INFECTION AND IMMUNITY, July 1989, p. 2021–2027
0019-9567/89/072021-07$02.00/0

Vol. 57, No. 7

Purification and Antimicrobial Properties of Three Defensins from Rat Neutrophils

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Received 1 February 1989/Accepted 27 March 1989

Three cysteine-rich cationic peptides, designated RatNP-1, RatNP-3, and RatNP-4, were purified from an acid extract of rat polymorphonuclear neutrophils, sequenced, and tested for antimicrobial activity. The peptides ranged from 29 to 32 amino acids in length (M_r, 3,252 to 3,825), and each contained all eight invariantly conserved "framework" residues that are characteristic of defensins. Each of the peptides killed *Escherichia coli* ML-35, *Acinetobacter calcoaceticus* HON-1, *Staphylococcus aureus* 502A, and *Candida albicans* 820 in vitro. RatNP-1, the most cationic rat defensin, was also the most potent. With this report, a total of 13 distinct defensins have been characterized in the polymorphonuclear leukocytes of four mammalian species. The existence of the defensin system in rats should facilitate investigations of the in vivo role of defensins in experimental infections.

Polymorphonuclear leukocytes (PMN) are major effectors in host defenses against bacteria and fungi. Their ability to kill ingested microorganisms depends on both the generation of toxic microbicidal oxidants (2, 9, 20) and the delivery of preformed antimicrobial proteins or peptides (12, 34, 36) from their cytoplasmic granules to phagosomes. A recently characterized family of small, cysteine-rich peptides (defensins) are prominent among the antimicrobial molecules contained within human, guinea pig, or rabbit PMN (13, 37–40, 42). Their relative abundance, widespread distribution, and broad antimicrobial spectrum—which includes bacteria (13, 39, 42), fungi (13, 23, 35, 39, 41), and some enveloped viruses (6, 13, 22, 39)—suggest that defensins may play significant roles in PMN-mediated host defenses.

Although defensins have not heretofore been described in rat PMN, Modrzakowski et al. reported that an extract prepared from rat PMN exerted antibacterial activity against certain bacteria (17, 18, 24, 25, 28), and they associated the activity of these extracts with small cationic peptides. Because rats are widely used in experimental models of infection, precise characterization of their antimicrobial effector molecules is desirable. In this communication, we describe the purification and primary structures of three rat PMN defensins and provide preliminary evidence of their broad antimicrobial spectrum.

MATERIALS AND METHODS

Purification of PMN. To elicit peritoneal neutrophils, Sprague-Dawley rats were injected intraperitoneally with normal saline solution containing 7.5% sodium caseinate (Eastman Kodak Co., Rochester, N.Y.) in a dose of 0.4 ml/10 g of body weight (3). Six hours later the rats were sacrificed, and peritoneal exudate cells were obtained by lavaging the peritoneal cavity of each rat with 40 ml of Dulbecco phosphate-buffered saline that contained 5 mM glucose and 5 U of heparin per ml (DPBS). The lavage fluids were centrifuged at 200 × *g* for 10 min at 4°C, and the pelleted cells were suspended in DPBS. Erythrocytes were removed by brief hypotonic lysis in ice-cold distilled water. After centrifugation, the PMNs were suspended in DPBS. After removal of samples for total and differential cell counts, the cells were pelleted and frozen at −20°C. Neutrophils accounted for 90 ± 3% of the cells, as assessed by examination of Wright Giemsa-stained slides. Cellular viability before freezing was 98 ± 1% by trypan blue exclusion.

Purification of rat neutrophil peptides. Rat peritoneal neutrophils (4.6×10^9 cells) obtained from a total of 18 rats were suspended in 17 ml of ice-cold 0.05 M sodium phosphate buffer (pH 6.6) that contained 2 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 10 μM pepstatin, 1 mM *n*-ethylmaleimide, and 2 mM EDTA (all obtained from Sigma Chemical Co., St. Louis, Mo.). This mixture was sonicated twice for 20 s each (Bronwill Biosonik IV, maximum power) and kept on ice for 30 min. Next, 9 ml of 20% cold acetic acid was added, and the mixture was sonicated again and stirred for 1 h at 4°C. This extracted sonicate was centrifuged at 27,000 × *g* for 20 min, and the insoluble residue was extracted as above.

