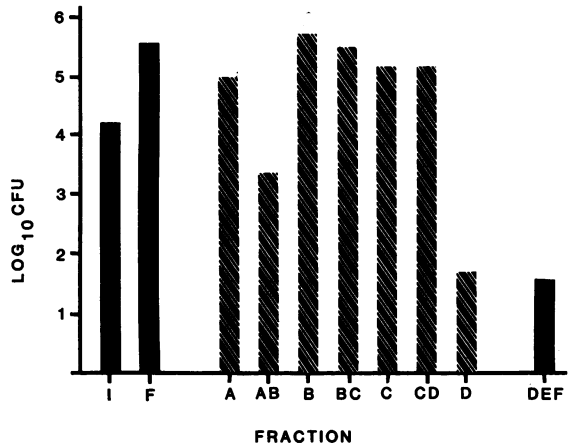

FIG. 1. Effect of PMN granule fractions and defensins on *A. calcoaceticus* HO-1. Bars I (initial) and F (final) depict, respectively, CFU before and after 2 h of control incubation, substituting diluent (phosphate-buffered saline) for granule fractions. Bars, A to D and bar DEF show CFU after 2 h of incubation with 100 μg of granule fractions A to D or defensin per ml. Log_{10} CFU of triplicate determinations varied within a range of less than 0.1.

nutrient addition were performed in 10 mM sodium phosphate buffer.

Protein studies. Protein concentration was measured by a modified Lowry technique (19) with chicken egg white lysozyme as the standard. A 1- to 4-μg amount of crude granule extract, each fraction, and purified defensins was subjected to electrophoresis on 10 to 30% gradient sodium dodecyl sulfate-polyacrylamide gels and acid-urea polyacrylamide gels (2). The amino acid composition of peak D was determined and compared with that of the defensins as described previously (13). A_{280} of equal Lowry concentrations of purified defensins and fraction D were compared.

RESULTS

Activity of PMN granule fractions against *A. calcoaceticus* HO-1. The chromatogram of the crude granule extract was similar to that obtained by Modrzakowski et al. (7, 8), displaying four peaks (A, B, C, and D) and three valleys (AB, BC, and CD). A total of 39 mg of protein was recovered and was found to be distributed among these fractions as follows: A, 5.3 mg (13.7%); AB, 2.8 mg (7.2%), B, 8.7 mg (22.5%); BC, 1.9 mg (4.9%); C, 5.5 mg (14.2%); CD, 3.1 mg (8.0%); D, 11.5 mg (29.7%).

At 100 μg/ml, fraction D demonstrated substantially more activity against *A. calcoaceticus* HO-1 than the other six granule protein extract fractions (Fig. 1). Fraction AB at 100 μg/ml displayed an intermediate level of activity between that of fraction D and the other fractions, which showed little difference from the control. We found no evidence of antimicrobial synergy between fraction D and other fractions, after combining 100 μg of fraction D per ml with 100 μg of each of the other fractions per ml (data not shown).

When crude granule protein extract was compared with fraction D at concentrations containing equivalent quantities of fraction D protein (Fig. 2), essentially all the antimicrobial activity of the granule extract against *A. calcoaceticus* HO-1 could be accounted for by its fraction D content. Analogous experiments with fraction AB showed that this fraction contributed little to the activity of the crude granule extract against strain HO-1 (data not shown).

Comparison of peak D with purified defensins. The microbicidal activities of purified defensins and fraction D against *A. calcoaceticus* HO-1 (Fig. 3) were found to be equivalent. Moreover, the activity of both purified defensins and peak D was unchanged when they were heated to 100°C for 30 min.

