Defensins

Natural Peptide Antibiotics of Human Neutrophils

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Abstract

We extracted a granule-rich sediment from normal human neutrophils and subjected it to chromatographic, electrophoretic, and functional analysis. The extract contained three small (molecular weight < 3,500) antibiotic peptides that were named human neutrophil peptide (HNP)-1, HNP-2, and HNP-3, and which will be referred to as "defensins." HNP 1-3, a mixture of the three defensins, killed *Staphylococcus aureus, Pseudomonas aeruginosa,* and *Escherichia coli* effectively in vitro when tested in 10 mM phosphate buffer containing certain nutrients, but it had little or no bactericidal activity in nutrient-free buffer. In contrast, the nutrient-free buffer supported a high degree of activity by HNP 1-3 against *Cryptococcus neoformans.* In addition to its antibacterial and antifungal properties, HNP 1-3 directly inactivated herpes simplex virus, Type 1. Two of the individual purified defensins, HNP-1 and HNP-2, were as microbicidal as the mixture HNP 1-3. HNP-3 was less active than the other defensins against most but not all of the microbes tested. Immunoperoxidase stains revealed HNP 1-3 to have a granular localization in the neutrophil's cytoplasm by light microscopy. Frozen thin section immunogold transmission electron microscopy showed HNP 1-3 to be localized in azurophil granules. These studies define a broad-spectrum antimicrobial system in human neutrophils. The defensin system may operate in conjunction with or independently from oxygen-dependent microbicidal processes to enable human neutrophils to inactivate and destroy potential pathogens.

Introduction

Granulocytes, key effector cells in host defenses against microbial infection, destroy invading microorganisms by two principal mechanisms. One of these depends on production of reactive oxygen intermediates (ROI)[1] by stimulated phagocytes. These ROI, such as H_2O_2 and $OH^{\cdot}$, can act directly or in concert with other granulocyte components, such as myeloperoxidase, to damage or kill ingested microbes (1-3).

In addition to such oxidative and peroxidative microbicidal mechanisms, granulocytes are equipped with antimicrobial mechanisms that can operate independently of ROI (3-5). Thus, neutrophils obtained from patients with chronic granulomatous disease (6-9) or normal neutrophils tested under anaerobic conditions (10-12) retain substantial efficacy against certain bacteria and fungi, even though their ability to generate reactive oxygen intermediates is precluded by disease or by design.

We recently described the biochemical composition and antimicrobial properties of a family of six structurally-related peptides purified from rabbit granulocytes (13, 14). The peptides were small (32-34 amino acids/molecule), uniformly rich in cysteine (6 residues/molecule), and variably rich in arginine (4-10 residues/molecule) (14). In vitro, the peptides displayed a range of antimicrobial activities against bacteria (13), fungi (15), and certain enveloped viruses (16).

Because such natural peptide antibiotics might contribute significantly to ROI-independent granulocyte-mediated defenses against infection, we examined human neutrophilic granulocytes (polymorphonucleated neutrophilic leukocytes; PMN) for the presence of analogous molecules. Based on our earlier studies with rabbit granulocytes, we specifically sought small (molecular weight < 4,000), cysteine-rich peptides with activity against herpes simplex virus, type 1 (HSV-1) in vitro. We herein describe the purification and antimicrobial properties of three such peptides, not previously known to exist in human PMN. The primary structures of these peptides and their homology to the previously-characterized low molecular weight antibiotic peptides of rabbit granulocytes (14) are described in a companion paper (17).

Methods

Purification of human neutrophil peptides (HNP). With approval from the University of California at Los Angeles Human Subjects Protection Committee, human neutrophils were obtained in single donor leukopheresis packs from a commercial supplier (Hemacare, Van Nuys, CA)

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1. *Abbreviations used in this paper:* AB, antibody buffer; AU-PAGE, acid urea polyacrylamide gel electrophoresis; BPI, bactericidal/permeability-increasing protein; CLCP, chymotrypsin-like cationic proteins; GE, granulocyte extracts; HNP, human neutrophil peptides; HPLC, high performance liquid chromatography; HSV-1, herpes simplex virus, type 1; LPS, lipopolysaccharide; PFU, plaque-forming units; ROI, reactive oxygen intermediates; RP-HPLC, reverse-phase high performance liquid chromatography; TBS, tris-buffered saline.

and were processed within 2 h of harvest. The cells were twice washed of platelets by low speed centrifugation (200 *g* for 10 min) and resuspension in Dulbecco's phosphate-buffered saline at 4°C. Contaminating erythrocytes were removed by a 45-s cold hypotonic lysis followed by low speed centrifugation. This left a pellet containing $1-3 \times 10^{10}$ leukocytes, >90% of which were neutrophils, with the remainder consisting mostly of lymphocytes and monocytes. Over 90% of the cells were viable by trypan blue exclusion.

The leukocytes were resuspended in 30 ml of 0.34 M sucrose (pH 7.4) and homogenized in a glass teflon pestle homogenizer (Thomas Scientific, Philadelphia, PA) until most cells appeared broken under phase-contrast microscopy. The cell debris and unbroken cells were removed by another low speed centrifugation, 200 *g* for 10 min, leaving a granule-rich supernatant. The residual cell pellet was subjected to three more cycles of homogenization and centrifugation in 0.34 M sucrose to yield additional granule-rich supernatants. The supernatants were combined and centrifuged at 27,000 *g* for 30 min at 4°C. The resulting pellets were pooled and extracted overnight in 100 ml of 10% acetic acid at 4°C. This extract was cleared at 27,000 *g* for 30 min and concentrated to ~10 ml in a vacuum centrifuge (Speed-Vac; Savant Instruments, Inc., Hicksville, NY).

The steps involved in purifying the defensins, HNP-1, HNP-2, and HNP-3, are summarized in Scheme I and described below. HNP 1-3 denotes a mixture of HNP-1, HNP-2, and HNP-3, as shown in Scheme I, step 3. When used for antimicrobial assays, HNP 1-3 was purified further by reverse-phase high performance liquid chromatography (RP-HPLC) under conditions resulting in the copurification of its components (see below).

Antibacterial assays. The bactericidal activity of human neutrophil peptides was tested against *Staphylococcus aureus* 502A, *Pseudomonas aeruginosa* PAO579, and *Escherichia coli* ATCC 29648. The organisms were maintained on trypticase soy agar plates, and organisms from a single colony were inoculated into 50 ml of trypticase soy broth (Difco Laboratories, Inc., Detroit, MI) and cultured overnight at 37°C. 1 ml of this intermediate culture was diluted with 49 ml of fresh nutrient broth and incubated for an additional 18 h at 37°C. A portion of this stationary phase culture was washed with 10 mM phosphate buffer, pH 7.4, and the concentration of colony-forming units (CFU) per milliliter was quantitated by measuring its absorbance at 620 nm with reference to previously determined standards. Incubation mixtures contained 1×10^5 bacterial CFU and 5 μg of HNP in 100 μl of 10 mM sodium phosphate buffer, pH 7.4. Control mixtures lacked HNP but contained a small amount of acetic acid (final concentration, 0.08 mM), equalling that introduced as a vehicle for HNP in the experimental mixtures. This incubation mixture was supplemented with various nutrients as indicated in the text. After incubation with HNP for 2 h at 37°C, timed samples were removed, serially diluted, spread on nutrient agar plates, and incubated for 48–72 h to allow full colony development.