The supernatants obtained from these extractions were pooled, dialyzed in Spectrapore 3 tubing (Spectrum Medical Industries, Inc., Los Angeles, Calif.) against 5% acetic acid and concentrated at a volume of 5 ml by vacuum centrifugation (Speed-Vac; Savant Instruments, Inc., Hicksville, N.Y.). This concentrate was chromatographed on a 2.5- by 120-cm column of Bio-Gel P-10 (Bio-Rad Laboratories, Richmond, Calif.) previously equilibrated with 5% acetic acid. We collected 5-ml fractions at a flow rate of 10 ml/h, with monitoring of A_{280}. Samples of alternate fractions were subjected to acid-urea polyacrylamide gel electrophoresis (AU-PAGE) (40). The peptides of interest eluted between fractions 86 and 149. Appropriate fractions were pooled,

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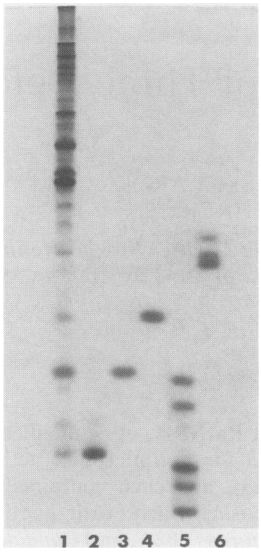

FIG. 1. AU-PAGE of rat, rabbit, and human defensins. Lanes: 1, 40 μg of rat neutrophil extract; 2, 2.0 μg of RatNP-1; 3, 2.0 μg of RatNP-3; 4, 2.0 μg of RatNP-4; 5, 1 μg each of rabbit defensins NP-1 (most cathodal), NP-2, NP-3 (a and b), NP-4, and NP-5; 6, 1.7 μg each of human defensins HNP-1 (most cathodal), HNP-2, and HNP-3.

lyophilized in a vacuum centrifuge, and further purified by reversed-phase high-performance liquid chromatography (RP-HPLC) on a C-18 column (4.6 by 250 mm, Vydac; The Separation Group, Hesperia, Calif.) with a water-acetonitrile gradient that contained 0.1% trifluoroacetic acid as previously described (42). Purity of the peptides was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), AU-PAGE, and RP-HPLC.

Composition analysis. Each peptide was reduced and subsequently alkylated with iodoacetamide in 6.0 M guanidine hydrochloride–0.5 M Tris hydrochloride (pH 8.1)–0.2 mM EDTA as previously described (15). The carboxamidomethylated peptides were desalted by RP-HPLC on a 4.6- by 250-mm C-18 column (Vydac). Performic acid-oxidized, carboxamidomethylated, or native peptides were hydrolyzed under vacuum in 5.7 N HCl for 24 to 72 h. Amino acids were quantitated as phenylthiocarbamyl derivatives by RP-HPLC on a C-18 column as previously described (40). The assay for free sulfhydryls was performed spectrophotometrically with Ellman reagent (16), and carbohydrate content was estimated by the phenol-sulfuric acid assay (8).

Sequence determination. The S-carboxamidomethylated peptides (2 to 4 nmol) were sequenced on a model 475A sequencing system (Applied Biosystems, Inc., Foster City, Calif.), as previously described (37). Confirmation of the carboxyl-terminal residues of the rat peptides was performed by amino acid analysis of residues liberated by carboxypeptidase Y (Pierce Chemical Co., Rockford, Ill.) treatment (1).

Antibacterial assays. We tested the bactericidal activity of rat neutrophil peptides against *Escherichia coli* ML-35, *Staphylococcus aureus* 502A, and *Acinetobacter calcoaceticus* HON-1 (ATCC 14987). The organisms were maintained on tryptic soy agar (Difco Laboratories, Detroit, Mich.). Experimental cultures were grown overnight in full-strength tryptic soy broth (TSB) at 37°C, diluted 1:50 into fresh TSB, and incubated for an additional 18 h at 37°C to prepare stationary-phase cultures. To prepare a mid-log-phase culture of *S. aureus*, an inoculum from an 18-h broth culture was diluted 1:50 with TSB and incubated for 3 h at 37°C with shaking. The concentration of CFU was estimated by spectrophotometry (A_{620}), referenced to previously determined standards. Assay mixtures contained 5×10^4 CFU of washed bacteria with or without 1 to 50 μg of rat neutrophil peptide per ml in a total of 50 μl, per time point, of 10 mM sodium phosphate buffer (pH 7.4) supplemented with TSB (1:100, vol/vol). Controls lacked defensins but were otherwise identical. Experimental and control mixtures were incubated for 2 h at 37°C in a shaking water bath. Thereafter, samples were appropriately diluted, spread on tryptic soy agar plates with a spiral spreader (Spiral Technologies, Rockville, Md.) (7, 14), and incubated for 24 to 48 h to allow full colony development. All experiments were performed in duplicate, with three independent readings obtained from each plate. The technique of spiral spreading afforded highly accurate and reproducible results over a 4-$\log_{10}$ range of CFU per milliliter. The coefficient of variation of individual values in these experiments [(standard deviation × 100)/mean] was approximately 4.2%. Means and standard errors of the means were calculated after $\log_{10}$ transformation of the primary data.