On acid-urea polyacrylamide gels, fraction D and purified defensins demonstrated an identical three-band electrophoretic pattern (Fig. 4), representing the three defensins (HNP1, HNP2, and HNP3). On a sodium dodecyl sulfate-gel (not shown), fraction D migrated between insulin chains (MW, ca. 3,000) and bovine trypsin inhibitor (MW, ca. 6,200), consistent with the known MW of defensins (13). At equivalent Lowry protein concentrations, the UV A_{280} of fraction D and purified defensins varied by less than 5%, suggesting that the two have similar amounts of amino acids absorbing at this wavelength (tyrosine and, to a lesser degree, tryptophan). Amino acid analysis of peak D and defensins revealed nearly identical composition (data not shown).

Modulation of defensin activity against *A. calcoaceticus* HO-1 by ambient conditions. The activity of defensins against

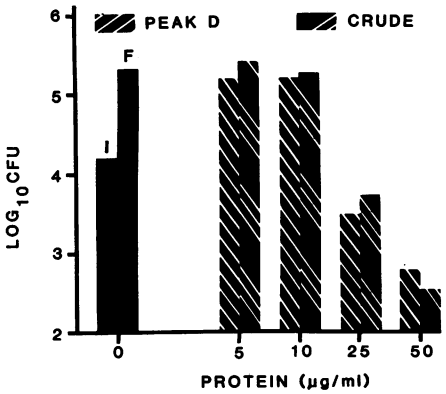

FIG. 2. Comparison of microbicidal activity of peak D versus PMN crude granule extract at the same peak D protein content. The horizontal axis shows the peak D protein content. Bars I (initial) and F (final), respectively, show CFU before and after 2 h of incubation with diluent (phosphate-buffered saline). Log_{10} CFU of triplicate determinations varied within a range of less than 0.1.

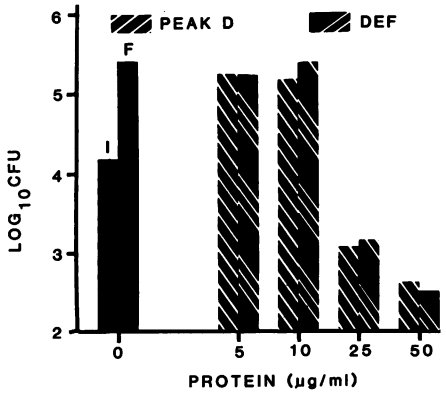

FIG. 3. Comparison of microbicidal activity of peak D versus defensins at the same protein concentrations. Bars I and F, respectively, show CFU before and after 2 h of incubation with diluent control (phosphate-buffered saline). The horizontal axis shows the total protein concentration of each test substance. Log_{10} CFU of triplicate determinations varied within a range of less than 0.1

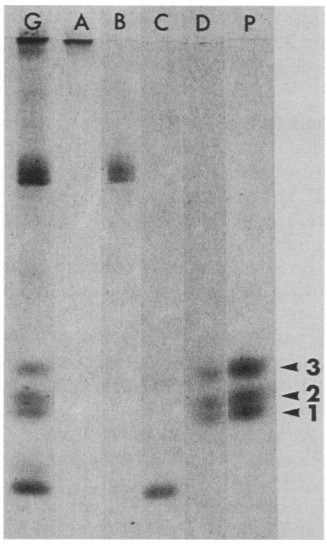

FIG. 4. Electrophoretogram of crude PMN granule extract (G), fractions A to D, and purified defensins (P). Approximately 10^6 cell equivalents of G and fractions A to D and 5 μg total of defensins were applied to a 12.5% polyacrylamide acid-urea gel. Arrows denote the positions of individual defensins, HNP1, HNP2, and HNP3. At 10^6 cell equivalents, the fractions AB, BC, and CD did not yield visible bands.

strain HO-1 was directly related to defensin concentration and duration of incubation (Fig. 5). Low pH and increasing ionic strength appeared to interfere with the antimicrobial activity of defensins (Fig. 6 and 7), but the addition of small amounts of nutrients (1:100 dilution of tryptic soy broth) markedly increased the killing of strain HO-1 even at high ionic strength (Fig. 7).