Antifungal assays. Cryptococcus neoformans C-384 (Type C) was maintained on Sabouraud's 2% dextrose agar plates. Test organisms were prepared by inoculating 10 ml of Sabouraud's 2% dextrose broth with a loopful of organisms from a single colony and incubating this culture for 18 h at 37°C. From this intermediate culture, 1 ml was removed and added to 50 ml of fresh Sabouraud's broth that was incubated at 37°C for an additional 18 h. The test organisms were washed twice by centrifugation in 10 mM sodium phosphate buffer, counted in a hemocytometer, and adjusted to the desired concentration in 10 mM sodium phosphate buffer. Incubation mixtures contained 1×10^5 CFU±5 μg of HNP in a final volume of 100 μl. These mixtures were incubated at 37°C for 20–240 min, and samples were removed, serially diluted, and spread on Sabouraud's 2% dextrose agar petri plates for 3–5 d to insure full colony development.

Antiviral assays. The activity of human neutrophil peptides against HSV-1, McIntyre strain, was tested in two systems. The first was qualitative and useful in monitoring the biological activity of the many fractions tested during our early studies. In these tests, each of the high performance liquid chromatography (HPLC)-purified, lyophilized fractions was dissolved in 100 μl of 0.01% acetic acid. A portion (40 μl) of this solution was mixed with 360 μl of Eagle's minimal essential medium (Gibco Laboratories, Grand Island, NY) containing 2% fetal bovine serum, 20 mM Hepes buffer (pH 7.3), antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), and HSV-1 (1×10^5 plaque-forming units (PFU)/ml). The final pH of this mixture was 7.25. After 30 min at room temperature, this mixture was placed over Vero cell monolayers in 24-well tissue culture plates and incubated for 24–48 h at 37°C with periodic monitoring by inverted phase microscopy. Unprotected infected monolayers developed characteristic moderate cytopathic effects by 24 h (polykaryon formation and mild cellular rounding) that progressed markedly by 48 h. Fractions containing HNP 1-3 were initially detected and their purification was followed by the marked protection they afforded from virally-induced cytopathic effects (data not shown).

The second assay was quantitative, and measured the direct neutralization of HSV-1 by the peptides. Various concentrations of HPLC-purified peptide in 180 μl of phosphate-buffered saline solution (PBS) were mixed with 20 μl of titered HSV-1 preparations (~4×10^6 PFU/ml in Eagle's minimal essential medium containing 2% vol/vol fetal bovine serum and antibiotics) and incubated for up to 4 h at 37°C. Control mixtures containing only HSV-1 and dilute acetic acid (the vehicle for HNP) in PBS were incubated in parallel at 37°C. At intervals, aliquots of the mixtures were serially diluted and viral PFU were titered in triplicate on Vero cell monolayers by standard techniques. In four representative experiments, the mean coefficient of variation (standard deviation/mean) of this assay procedure was 20.9%.

Immunoperoxidase staining. HNP 1-3 was conjugated to ovalbumin (Sigma Chemical Co., St. Louis, MO) by glutaraldehyde (18), mixed with complete Freund's adjuvant (Difco Laboratories, Inc.), and used to immunize rabbits. Sera were fractionated on DEAE cellulose to provide an IgG fraction used for subsequent staining. Preimmune serum, similarly fractionated, served as the control. Mixed peripheral blood leukocytes were prepared by sedimenting 10 ml of heparinized venous blood that had been mixed with 5 ml of 3% dextran in normal saline. The cells were washed with PBS and were deposited on glass slides with a cytocentrifuge (Shandon Southern Instruments, Inc., Sewickley, PA). Slides were air-dried and then fixed for 10 min in PBS containing 10% formalin, and washed with tris-buffered saline (TBS; 20 mM tris, pH 7.5, 500 mM NaCl). To inactivate endogenous peroxidase activity, the slides were treated with 0.1 M periodic acid for 5 min, rinsed with distilled water, and treated with 0.02% sodium borohydride for 2 min (19). The slides were washed with TBS and incubated for 24 h with a 1:800 dilution of rabbit anti-HNP-IgG or a 1:200 dilution of preimmune IgG (control) in antibody buffer (AB) consisting of 0.05% Tween 20, 0.01% thimerosal, and 1% gelatin in TBS. After this primary incubation, they were washed with TBS containing 0.05% Tween and incubated for an additional 24 h with a 1:2,000 dilution of peroxidase-labeled goat anti-rabbit IgG (Cappel Laboratories, Cochranville, PA). After extensive washing, first with TBS/0.05% Tween and then with TBS, the immunoperoxidase stain was developed by placing the slides in TBS/0.015% H_2O_2/0.05% 1-chloronaphthol (Bio-Rad Laboratories, Richmond, CA) for 10 min. The

Outline of the Purification Procedure

1. Purify granulocytes by leukopheresis
   —homogenize in 0.34 M sucrose
   —clear nuclei and debris at 200 *g*
2. Centrifuge at 27,000 *g* for 20 min
   —extract sediment with 10% acetic acid
   —concentrate extract 10-fold under vacuum
3. First chromatography on Biogel P-10 (HNP 1–3)
4. Ion-exchange and reverse-phase HPLC (HNP-3)
5. Second chromatography on Biogel P-10 (HNP-1 / HNP-2)
6. Final reverse-phase HPLC

SCHEME I

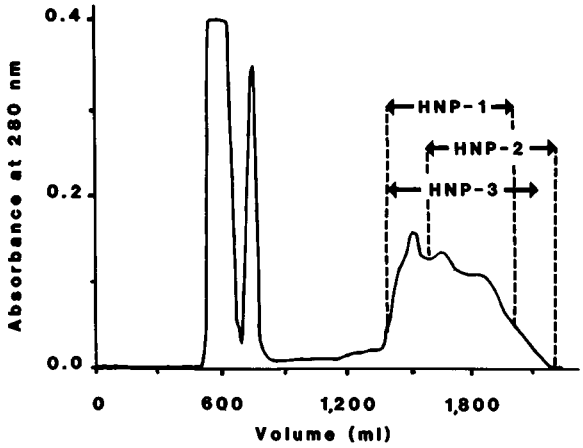

Figure 1. Chromatography of PMN extract. ~15 ml of the 10-fold concentrated extract (step 2b of Scheme I) was chromatographed on a 137 cm × 3.8 cm column of Biogel P10. HNP 1–3 emerged as a late and incompletely resolved series of peaks as shown.