Antifungal assays. *Candida albicans* 820 and *Cryptococcus neoformans* A-383 (kindly provided by D. H. Howard, University of California, Los Angeles), were maintained on Sabouraud 2% dextrose agar plates. Experimental cultures were started from single agar colonies that were grown for 18 h in 50 ml of Sabouraud 2% dextrose broth at 37°C, diluted 1:50 in fresh Sabouraud broth, and incubated at 37°C for an additional 18 h. The test organisms were washed twice in 10 mM sodium phosphate buffer (pH 7.4), counted in a hemacytometer, and adjusted as required. Assay mixtures contained 5×10^4 CFU with or without 1 to 50 μg of rat neutrophil peptide per ml in a final volume of 50 μl of 10 mM sodium phosphate buffer (pH 7.4). These mixtures were incubated at 37°C for 2 h in a shaking water bath. For colony

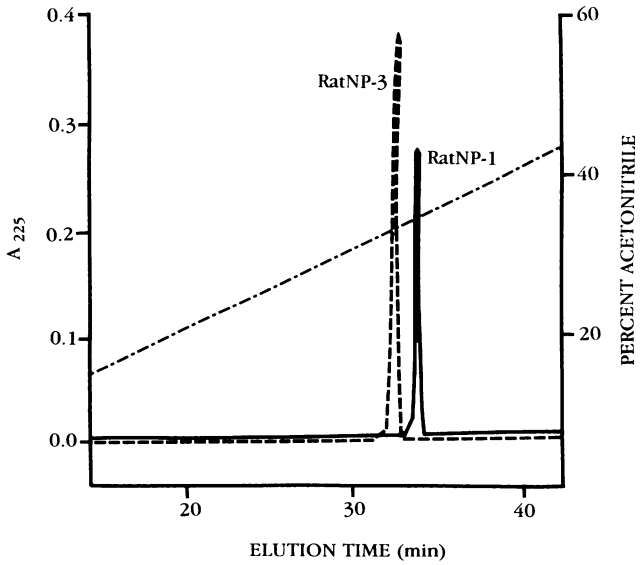

FIG. 2. Chromatogram of purified rat defensins. Purified RatNP-1 and -3 (10 and 20 μg, respectively) were chromatographed on a 4.6- by 250-mm C-18 column by using a 0 to 60% gradient of acetonitrile over 60 min at 1 ml/min. Trifluoroacetic acid (0.1%) was used as an ion-pairing agent. Elution of the rat peptides was monitored spectrophotometrically by A_{225}.

TABLE 1. Amino acid composition and molecular weights[a] of rat defensins

Amino acid	Composition (no. of residues)[b]		
	RatNP-1	RatNP-3	RatNP-4
Cys[c]	4.9 (6)[d]	5.5 (6)	5.3 (6)
Asp		1.2 (1)	1.0 (1)
Glu	0.8 (1)	1.0 (1)	1.0 (1)
Ser	1.2 (1)	3.9 (4)	1.0 (1)
Gly	3.9 (4)	3.0 (3)	4.6 (5)
Arg	7.9 (9)	5.3 (6)	4.4 (5)
Thr	2.1 (2)	1.0 (1)	1.0 (1)
Ala	1.1 (1)	1.0 (1)	2.7 (3)
Tyr	2.2 (3)	0.8 (1)	1.8 (2)
Val	1.3 (1)		1.1 (1)
Ile	1.1 (1)	1.0 (1)	1.9 (2)
Leu	1.9 (2)	3.0 (3)	2.9 (3)
Phe	0.9 (1)	1.0 (1)	

[a] Molecular weights (calculated from sequence analysis data) of RatNP-1, -3, and -4 were 3,825, 3,252, and 3,329, respectively.
[b] Values determined from analysis of 72-h hydrolysates.
[c] Cysteine was determined both as cysteic acid and *S*-carboxymethyl cysteine.
[d] Numbers within parentheses indicate residues determined by sequence analysis.

counting, samples were removed, appropriately diluted, and spread on Sabouraud agar plates with a spiral spreader. *C. albicans* colonies were counted 24 to 48 h later; *C. neoformans* colonies were counted after 72 h.

Direct viral neutralization. The ability of rat granulocyte defensins to directly inactivate type 1 herpes simplex virus (McIntyre strain) was tested as previously described (6, 22).

RESULTS

Peptide purification. AU-PAGE revealed that our initial acid extract of rat PMN contained five prominent cationic peptides (Fig. 1) that were designated rat neutrophil peptide 1 (RatNP-1) through -5, in order of their relative cathodal migration. Two of these peptides, RatNP-2 and -5, were substantially less abundant than the others and were not available in sufficient quantities for sequencing and functional testing. To determine whether any of the other three peptides was a defensin, we chromatographed the crude extract on Bio-Gel P-10 and examined their contents by AU-PAGE and SDS-PAGE. The fractions containing RatNP-1, -3, and -4 of the crude extract were purified to homogeneity by RP-HPLC (Fig. 2). The purity of the three peptides was confirmed by AU-PAGE (Fig. 1), RP-HPLC (Fig. 2), and SDS-PAGE (data not shown).