DISCUSSION

Defensins, the most recently characterized antimicrobial peptides of human PMN, were shown to account for nearly all of the activity of crude PMN granule extract against *A. calcoaceticus* HO-1. The activity of purified defensins against strain HO-1 was found to increase with increasing defensin dose, longer duration of incubation, lower ionic strength, and the addition of a dilute mixture of nutrients. Similar dependence of defensin-mediated antibacterial activity on simple or complex nutrients was found previously with strains of *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* (2). The molecular basis of defensin action, as yet unknown, is under active investigation. Despite decreased activity against strain HO-1 at higher

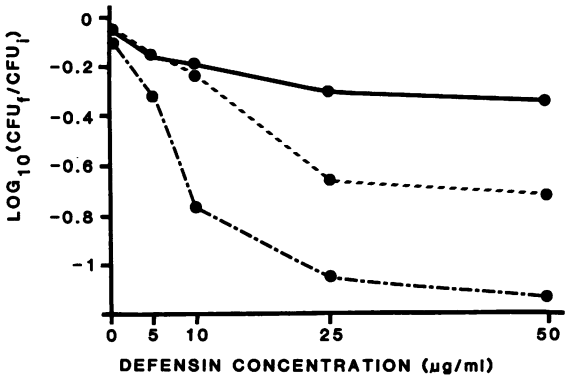

FIG. 5. Time and dose dependence of *A. calcoaceticus* HO-1 killing by defensins in nonnutritive media. The vertical axis shows the decrease in $\log_{10}$ CFU after incubation, relative to the preincubation defensin-free control. The horizontal axis shops defensin concentration in the assay medium (10 mM sodium phosphate [pH 7.4]). The decreased killing of strain HO-1 compared with that shown in Fig. 1 to 3 reflects the use of a nonnutritive medium in this assay (see Fig. 7). Log_{10} CFU of triplicate determinations varied within a range of less than 0.1. Symbols: — · — · , 120 min; – – –, 60 min; ———, 20 min.

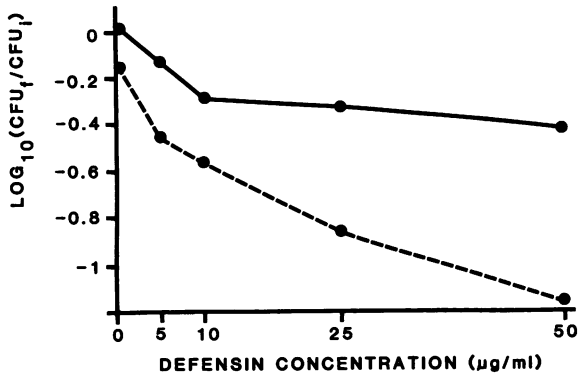

FIG. 6. Effect of pH on microbicidal activity of defensins in nonnutritive media. The vertical axis shows the decrease in $\log_{10}$ CFU after incubation for 2 h, relative to preincubation defensin-free control. The horizontal axis shows defensin concentration in the assay medium (10 mM sodium phosphate at the indicated pH). As in Fig. 5, the decreased killing of *A. calcoaceticus* HO-1 compared with that shown in Fig. 1 to 3 reflects the use of a nonnutritive medium in this assay. Log_{10} CFU of triplicate determinations varied within a range of less than 0.1. Symbols: – – –, pH 7.4; ———, pH 5.8.