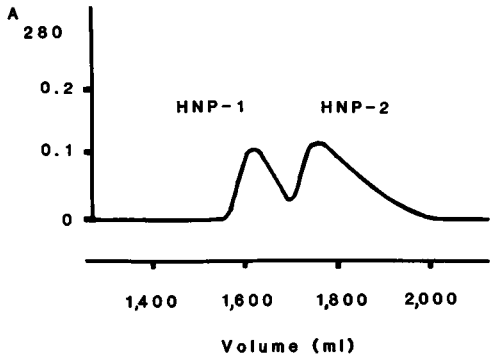

Figure 3. Chromatographic resolution of HNP-1 and HNP-2. The mixture of HNP-1 and HNP-2 obtained by ion exchange HPLC was desalted on a Vydac C18 column, lyophilized, resuspended in 1% acetic acid, and applied to a 137 cm × 3.8 cm column containing Biogel P10. The two peptides were largely resolved by this procedure, and the overlap regions of the effluent were purified by recycling them through the same column.

slides were washed with water, and counterstained with 0.01% acridine orange to show nuclear morphology.

Electron microscopy. Normal human leukocytes sedimented in dextran were washed in Hanks' solution and subsequently processed for frozen thin sections as described by Stenberg et al. (20). Briefly, the cells were fixed in 0.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h at 22°C and were washed well in the same buffer containing 10% (wt/vol) sucrose. The cells were infiltrated for 30 min with 2.1 M sucrose, embedded in the sucrose solution, frozen, and stored in liquid nitrogen. Sections were cut on a Reichert ultracut E. The frozen thin section techniques described by Tokuyasu (21) were used with the modifications for the use of colloidal gold described by Griffiths et al. (22). The primary antibody was used at a 1:4,000 dilution and immunogold probe, Protein A-5, from Janssen Pharmaceutica Beerse, Belgium was used at a 1:50 dilution. The control for these experiments was nonimmune, purified rabbit IgG or buffer in place of the primary antiserum.

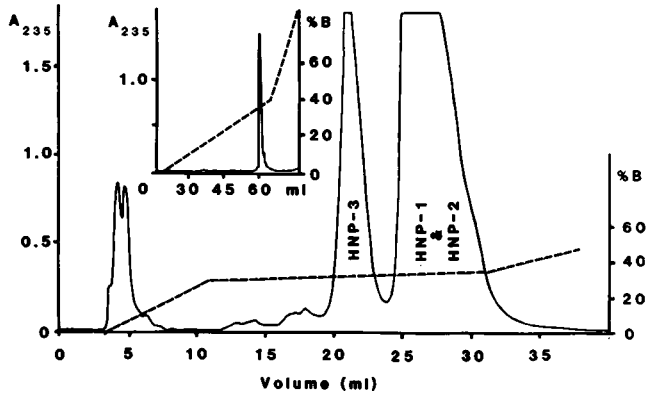

Figure 2. HPLC of HNP 1–3. *Main panel:* HNP 1–3, after P10 chromatography (Scheme I) was separated on a carboxymethylsilica column (Bio-Sil TSK IEX-535CM, 150 mm × 6 mm; Bio-Rad Laboratories), using a salt gradient. Buffers A and B contained 0.05 M sodium phosphate/10% acetonitrile, pH 7.0, and Buffer B also contained 1 M NaCl. The interrupted line shows the percent of Buffer B, and the solid line shows OD_{235}. *Inset:* an aliquot of the HNP-3 peak obtained by ion-exchange HPLC was chromatographed on a Vydac C-18 column. In this system, Buffer A contained 0.1% trifluoroacetic acid in water and Buffer B consisted of 0.1% trifluoroacetic acid in acetonitrile. The interrupted line shows percent B in the gradient.

Results

Peptide purification. Our initial fractionation of the concentrated granule extract was accomplished by gel permeation chromatography on a P-10 Biogel column (Bio-Rad Laboratories). Three peptides satisfying our initial search criteria coeluted (Fig. 1). They were named according to their relative cationic mobility on acid urea polyacrylamide gel electrophoresis (AU-PAGE): HNP-1, greatest mobility; HNP-2, intermediate mobility; and

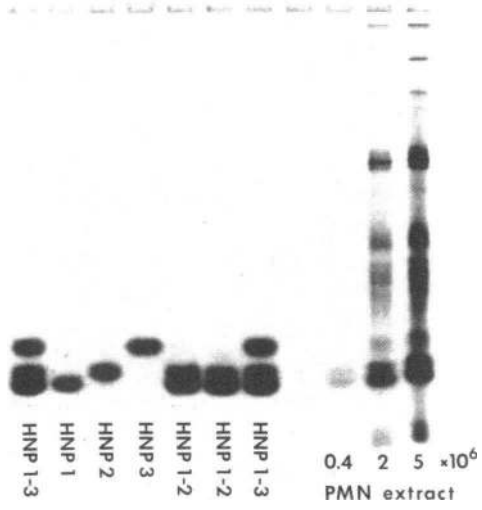

Figure 4. AU-PAGE of human neutrophil peptides. The lanes are numbered from the left. Lanes 1 and 7, 6 μg of HNP 1–3; Lanes 2–4, 2 μg each of HNP-1, HNP-2, and HNP-3; Lanes 5 and 6 contained 4 μg each of different batches of HNP 1–2. Lane 8 is blank; Lanes 9–11 contained crude PMN extract equivalent to 0.4, 2, and 5 × 10^6 neutrophils. Coomassie Brilliant Blue stain. The electrophoretogram of HNP-1, HNP-2, and HNP-3 in crude neutrophil extracts was unaffected by preincubation of PMN with 1 mM diisopropylfluorophosphate, a potent inhibitor of serine proteases (data not shown). From this and similar gels the total amount of extracted HNP 1–3 is estimated at 1–3 μg/10^6 PMN.

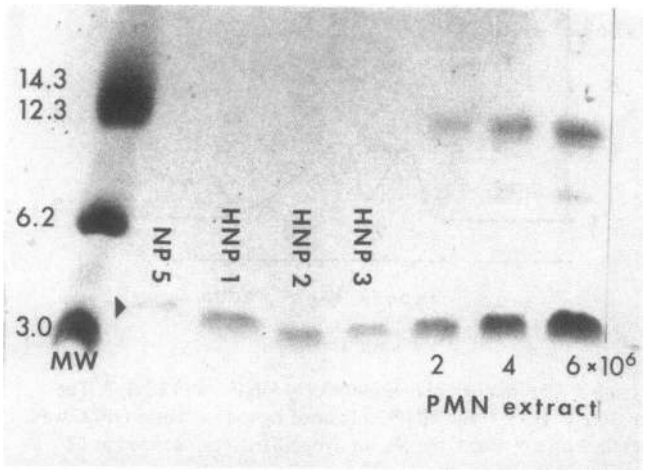

Figure 5. SDS-PAGE of human neutrophil peptides. From the left, lane 1, molecular weight marker peptides (Biotech Research Laboratories, Inc., Rockville, MD), molecular weights in kD as shown; Lane 2, 1 μg of rabbit NP-5 (M_r, 3,385), Lanes 3–5, 1 μg each of purified HNP peptides as shown; Lanes 6–8, crude neutrophil extract, corresponding to 2, 4, or 6 × 10^6 PMN-equivalents. Coomassie Brilliant Blue stain.