Characterization of rat peptides. The amino acid composition of RatNP-1, -3, and -4 revealed that each had a relatively high content of cysteine and arginine (Table 1), as had previously been noted for rabbit, human, and guinea pig defensins (37, 39, 40). Lysine and tryptophan residues were absent. RatNP-4 lacked the phenylalanine residue present in RatNP-1 and -3. Although RatNP-2 and -5 were not fully purified and sequenced, preliminary amino acid analyses suggest that these may also prove to be defensins (data not shown). In other experiments, we found that these rat neutrophil peptides were especially abundant in subcellular granule-rich fractions of rat PMN, consistent with the localization of defensins in human, rabbit, and guinea pig leukocytes (data not shown).

The molecular weights of RatNP-1, -3, and -4 as estimated by SDS-PAGE (data not shown) were consistent with the values obtained from the amino acid composition and sequence data shown in Table 1. Figure 3 compares the primary amino acid sequences of RatNP-1, -3, and -4 with the primary amino acid sequences of 10 other known defensins (37, 39, 40); note that all six cysteines in each rat peptide align precisely with the invariant cysteine residues of the other defensins. Figure 4 shows the remarkable similarity of RatNP-4 to the human defensin HNP-1; 17 of their residues coincided precisely, and another 6 were conservatively replaced. Given their size, cationicity, abundance in granulocytes, homologies, and especially their fully conserved cysteine motif, it is clear that RatNP-1, -3, and -4 are defensins.

Antimicrobial activity. Because the previously described members of the defensin family exerted antimicrobial activity in vitro, we examined the effects of the three rat defensins on selected bacteria and fungi (Table 2). Each rat defensin was more potent against *E. coli* ML-35 than the human PMN defensin HNP-1. Note that the relative efficacy of these rat defensins was in the same order of their net cationicity (positive charge): RatNP-1 > RatNP-2 > RatNP-4 (Fig. 5). All three rat neutrophil peptides also killed >90% of *A. calcoaceticus* in 2 h when tested at 50 μg/ml under our standard incubation conditions (data not shown).

Midlogarithmic-phase *S. aureus* 502A was also highly sensitive to the bactericidal activity of RatNP-1 (Table 2,

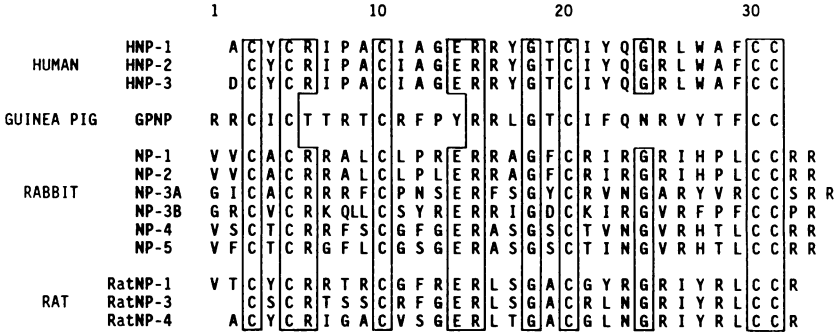

FIG. 3. Primary structures of rat, rabbit, human, and guinea pig defensins. The amino acid sequences of the defensins are shown with maximal alignment and enclosure of the framework residues shared by all of the peptides. Abbreviations: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine. Residue numbering is indexed to amino acid 1 of the rabbit defensins.

FIG. 4. Comparison of rat (RatNP-4) and human (HNP-1) defensins. Amino acid residues (first 2 lines) are shown in single-letter code as defined in the legend to Fig. 3. The identical residues are enclosed; c, conservative substitutions.

Fig. 6). At a concentration of 1 μg/ml, RatNP-1 caused a 2-$\log_{10}$-unit decrease in *S. aureus* CFU per milliliter, whereas 2.5 μg/ml reduced the viable counts by 5 $\log_{10}$ units. RatNP-1 was also effective against stationary-phase *S. aureus*; however, 10 times more (25 μg/ml) RatNP-1 was necessary to decrease the CFU per milliliter by 5 $\log_{10}$ units (data not shown). Although 50 μg of either RatNP-3 or -4 per ml killed approximately 2 $\log_{10}$ units of midlogarithmic *S. aureus* 502A, neither peptide (50 μg/ml) was effective against stationary-phase *S. aureus* 502A (Table 2).

RatNP-1 was also more potent against *C. albicans* than was RatNP-3, RatNP-4 or the human defensin HNP-1 (Fig. 7). At a concentration of 5 μg/ml, RatNP-1 caused approximately a 2-$\log_{10}$ unit (98%) decrease in *Candida* CFU per milliliter, and at 25 μg/ml the viable counts were reduced by 4 $\log_{10}$ units. Although RatNP-3 and -4 were not active at concentrations between 1 and 5 μg/ml, higher concentrations caused significant killing. At a concentration of 10 μg/ml, both RatNP-3 and -4 killed >97% of *C. neoformans* (Fig. 8), suggesting that this fungus might be more susceptible to defensins than was *C. albicans*. We lacked sufficient RatNP-1 to test this defensin against *C. neoformans*.