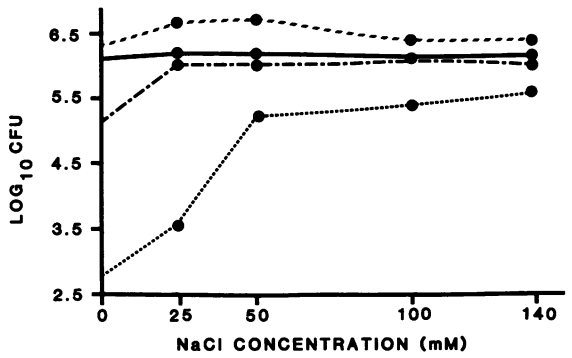

FIG. 7. Effect of salt concentration and nutrient mix on defensin activity against *A. calcoaceticus* HO-1. The horizontal axis shows the NaCl concentration in the incubation mixture, which also contained 10 mM sodium phosphate buffer (pH 7.4). Lines i-TSB (———) and f-TSB (– – –) (defensin-free controls) show, respectively, initial and final $\log_{10}$ CFU during incubation in a 1:100 dilution of tryptic soy broth, at increasing salt concentrations. The difference between f-TSB and i-TSB represents the amount of bacterial growth supported by this dilute medium. In the absence of triptic soy broth (lines not shown), no growth occurred and both pre- and postincubation CFU were identical to line i-TSB. Lines DEF-TSB (· · ·) and DEF (– · – ·) show the effect of salt concentration on microbicidal activity of 50 μg of defensin per ml, in the presence (DEF-TSB) or absence (DEF) of a 1:100 dilution of tryptic soy broth. All incubations were performed at 37°C for 2 h. Duplicate $\log_{10}$ CFU differed by less than 0.1.

ionic strengths, significant defensin-mediated killing persisted even at 150 mM NaCl in the presence of dilute nutrients. Defensins thus could retain their bactericidal activity against selected organisms even if the intraphagolysosomal ionic strength (as yet unknown) equalled that of serum. Although killing of strain HO-1 occurred under experimental conditions even at defensin concentrations as low as 25 μg/ml, our calculations (3) suggest that the intraphagolysosomal concentration of defensins may be as much as 100-fold higher. These higher concentrations may provide a margin of safety against known defensin inhibitors such as low pH, high ionic strength, and the presence of certain serum components.

Previous studies have partially determined the composition of PMN granule extract fractionated by gel permeation chromatography (7): fraction A contains myeloperoxidase; fraction B contains proteases (including elastase and cathepsin G); fraction C contains lysozyme; and fraction AB contains at least two distinct bactericidal proteins, a 57- to 59-kilodalton protein or group of proteins (4, 14, 18) and a 37-kilodalton cationic protein (14). We have shown that the lowest-MW protein fraction, fraction D, is a mixture of the three defensins, HNP1, HNP2, and HNP3. Measurements of defensin concentration in PMN granules by enzyme immunoassay and polyacrylamide gel densitometry revealed that defensins constitute about 10% of total PMN granule protein (W. G. Rice, J. M. Kinkade, Jr., T. Ganz, M. E. Selsted, R. I. Lehrer, and R. T. Parmley, Blood **66**:91A, 1985). Preferential solubilization of defensins may account for their higher concentration (30% of total protein) in the crude granule extract.

The individual PMN granule protein fractions demonstrate selective microbicidal activity against various organisms. Fraction AB and its several purified components (BPI, 57-kilodalton CAP, BP, and 37-kilodalton CAP) appear particularly potent against certain gram-negative bacteria including *Salmonella typhimurium*, *E. coli*, and *P. aeruginosa* but are not active against gram-positive organisms (1, 4, 7, 10, 14, 17, 18) and appeared only minimally active against *A. calcoaceticus* HO-1 in this study. Cathepsin G (also called chymotrypsinlike cationic protein), contained in fraction B, is broadly microbicidal against gram-positive and gram-negative bacteria (9) and fungi (5, 6). Recently, cathepsin G was also found to be highly active against *Neisseria gonorrhoeae* (15). Fraction C, consisting chiefly of lysozyme, is effective against several gram-positive bacteria but not against gram-negative bacteria (7). Fraction D and its three purified components, defensins HNP1, HNP2, and HNP3, are active against several gram-positive and gram-negative bacteria, fungi, and certain enveloped viruses (2) and are particularly potent against strain HO-1. Individual PMN granule proteins with differing antimicrobial specificities may thus contribute to a broad-spectrum antimicrobial environment in the phagolysosome.

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