HNP-3, least cathodal. Separation of HNP-3 from HNP-1 and HNP-2 was accomplished by high performance liquid ion exchange chromatography on a carboxymethyl silica column (Biosil TSK-CM; Bio-Rad Laboratories) in 50 mM sodium phosphate/10% acetonitrile, pH 7.0, eluted with a sodium chloride gradient (Fig. 1). This was followed by desalting and purification by RP-HPLC over a C-18 alkyl silica column (Vydac; Rainin Instrument Co., Inc., Woburn, MA) in 0.1% trifluoroacetic acid, with elution by an acetonitrile gradient (Fig. 2, inset). HNP-1 was separated from HNP-2 by recycling the mixture on a 150-cm-long Biogel P-10/1% acetic acid gel permeation system, followed by RP-HPLC as before (Fig. 3). The fractionation was monitored and the purity of each peptide was assessed by AU-PAGE and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Figs. 4 and 5). The overall purification procedure is summarized in Scheme I.

Antibacterial activity. The effects of exposing *S. aureus* 502A to HNP 1–3 are shown in Table I. Note that whereas the bacteria were unaffected by the defensins in 10 mM phosphate buffer, addition of either 5 mM glucose or 1% nutrient (trypticase soy) broth sensitized the bacteria to the peptides. In other experiments we observed that 0.05 mM glucose was as effective as 5 mM glucose in sensitizing *S. aureus* exposed to HNP 1–3 for 2 h (data not shown). Neither 5 mM pyruvate nor a mixture of six amino acids replaced glucose or trypticase soy broth in sensitizing the staphylococci to HNP 1–3.

The susceptibility of two gram-negative bacteria to HNP 1–3 is shown in Table II. Consonant with the observations on *S. aureus*, neither *P. aeruginosa* nor *E. coli* was killed after exposure to 50 μg/ml of HNP 1–3 in 10 mM phosphate buffer, and both were susceptible when the incubation mixture contained 1% trypticase soy broth. In contrast to the experiments with *S. aureus*, addition of glucose had little or no effect in inducing sensitivity to the peptides.

Antifungal activity. We tested the activity of HNP 1–3 against *C. neoformans* C-384. Unlike the aforementioned bacteria, this organism was exquisitely susceptible to HNP 1–3 in nutrient-free buffer. Fig. 6 shows that killing was extensive and time-dependent, with a reduction of >3 $\log_{10}$ CFU/ml, relative to control, after 4 h of incubation. Colony counts of the controls remained essentially unaltered during the 4-h incubation period.

Antiviral effects. The direct inactivation of HSV-1 by HNP 1–3 is illustrated in Fig. 7, a single, but representative, experiment. Note that the initial rapid fall in viral PFU/ml was followed by a more gradual decline that continued for the duration of the experiment (4 h).

Antimicrobial effects of individual purified defensins. The antimicrobial activity of the individual purified defensins, HNP-1, HNP-2, and HNP-3, was compared with that of the mixture HNP 1–3, as shown in Table III. HNP-1 and HNP-2 were as active as HNP 1–3. HNP-3 was less microbicidal than the other

Table I. Effects of Substrates on Susceptibility of S. aureus to HNP

Experiment	Addition	Concentration	Control	+HNP 1–3	Δ $\log_{10}$
				50 μg/ml	
1	None	—	4.9 × 10^5	5.0 × 10^5	−0.01
	Glucose	5 mM	4.9 × 10^5	2.5 × 10^3	2.29
	Pyruvate	5 mM	1.0 × 10^5	2.2 × 10^5	−0.34
	TSB	1:100	1.5 × 10^6	1.1 × 10^3	3.13
	Amino acid	2.5 mM each	6.5 × 10^5	5.1 × 10^5	0.11
2	None	—	1.1 × 10^6	1.1 × 10^6	0.00
	Glucose	5 mM	8.3 × 10^5	3.3 × 10^4	1.40
3	None	—	7.7 × 10^5	7.7 × 10^5	0.00
	Glucose	5 mM	7.4 × 10^5	3.2 × 10^4	1.37
	TSB	1:100	2.1 × 10^6	2.5 × 10^3	2.90
	TSB + glucose	1:100 + 5 mM	2.8 × 10^6	3.8 × 10^3	2.87

The incubation medium was 10 mM phosphate buffer, pH 7.4, supplemented with the indicated additions. Incubations were conducted for 2 h at 37°C with 18 h *S. aureus* cultures grown in trypticase soy broth (TSB). Input concentrations (mean±SEM × 10^6 CFU/ml) were 1.12±0.17 × 10^6 (Experiment 1), 1.52±0.13 × 10^6 (Experiment 2), and 9.3±0.3 × 10^5 (Experiment 3). The mixed amino acids contained alanine, glutamine, histidine, leucine, methionine, and tryptophan (2.5 mM each). Data show mean CFU/ml after 2 h, and $\log_{10}$ reduction of HNP-treated bacteria relative to control. The sensitizing effect of glucose is established within 20 min (data not shown).

Table II. Effects of Substrates on Susceptibility of Pseudomonas aeruginosa and Escherichia coli to HNP

Experiment	Addition	Concentration	Control	+HNP 1-3	$\Delta\log_{10}$
				50 μg/ml	
Pseudomonas aeruginosa PAO 579					
1	None	—	1.0×10^6	1.1×10^6	−0.04
	Glucose	5 mM	1.3×10^6	7.7×10^5	0.23
	TSB	1:100	1.1×10^6	1.7×10^4	1.81
	TSB + glucose	1:100 + 5 mM	1.8×10^6	2.3×10^4	1.89
2	None	—	1.2×10^6	1.0×10^6	0.08
	Glucose	5 mM	1.1×10^6	7.4×10^5	0.17
	TSB	1:100 + 5 mM	5.0×10^5	5.5×10^4	0.96
Escherichia coli ATCC 29648					
3	None	—	1.4×10^6	1.2×10^6	0.07
	Glucose	5 mM	1.2×10^6	1.3×10^6	−0.03
	TSB	1:100	6.1×10^6	1.4×10^3	3.64
	Pyruvate	5 mM	1.5×10^6	1.1×10^6	0.13
4	None	—	1.2×10^6	1.0×10^6	0.08
	Glucose	5 mM	1.1×10^6	1.2×10^6	−0.04
	TSB	1:100	6.4×10^6	1.0×10^3	3.81
	Pyruvate	5 mM	1.4×10^6	1.2×10^6	0.07

The incubation medium was 10 mM phosphate buffer, pH 7.4, supplemented with the indicated additions. Incubations were conducted for 2 h at 37°C with overnight (18 h) cultures of bacteria that had been grown in trypticase soy broth (TSB). The input concentrations (mean±SEM × 10^6 CFU/ml) were as follows: 1.65±0.1, experiment 1; 1.49±0.03, experiment 2; 1.48±0.02, experiment 3; and 1.25±0.03, experiment 4. Data show mean surviving CFU/ml after 2-h incubation and $\log_{10}$ decrease in CFU/ml relative to control incubated without HNP for 2 h.

defensins against *C. neoformans* C-384, *E. coli* ML-35, and *S. aureus* 502A. The activity of HNP-3 against HSV-1 (McIntyre) and *E. coli* ATCC 29648 was similar to that of the other defensins.