The three rat defensins were also tested for their ability to directly inactivate herpes simplex virus type 1 (McIntyre strain). RatNP-1 (50 μg/ml) caused modest direct viral neutralization, reducing the number of surviving herpes simplex virus type 1 PFU per milliliter by 90% after 60 min. RatNP-3 and -4 were inactive under these conditions (data not shown).

DISCUSSION

Since Metchnikoff described the role of PMN in defense against infection (reviewed in reference 27), much effort has focused on understanding the biochemical basis of their antimicrobial activity. PMN antimicrobial mechanisms may be classified as either oxidative or nonoxidative. The oxidative mechanisms depend on the production of toxic oxidants, such as H_2O_2, OH^-, OCl^-, and chloramines, by stimulated PMN (2, 20). These oxidants act directly or in concert with other PMN components to destroy invading microbes (2, 20).

The nonoxidative antimicrobial mechanisms of PMN arise, in part, from the effects of antimicrobial proteins or peptides sequestered within the cytoplasmic granules of the PMN (12, 34). Among these antimicrobial effector molecules are lysozyme (5, 11), cathepsin G (29–31), bactericidal permeability-increasing protein (BPI) (10) and related or additional factors (19, 21), and defensins (13, 37–39, 42). Additional types of antimicrobial peptides have recently been reported to exist in bovine PMN (26, 33).

The structural and functional attributes of the three rat peptides described in this report establish their identity as members of the defensin peptide family. Like other defensins (6, 13, 35, 39, 41, 42), the rat peptides displayed antimicrobial activity against a broad spectrum of microbial targets. These included *S. aureus* 502A, two gram-negative bacteria, two fungi, and, to a lesser extent, an enveloped virus. Because we purified RatNP-1, -3, and -4 to homogeneity and we used different microbial targets and assay conditions, it is not possible to compare our studies directly with those of Modrzakowski et al. (17, 18, 25, 28). Nevertheless, our data confirm their report that cationic, low-molecular-weight antimicrobial peptides exist in rat PMN.

The rat peptides described in this report retain all of the eight framework residues common to the other 10 known members of the defensin family (37, 39, 40). Six of these conserved amino acids are half-cystines, whose intramolecular disulfide bonds shape and stabilize the fold of the polypeptide backbone. Arginine-15 and glycine-18 are also conserved. Conservation of these eight framework residues, particularly the six half-cystines, provides a hallmark for the defensin family. The disulfide pairing (39a) and the backbone

TABLE 2. Effects of purified rat peptides (defensins) on test organisms[a]

Organism	Peptide	TSB	*n*	Mean CFU/ml		$\log_{10}$ reduction[b]
				Control[c]	Treated	
S. aureus 502A (18 h)	RatNP-1	+	1	1.5×10^6	$<10^2$	>4.17
	RatNP-3	+	3	1.4×10^6	8.9×10^5	0.20 ± 0.07
	RatNP-4	+	3	1.4×10^6	9.1×10^5	0.13 ± 0.10
S. aureus 502A (midlogarithmic)	RatNP-1	+	2	2.0×10^6	$<10^2$	>4.30
	RatNP-3	+	3	2.0×10^6	2.5×10^4	1.90 ± 0.20
	RatNP-4	+	3	2.0×10^6	1.3×10^4	2.20 ± 0.10
E. coli ML-35 (18 h)	RatNP-1	+	1	1.9×10^7	$<10^2$	>5.28
	RatNP-3	+	4	1.3×10^7	1.7×10^2	4.37 ± 0.12
	RatNP-4	+	4	1.3×10^7	1.6×10^3	3.89 ± 0.19
C. albicans 820 (18 h)	RatNP-1	−	1	1.2×10^6	$<10^2$	>4.08
	RatNP-3	−	3	1.2×10^6	7.6×10^3	2.19 ± 0.27
	RatNP-4	−	3	1.2×10^6	1.5×10^3	2.91 ± 0.04
C. neoformans A-383 (18 h)	RatNP-3	−	3	1.3×10^6	1.7×10^2	3.89 ± 0.08
	RatNP-4	−	3	1.3×10^6	2.7×10^2	3.13 ± 0.43

[a] Stationary-phase (18-h) or midlogarithmic-phase organisms, approximately 10^6 CFU/ml, were incubated for 2 h at 37°C with 50 μg of rat peptide per ml in 10 mM sodium phosphate buffer with (+) or without (−) supplementation with 1% (vol/vol) TSB.

[b] $\log_{10}$ decrease from incubated controls after 2 h (mean ± standard error of the mean).

[c] Controls were incubated for 2 h in defensin-free medium as described in Materials and Methods.

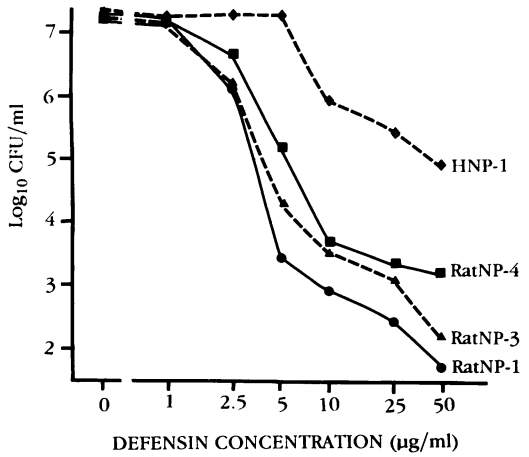

FIG. 5. Antibacterial activity of rat defensins against *E. coli* ML-35. Stationary-phase (18-h) organisms (10^6 CFU/ml) were incubated with the indicated concentrations of RatNP-1 (●), RatNP-3 (▲), RatNP-4 (■), and HNP-1 (◆) in 10 mM sodium phosphate buffer supplemented with TSB (pH 7.4) for 2 h at 37°C. Data for RatNP-3 and -4 are mean values of three experiments performed with each. Single experiments were done with RatNP-1 and HNP-1.

structure of several defensins (4, 32) have been described elsewhere.

In addition to possessing the eight framework residues common to all defensins, rat defensins shared three additional amino acids with their human and rabbit congeners: arginine-6, glutamic acid-14, and glycine-24 (Fig. 3). When compared with each other, the three rat peptides were homologous in 18 of their 29 to 32 residue positions (Fig. 3).

RatNP-4 was remarkably similar to the human defensins. When RatNP-4 (31 residues) was aligned with human HNP-1 (30 residues), 17 amino acids were identical and 7 were conservatively substituted (Fig. 4). Overall, the sequence homology among these defensins was as follows: rat to rat, 62 to 68% identical; rat to human, 38 to 57% identical; rat to rabbit, 32 to 58% identical; rat to guinea pig, 32 to 42% identical.

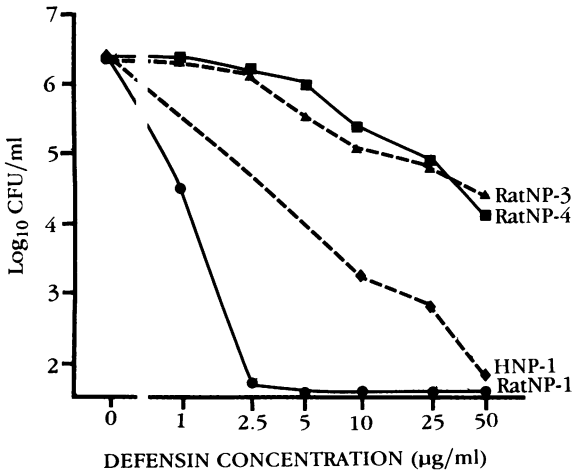

FIG. 6. Antibacterial activity of rat defensins against *S. aureus* 502A. Mid-log-phase organisms (10^6 CFU/ml) were incubated with the indicated concentrations of RatNP-1 (●), RatNP-3 (▲), RatNP-4 (■), and HNP-1 (◆) in 10 mM sodium phosphate buffer supplemented with TSB (pH 7.4) for 2 h at 37°C. Data for RatNP-3 and -4 are means of two experiments with each. Single experiments were done with RatNP-1 and HNP-1.

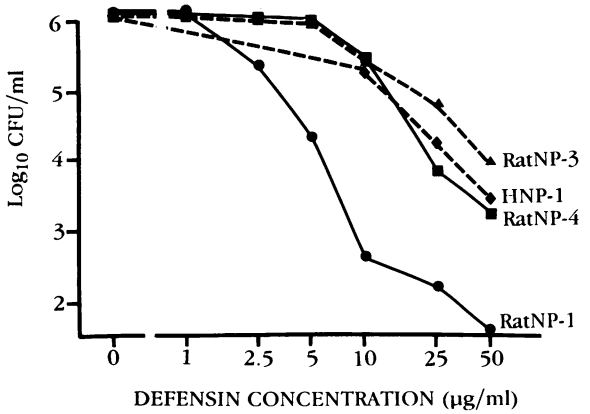

FIG. 7. Antifungal activity of rat defensins against *C. albicans* 820. Blastoconidia (10^6 CFU/ml) were incubated for 2 h at 37°C with the indicated concentrations of RatNP-1 (●), RatNP-3 (▲), RatNP-4 (■), and HNP-1 (◆) in 10 mM sodium phosphate buffer (pH 7.4). Data for RatNP-3 and -4 are means of two experiments with each. Single experiments were done with RatNP-1 and HNP-1.

Although our limited quantities of purified rat defensins precluded more exhaustive functional testing, our experiments established that members of the newly characterized rat defensin family exerted microbicidal activity against selected gram-positive and gram-negative bacteria and certain fungi in vitro. RatNP-1 appeared to be the most potent of these rat defensins, perhaps because it was also the most cationic. This behavior was consistent with our findings that the relative bactericidal (13, 42) and fungicidal (6, 13, 23, 35, 41) efficacy of human and rabbit defensins correlated with the net positive charge of the peptides. Our finding that stationary-phase *S. aureus* was considerably less susceptible to killing by rat defensins than midlogarithmic-phase organisms suggests that target cell growth and/or metabolism modulates susceptibility to rat PMN defensins. We have observed similar results when human PMN defensins were tested against *S. aureus* 502A and *E. coli* ML-35 (unpublished data) and *C. albicans* (13).