Immunocytochemistry. Fig. 8 shows normal human PMNs that have been stained by an immunoperoxidase procedure with antibody to HNP 1–3. Note that the reaction product is cytoplasmic and granular, suggesting that the HNPs are localized to the cell's granules. Neither eosinophils nor lymphocytes contained reaction product. Controls performed with preimmune serum or without antiserum showed no peroxidase staining.

Fig. 9 shows the localization of HNP 1–3 in a transmission electron micrograph of a frozen thin section of a human PMN. Leukocytes incubated with antiserum to HNP 1–3 and protein A-gold 5 showed labeling in the azurophil granules and not in the smaller specific granules. In this type of preparation the large azurophil granules appear electron-lucent, probably because their contents have been partially solubilized. The tendency of azurophil granules to be easily extracted has been pointed out in earlier electron microscopic studies (23, 24). No labeling was seen when normal rabbit serum or buffer replaced the primary antibody.

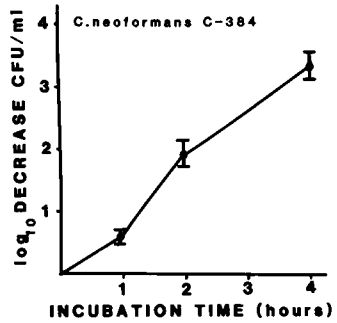

Figure 6. Effect of HNP 1–3 on *C. neoformans*, strain C-384. Test organisms, 1×10^6 CFU/ml, were incubated for the indicated times at 37°C with 50 μg/ml of HNP 1–3 in unsupplemented 10 mM phosphate buffer, pH 7.4. Note that fungal killing is represented on a logarithmic scale. Data represent mean±SEM $\log_{10}$ reduction of colony count, relative to controls incubated in peptide-free buffer, and are derived from nine separate experiments.

Discussion

The ability of PMN to kill microorganisms is a key component of host defenses against infection. The microbicidal properties of PMN arise from two fundamentally distinct mechanisms. Best understood are the mechanisms linked to production of ROI by PMN (reviewed in 2, 3, 25). The production of molecules such as $OH^{\cdot}$, H_2O_2, HOCl, and reactive chloramines equips the PMN with potent, nonspecific antimicrobial effectors that can act against diverse potential pathogens.

In addition, human PMN clearly possess antimicrobial mechanisms that can act independently of ROI (reviewed in 3, 4, 5). PMN from patients with chronic granulomatous disease (CGD) fail to generate ROI, yet they kill *Salmonella typhimu-*

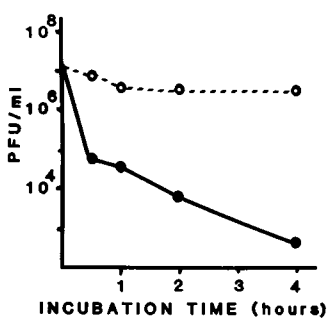

Figure 7. Antiviral activity of HNP 1–3. HSV-1 (McIntyre strain) was incubated with (●) or without (○) the addition of 50 μg/ml of HNP 1–3 for up to 4 h at 37°C under conditions fully described elsewhere (16). Viral PFUs were titered on Vero cells after suitable dilution.

Table III. Effects of Purified HNP-1, HNP-2, and HNP-3 on Test Organisms

Experiment	Input	Decrease (log_{10} units) in concentration of test organisms relative to input				
		Control	HNP 1-3	HNP-1	HNP-2	HNP-3
	log					
C. neoformans C-384						
1	6.23	0.01	3.08	3.21	3.00	1.08
2	6.04	0.04	1.68	2.11	1.72	0.52
E. coli ATCC 29648						
1	6.15	−0.79	3.52	3.22	3.43	3.61
2	6.23	−0.79	3.55	2.04	3.60	3.47
E. coli ML-35						
1	6.28	−0.65	2.67	2.40	2.75	0.64
2	6.20	−0.84	1.46	1.91	1.30	0.10
P. aeruginosa PAO 579						
1	6.24	0.31	1.16	1.20	1.39	1.07
2	6.45	0.22	0.47	0.64	0.91	0.35
S. aureus 502A						
1	5.97	−0.72	1.65	2.14	1.63	0.23
2	6.22	−0.37	2.13	2.27	1.95	0.55
HSV-1 (McIntyre)						
1	6.79	0.18	2.62	2.27	2.72	2.36
2	6.62	NT	2.36	2.17	1.90	2.28
3	6.76	0.02	1.75	1.77	1.74	1.34

Data are shown in log_{10} units. The input column shows the absolute concentration of bacteria or fungi in CFU/ml or of HSV-1 in PFU/ml at the outset of the incubation. The remaining data show the decrease in CFU/ml, relative to the input, after a 2-h incubation of fungi or bacteria with 50 μg/ml of a mixture of the three defensins (HNP 1-3) or with 50 μg/ml of the individual peptides. *C. neoformans* was incubated in nutrient-free 10 mM phosphate buffer, pH 7.4, whereas bacteria were incubated in that buffer supplemented with 1% vol/vol trypticase soy broth. A minus sign indicates that the CFU/ml at 2 h was higher than the input (i.e., that growth had occurred). Experiments with HSV-1 were conducted by exposing the indicated concentration of viruses to 25 μg/ml of HNP 1-3 or the individual peptide species for 60 min at 37°C in Dulbecco's phosphate-buffered saline. The HNP 1-3 used in this study was reconstituted by mixing the individual purified peptides in a ratio of 1:1:0.5 (HNP-1/HNP-2/HNP-3).

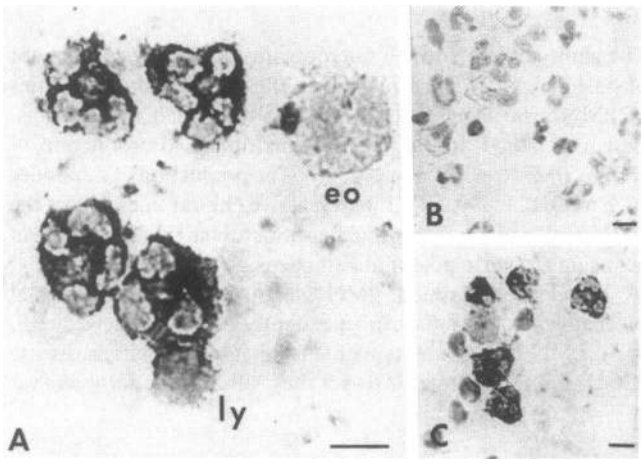

Figure 8. Immunoperoxidase stain. Normal peripheral blood cells were treated to inactivate any endogenous peroxidase and then exposed to rabbit anti HNP 1-3 IgG (*A* and *C*) or preimmune rabbit IgG (*B*). After treatment with peroxidase-conjugated second antibody (goat anti-rabbit IgG), the slides were developed as described in the text. *A* shows four neutrophils that stain positively for HNP 1-3 and a lymphocyte (ly) and eosinophil (eo) that did not stain. *B* and *C* show lower power views of cells immunostained with preimmune (*B*) or anti-HNP 1-3 IgG (*C*). An acridine orange counterstain was used to display nuclear morphology. The bar in the lower right corner of each panel represents 10 μm.

rium (8, 9), *Neisseria gonorrhoeae* (7), and *Candida parapsilosis* and *C. pseudotropicalis* (6) with considerable efficacy in vitro. Stringently anaerobic conditions preclude ROI production by PMN. Yet, under anaerobic conditions, normal human PMN retain substantial activity against many bacteria (10-12) including *E. coli, Klebsiella pneumoniae, S. typhimurium, S. aureus, Staphylococcus epidermidis, P. aeruginosa,* and *Streptococcus faecalis* (10-12). Finally, obligate anaerobic bacteria, such as *Bacteroides fragilis* and *B. vulgatus,* are killed by human PMN under strictly anaerobic conditions (10, 12, 26, 27).