With this report, a total of 13 defensins have now been found in the PMN of four mammalian species: rabbits, guinea pigs, humans, and rats. It appears that defensins are

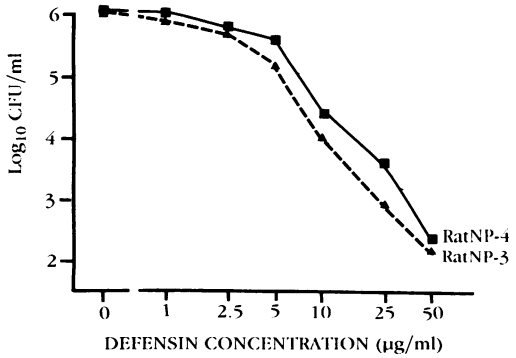

FIG. 8. Antifungal activity of rat defensins against *C. neoformans* A-383. Stationary-phase organisms (10^6 CFU/ml) were incubated with increasing concentrations of RatNP-3 (▲) and RatNP-4 (■) in 10 mM sodium phosphate buffer (pH 7.4) for 2 h at 37°C. Data for RatNP-3 and RatNP-4 are means of three experiments with each.

widely distributed within the PMN of diverse species. Because these 13 defensins diverge markedly in their antimicrobial spectrum and potency, such differences may contribute to interspecies variations in resistance to given pathogens. The ability to compare defensins from the phagocytic cells of different mammalian species can provide a powerful tool for investigating host resistance to pathogenic microorganisms.

ACKNOWLEDGMENTS

We thank Sammy Saab and Anne Barton for their helpful cooperation with these experiments and Erzsebet Huffman for preparing the manuscript. The careful attention of the University of California, Los Angeles, Chancellor's Animal Research Committee also deserves mention.

These studies were supported in part by grants from the Will Rogers Foundation, Endowment for Pulmonary Research, the National Institutes of Health (Public Health Service grants HL 35640 [to T. Ganz], AI 16252, AI 22839 [to R. I. Lehrer], and AI 22931), and the Office of Naval Research (grant N00014 86 K 0525 to M. E. Selsted).