The molecular mechanisms of O_2-independent microbicidal activity in PMN have not yet been fully defined. Among the best characterized potential effectors are a group of antimicrobial peptides known to be abundant in rabbit granulocytes, but not heretofore shown to have counterparts in human PMN. Hirsch (28) showed in 1956 that a crude mixture of proteins, "phagocytin," obtained by extracting rabbit granulocytes with dilute acid, exerted bactericidal activity in vitro, and suggested that its active principles resided in the cytoplasmic granules of rabbit PMN (29). Zeya and Spitznagel (30, 31) greatly refined these observations, demonstrating that "phagocytin" contained a family of small proteins, "lysosomal cationic proteins," that were rich in arginine and cysteine, and displayed specificity in their bactericidal spectrum. We recently purified these rabbit PMN "lysosomal cationic peptides" to homogeneity, and established their amino acid sequences (14). The peptides are composed of

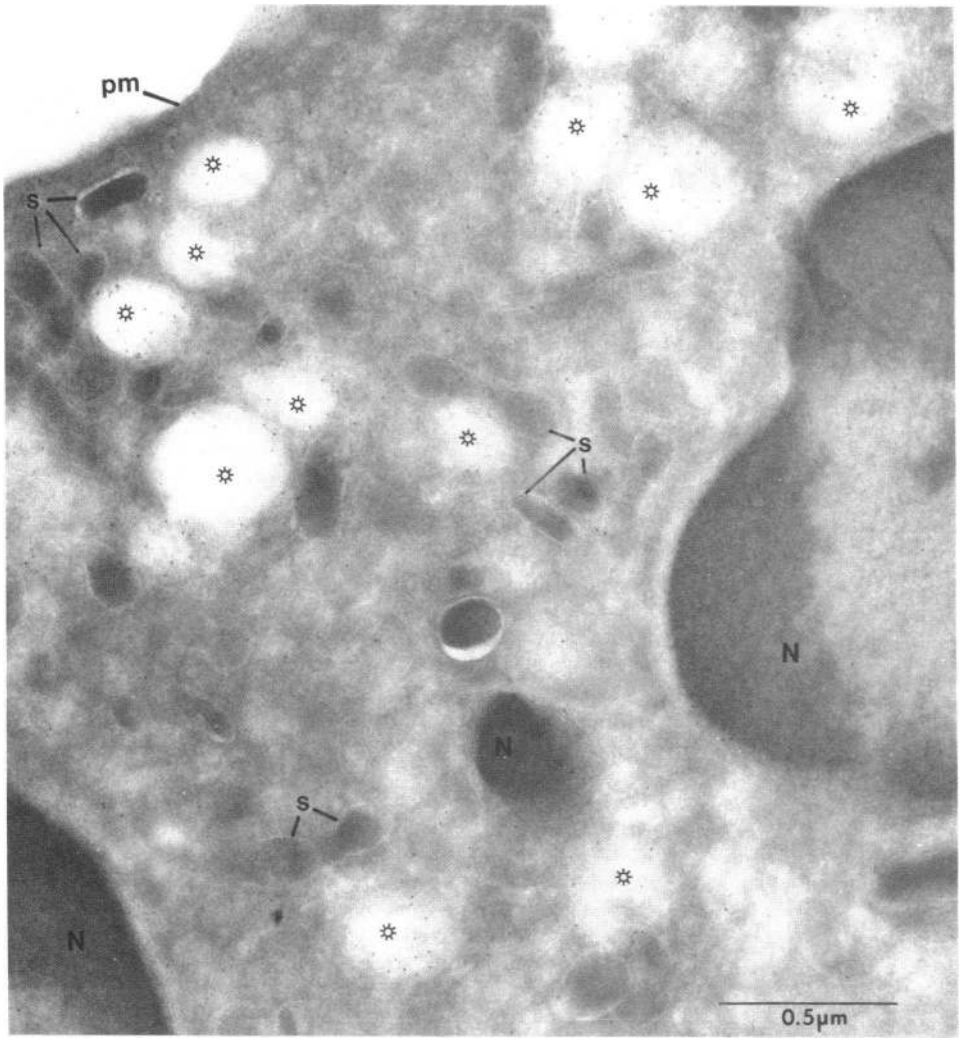

Figure 9. Transmission electron micrograph showing the localization of antigen (HNP 1–3) with immunogold label in a human neutrophil fixed and prepared for frozen thin section immunocytochemistry. Note the immunogold label in large extracted granules (•), which are believed to be the morphologic counterpart of the peroxidase-positive azurophil granule population. The specific granule population, denoted by "s," is better preserved morphologically and lacks immunogold label. N, nucleus; pm, plasma membrane.

32–34 amino acid residues/molecule. Although variably arginine-rich, all six peptides contain a strictly conserved, nonconsecutive framework composed of 11 amino acid residues. Some of the rabbit granulocyte peptides have been crystallized (32) and are being analyzed by x-ray crystallography. The antimicrobial spectrum of the purified rabbit granulocyte peptides includes aerobic bacteria (13), fungi (15, 33), and certain enveloped viruses (16), such as HSV-1.

Human PMN contain a group of chymotrypsin-like cationic proteins (CLCP) that exhibit microbicidal properties against gram-positive and gram-negative bacteria (34–36) and fungi (37). Their microbicidal effects do not depend on proteolytic activity, for heating abolishes the enzymatic activity of CLCP without decreasing its antimicrobial properties (35). Another antimicrobial constituent of granulocytes, described by Elsbach et al. (38), is a distinctive cationic (pI > 9.6) protein, that was named for its bactericidal/permeability-increasing properties (BPI). Present in rabbit and human granulocytes, its actions are reportedly specific for certain gram-negative bacteria. Both BPI (50,000–58,000 mol wt) and CLCP (25,000–28,000 mol wt) are considerably larger than the six small rabbit granulocyte cationic peptides (38, 39), or their human counterparts, the defensins (3,300–3,900 mol wt).

At least two other components of PMN granules, lysozyme (muramidase) and (apo)lactoferrin, may also contribute to microbicidal activity. Lysozyme, discovered by Fleming in 1922, hydrolyses the beta 1–4 linkage between *N*-acetylglucosamine and *N*-acetylmuramic acid residues in bacterial cell wall peptidoglycans. Lysozyme can kill a limited spectrum of gram-positive bacteria (e.g., *Micrococcus lysodeikticus* and *Bacillus megaterium* [1]) and fungi (e.g., *Coccidioides immitis* [40] and *C. neoformans* [41]) in vitro. Apolactoferrin may kill certain susceptible bacteria directly, e.g., *Streptococcus mutans* and *Vibrio cholerae* (42). In addition, lactoferrin may interact with ROI to facilitate generation of hydroxyl radicals from superoxide and hydrogen peroxide (43, 44).

Studies with partially purified granule extracts (GE) have also yielded valuable insights into the potential antimicrobial constituents of PMN. Spitznagel and his associates extracted human PMN granules with 0.2 M acetate buffer, pH 4.0, and tested the bactericidal activity of this GE. Using defined outer-membrane mutants, Rest et al. (44, 45) found that *S. typhimurium* LT2 and other *Enterobacteriaceae* became increasingly susceptible to human GE as the carbohydrate content of their lipopolysaccharide (LPS) decreased. Bacteria grown with aeration to log phase were killed by GE from 10 to 15 times more effectively than bacteria grown to stationary phase under static conditions. Bactericidal activity of human GE against *Enterobacteriaceae* was time, temperature, pH, and concentration-dependent (46). Modrzakowski et al. (47) fractionated GE by Sephadex

G-100 column chromatography, and reported that several of its fractions showed this pattern of activity. Analysis of GE by PAGE electrophoresis revealed it to be a complex mixture of at least 20 molecular species (48; and Fig. 1). They purified an especially active fraction, "Valley AB," and reported the apparent molecular weight of its active component to be ~37,000 (48). Cationic proteins responsible for bactericidal activity could be removed by absorption to purified bacterial LPS. More recently, Shafer et al. (48) provided evidence that cationic peptides bound to the lipid A moiety of LPS by ionic and hydrophobic bonds. The relationship of this "Valley AB" protein to BPI remains to be established (49).

The three cysteine-rich antimicrobial peptides (defensins) described in this report are, by amino acid sequence determination, homologous to the family of six low molecular weight cationic peptides of rabbit PMN (14). Specifically, they are composed of 29 (HNP-2) or 30 (HNP-1 and HNP-3) amino acid residues and retain all of the 11 "framework" residues common to the rabbit peptides (17). Additional studies are required before we can assess the overall contributions of HNP-1, HNP-2, and HNP-3 to PMN-mediated human host defenses. However, the presence of antibiotic peptides in the cytoplasmic granules of PMN, cells whose primary responsibility is in defense against microbial infection, is unlikely to be coincidental. Our finding that the human PMN's defensins are contained in large, myeloperoxidase-containing azurophil granules is consistent with earlier studies defining the localization of the low molecular weight cationic peptides of rabbit PMN (50). The defensins and their homologs in leukocytes of other species are likely to endow PMNs with the ability to kill a wide variety of potential pathogens.

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References

1. Klebanoff, S. J., and R. A. Clark. 1978. The neutrophil: function and clinical disorders. North Holland Publishing Company, Amsterdam. 409–466.

2. Babior, B. M. 1980. The role of oxygen radicals in microbial killing by phagocytes. *In* The reticuloendothelial system. A comprehensive treatise, vol. II. Biochemistry and metabolism. A. J. Sbarra and R. R. Strauss, editors. Plenum Publishing Corp., New York. 339–354.

3. Root, R. K., and M. S. Cohen. 1981. The microbicidal mechanisms of human neutrophils and eosinophils. *Rev. Infect. Dis.* 3:565–598.

4. Spitznagel, J. K. 1984. Nonoxidative antimicrobial reactions of leukocytes. *Contemp. Top. Immunobiol.* 14:283–343.

5. Elsbach, P., and J. Weiss. 1983. A reevaluation of the roles of the O_2-dependent and O_2-independent microbicidal systems of phagocytes. *Rev. Infect. Dis.* 5:843–853.

6. Lehrer, R. I. 1972. Functional aspects of a second mechanism of candidacidal activity in human neutrophils. *J. Clin. Invest.* 51:2566–2572.

7. Rest, R. F., S. M. Fischer, Z. Z. Ingham, and J. F. Jones. 1982. Interactions of *Neisseria gonorrhoeae* with human neutrophils: effects of serum and gonococcal opacity on phagocyte killing and chemiluminescence. *Infect. Immun.* 36:737–744.

8. Spitznagel, J. K., and N. Okamura. 1983. Oxygen-independent microbicidal mechanisms of human polymorphonuclear leukocytes. *Adv. Exp. Med. Biol.* 162:5–17.

9. Weiss, J., P. Elsbach, I. Olsson, and H. Odeberg. 1978. Purification and characterization of a potent bactericidal and membrane active protein from the granules of human polymorphonuclear leukocytes. *J. Biol. Chem.* 253:2664–2672.

10. Mandell, G. L. 1974. Bactericidal activity of aerobic and anaerobic polymorphonuclear neutrophils. *Infect. Immun.* 9:337–341.

11. Okamura, N., and J. K. Spitznagel. 1982. Outer membrane mutants of *Salmonella typhimurium* LT2 have lipopolysaccharide resistance to the bactericidal activity of anaerobic human neutrophils. *Infect. Immun.* 36:1086–1095.

12. Vel, W. A. C., F. Namavar, J. J. Verweij, A. N. B. Pubben, and D. M. McLaren. 1984. Killing capacity of human polymorphonuclear leukocytes in aerobic and anaerobic conditions. *J. Med. Microbiol.* 18: 173–180.

13. Selsted, M. E., D. Szklarek, and R. I. Lehrer. 1984. Purification and antibacterial activity of antimicrobial peptides of rabbit granulocytes. *Infect. Immun.* 45:150–154.

14. Selsted, M. E., D. M. Brown, R. J. DeLange, S. S. L. Harwig, and R. I. Lehrer. 1985. Primary structures of six antimicrobial peptides of rabbit peritoneal neutrophils. *J. Biol. Chem.* 260:4579–4584.

15. Selsted, M. E., D. Szklarek, T. Ganz, and R. I. Lehrer. 1985. Activity of rabbit leukocyte peptides against *Candida albicans*. *Infect. Immun.* 49:202–206.

16. Lehrer, R. I., K. Daher, T. Ganz, and M. E. Selsted. 1985. Direct inactivation of viruses by MCP-1 and MCP-2, natural peptide antibiotics from rabbit leukocytes. *J. Virol.* 54:467–472.

17. Selsted, M. E., S. L. S. Harwig, T. Ganz, J. W. Schilling, and R. I. Lehrer. 1985. Primary structures of three human neutrophil defensins. *J. Clin. Invest.* 76:1436–1439.

18. Reichlin, M. 1980. Use of glutaraldehyde as a coupling agent for proteins and peptides. *In* Methods in Immunology, Vol. 70. Immunochemical techniques. H. Van Vunakis and J. J. Langone, editors. Academic Press, Inc., New York. 159–165.

19. Heyerman, E., and A. M. Neville. 1977. A shorter immunoperoxidase technique for the demonstration of carcinoembryonic antigen and other cell products. *J. Clin. Pathol. (Lond.).* 30:138–140.

20. Stenberg, P. E., M. A. Shuman, S. P. Levine, and D. F. Bainton. 1984. Redistribution of alpha-granules and their contents in thrombin-stimulated platelets. *J. Cell Biol.* 98:748–760.

21. Tokuyasu, K. T. 1980. Immunochemistry on ultrathin frozen sections. *Histochem. J.* 12:381–403.

22. Griffiths, G., R. Brands, B. Burke, D. Louvard, and G. Warren. 1982. Viral membrane proteins acquire galactose in trans Golgi cisternae during intracellular transport. *J. Cell Biol.* 95:781–792.

23. Bainton, D. F., J. L. Ullyot, and M. G. Farquhar. 1971. The development of neutrophilic polymorphonuclear leukocytes in human bone marrow. *J. Exp. Med.* 134:907–934.

24. Cramer, E., K. B. Pryzwansky, J-L. Villeval, U. Testa, and J. Breton-Gorius. 1985. Ultrastructural localization of lactoferrin and myeloperoxidase in human neutrophils by immunogold. *Blood.* 2:423–432.

25. Roos, D. 1980. The Cell Biology of Inflammation. G. Weissman, editor. Elsevier/North Holland, Amsterdam. 337–385.

26. Bjornson, A. B., W. A. Altemeier, and H. S. Bjornsen. 1976. Comparison of the in vitro bactericidal activity of human serum and leukocytes against *Bacteroides fragilis* and *Fusobacterium mortiferum* in aerobic and anaerobic environments. *Infect. Immun.* 14:843–847.

27. Ingham, H. R., P. R. Sisson, R. L. Middleton, K. K. Narang, A. A. Codd, and J. B. Selkon. 1981. Phagocytosis and killing of bacteria in aerobic and anaerobic conditions. *J. Med. Microbiol.* 14:391–399.

28. Hirsch, J. G. 1956. Phagocytin: a bactericidal substance from polymorphonuclear leukocytes. *J. Exp. Med.* 103:589–611.

29. Hirsch, J. G. 1960. Antimicrobial factors in tissues and phagocytic cells. *Bacteriol. Rev.* 21:133–140.

30. Zeya, H. I., and J. K. Spitznagel. 1966. Cationic proteins of poly-

morphonuclear leukocyte lysosomes. II. Composition, properties and mechanism of antibacterial action. *J. Bacteriol.* 91:755–762.

31. Zeya, H. I., and J. K. Spitznagel. 1968. Arginine-rich proteins of polymorphonuclear leukocyte lysosomes. Antimicrobial specificity and biochemical heterogeneity. *J. Exp. Med.* 12:927–941.

32. Westbrook, E. M., R. I. Lehrer, and M. E. Selsted. 1984. Characterization of two crystal forms of neutrophil cationic protein NP2, a naturally occurring broad-spectrum antimicrobial agent from leukocytes. *J. Mol. Biol.* 178:783–785.

33. Lehrer, R. I., K. M. Ladra, and R. B. Hake. 1975. Nonoxidative fungicidal mechanisms of mammalian granulocytes: demonstration of components with candidacidal activity in human, rabbit and guinea pig leukocytes. *Infect. Immun.* 11:1226–1234.

34. Odeberg, H., and I. Olsson. 1975. Mechanisms for the microbicidal activity of cationic proteins of human granulocytes. *Infect. Immun.* 14: 1269–1275.

35. Odeberg, H., and I. Olsson. 1975. Antibacterial activity of cationic proteins from human granulocytes. *J. Clin. Invest.* 56:1118–1124.

36. Olsson, I., and P. Venge. 1974. Cationic proteins of human granulocytes. II. Separation of the cationic proteins of the granules of leukemic myeloid cells. *Blood.* 44:235–246.

37. Drazin, R. E., and R. I. Lehrer. 1977. Fungicidal properties of a chymotrypsin-like cationic protein from human neutrophils: adsorption to *Candida parapsilosis. Infect. Immun.* 17:382–388.

38. Elsbach, P., J. Weiss, R. C. Franson, S. Beckerdite-Quagliata, A. Schneider, and L. Harris. 1979. Separation and purification of a potent bactericidal/permeability increasing protein and a closely related phospholipase A_2 from rabbit polymorphonuclear leukocytes. Observations on their relationship. *J. Biol. Chem.* 254:11000–11009.

39. Olsson, I., H. Odeberg, J. Weiss, and P. Elsbach. 1978. Bactericidal cationic proteins of human granulocytes. *In* Neutral Proteases of Human Polymorphonuclear Leukocytes. K. Havemann and A. Janoff, editors. Urban & Schwarzenberg, Inc., Baltimore-Munich.

40. Collins, M. S., and D. Pappagiannis. 1974. Inhibition by lysozyme of the growth of the spherule phase of *Coccidioides immitis* in vitro. *Infect. Immun.* 10:616–623.

41. Gadebusch, H. H., and A. G. Johnson. 1966. Natural host resistance with *Cryptococcus neoformans.* IV. The effect of some cationic proteins on the experimental disease. *J. Infect. Dis.* 116:551–565.

42. Arnold, R. R., M. F. Cole, and J. R. McGhee. 1977. A bactericidal effect for human lactoferrin. *Science (Wash. DC).* 197:263–265.

43. Ambruso, D. R., and R. B. Johnston, Jr. 1981. Lactoferrin enhances hydroxyl radical production by human neutrophils, neutrophil particulate fractions, and an enzymatic generating system. *J. Clin. Invest.* 67:352–360.

44. Winterbourn, C. C. 1983. Lactoferrin-catalysed hydroxyl radical production. Additional requirement for a chelating agent. *Biochem. J.* 210:15–19.

45. Rest, R. F., M. H. Cooney, and J. K. Spitznagel. 1978. Bactericidal activity of specific and azurophil granules from human neutrophils: Studies with outer-membrane mutants of *Salmonella typhimurium* LT-2. *Infect. Immun.* 19:131–137.

46. Rest, R. F., M. H. Cooney, and J. K. Spitznagel. 1977. Susceptibility of lipopolysaccharide mutants to the bactericidal action of human neutrophil lysosomal fraction. *Infect. Immun.* 16:145–151.

47. Modrzakowski, M. C., M. H. Cooney, L. E. Martin, and J. K. Spitznagel. 1979. Bactericidal activity of fractionated granule contents from human polymorphonuclear leukocytes. *Infect. Immun.* 23:589–591.

48. Modrzakowski, M. C., and J. K. Spitznagel. 1979. Bactericidal activity of fractionated granule contents from human polymorphonuclear leukocytes: antagonism of granule cationic proteins by lipopolysaccharide. *Infect. Immun.* 25:597–602.

49. Shafer, W. M., S. G. Casey, and J. K. Spitznagel. 1984. Lipid A and resistance of *Salmonella typhimurium* to antimicrobial granule proteins of human neutrophil granules. *Infect. Immun.* 43:834–838.

50. Zeya, H. I., and J. K. Spitznagel. 1971. Isolation of polymorphonuclear leukocyte granules from rabbit bone marrow. *Lab. Invest.* 24:237–245.