LITERATURE CITED

1. **Allen, G.** 1981. Preliminary characterization of the protein, p. 41–42. *In* F. S. Work and R. H. Burdon (ed.), Sequencing of proteins and peptides. Elsevier/North-Holland Publishing Co., Amsterdam.
2. **Babior, B. M.** 1980. The role of oxygen radicals in microbial killing by phagocytes, p. 339–354. *In* A. J. Sbarra and R. R. Strauss (ed.), The reticuloendothelial system. A comprehensive treatise, vol. 2. Biochemistry and metabolism. Plenum Publishing Corp., New York.
3. **Babior, B. M., and H. J. Cohen.** 1981. Measurement of neutrophil function: phagocytosis, degranulation, the respiratory burst and bacterial killing, p. 1–28. *In* M. J. Cline (ed.), Leukocyte function. Churchill Livingstone, Ltd., New York.
4. **Bach, A. C., M. E. Selsted, and A. Pardi.** 1987. Two-dimensional NMR studies of the antimicrobial peptide NP-5. Biochemistry **26**:4389–4397.
5. **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.
6. **Daher, K. A., M. E. Selsted, and R. I. Lehrer.** 1986. Direct inactivation of viruses by human granulocyte defensins. J. Virol. **60**:1068–1074.
7. **Donnelly, C. B., J. E. Giochrist, J. T. Peeler, and J. E. Campbell.** 1976. Spiral plate count method for the examination of raw and pasteurized milk. Appl. Environ. Microbiol. **32**:21–27.
8. **Dubois, M., K. A. Gilles, P. A. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350–356.
9. **Elsbach, P., and J. Weiss.** 1983. A reevaluation of the roles of O_2-dependent and O_2-independent microbicidal systems of phagocytes. Rev. Infect. Dis. **5**:843–853.
10. **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.
11. **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.
12. **Ganz, T., M. E. Selsted, and R. I. Lehrer.** 1986. Antimicrobial activity of phagocyte granule proteins. Semin. Res. Infect. **1**:107–117.
13. **Ganz, T., M. E. Selsted, D. Szklarek, S. S. L. Harwig, K. Daher, and R. I. Lehrer.** 1985. Defensins: natural peptide antibiotics of human neutrophils. J. Clin. Invest. **76**:1427–1435.
14. **Giochrist, J. E., C. B. Donnelly, J. T. Peeler, and J. E. Campbell.** 1977. Collaborative study comparing the spiral plate and aerobic plate count methods. J. Assoc. Off. Anal. Chem. **60**:807–812.
15. **Glazer, A. M., R. J. DeLange, and D. S. Sigman.** 1975. Modification of protein side-chains: group-specific reagents, p. 103–104. *In* T. S. Work and E. Work (ed.), Chemical modification of proteins. Elsevier/North-Holland Publishing Co., Amsterdam.
16. **Glazer, A. M., R. J. DeLange, and D. S. Sigman.** 1975. Modification of protein side-chains: group-specific reagents, p. 113–114. *In* T. S. Work and E. Work (ed.), Chemical modification of proteins. Elsevier/North-Holland Publishing Co., Amsterdam.
17. **Hodinka, R. L., and M. C. Modrzakowski.** 1983. Bactericidal activity of granule contents from rat polymorphonuclear leukocytes. Infect. Immun. **40**:139–146.
18. **Hodinka, R. L., and M. C. Modrzakowski.** 1986. Granule contents from rat polymorphonuclear leukocytes: antimicrobial properties and characterization. Can. J. Microbiol. **32**:498–504.
19. **Janoff, A., and J. Scherer.** 1968. Mediators of inflammation in leukocyte lysosomes. IX. Elastinolytic activity in granules of human polymorphonuclear leukocytes. J. Exp. Med. **128**:1137–1155.
20. **Klebanoff, S. J., and R. A. Clark.** 1978. The neutrophil: function and clinical disorders, p. 409–466. Elsevier/North Holland Publishing Co., Amsterdam.
21. **Leffell, M. S., and J. K. Spitznagel.** 1972. Association of lactoferrin with lysozyme in granules of human polymorphonuclear leukocytes. Infect. Immun. **6**:761–765.
22. **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.
23. **Lehrer, R. I., D. Szklarek, T. Ganz, and M. E. Selsted.** 1985. Correlation of binding of rabbit granulocyte peptides to *Candida albicans* with candidacidal activity. Infect. Immun. **49**:207–211.
24. **Loeffelholz, M. J., and M. C. Modrzakowski.** 1986. Isolation of cationic peptides from rat polymorphonuclear leukocyte granule contents using fast protein liquid chromatography. Anal. Biochem. **158**:377–381.
25. **Loeffelholz, M. J., and M. C. Modrzakowski.** 1988. Antimicrobial mechanisms against *Acinetobacter calcoaceticus* of rat polymorphonuclear leukocyte granule extract. Infect. Immun. **56**:552–556.
26. **Marzari, R., B. Scaggiante, B. Skerlavaj, M. Bittolo, R. Gennaro, and D. Romeo.** 1988. Small, antibacterial and large, inactive peptides of neutrophil granules share immunoreactivity to a monoclonal antibody. Infect. Immun. **56**:2193–2197.
27. **Metchnikoff, E.** 1905. Immunity in infective diseases. Cambridge University Press, Cambridge.
28. **Modrzakowski, M. D., D. Dosch-Meier, and R. L. Hodinka.** 1983. Effect of rat polymorphonuclear leukocyte granule components on the growth and survival of *Pseudomonas aeruginosa* and *Salmonella typhimurium*. Can. J. Microbiol. **29**:1339–1343.
29. **Odeberg, H., and I. Olsson.** 1975. Mechanisms for the microbicidal activity of cationic proteins of human granulocytes. Infect. Immun. **14**:1269–1275.
30. **Odeberg, H., and I. Olsson.** 1975. Antibacterial activity of cationic proteins from human granulocytes. J. Clin. Invest. **56**:1118–1124.
31. **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.
32. **Pardi, A., D. R. Hare, M. E. Selsted, R. D. Morrison, D. A. Bassolino, and A. C. Bach.** 1988. Solution structures of the rabbit defensin NP-5. J. Mol. Biol. **201**:625–636.
33. **Romeo, D., B. Skerlavaj, M. Bolognesi, and R. Gennaro.** 1988. Structure and bactericidal activity of an antibiotic dodecapeptide purified from bovine neutrophils. J. Biol. Chem. **263**: 9573–9575.
34. **Root, R. K., and M. S. Cohen.** 1981. The microbicidal mechanisms of human neutrophils and eosinophils. Rev. Infect. Dis. **3**:565–598.
35. **Segal, G. P., R. I. Lehrer, and M. E. Selsted.** 1985. In vitro effect of phagocyte cationic peptides of *Coccidioides immitis*. J.

Infect. Dis. **151**:890–894.
36. **Selsted, M. E.** 1988. Nonoxidative killing by neutrophils. Ann. Intern. Med. **109**:127–142.
37. **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**: 4579–4585.
38. **Selsted, M. E., D. M. Brown, R. J. DeLange, and R. I. Lehrer.** 1983. Primary structures of MCP-1 and MPC-2, natural peptide antibiotics of rabbit lung macrophages. J. Biol. Chem. **258**: 14485–14489.
39. **Selsted, M. E., and S. S. L. Harwig.** 1987. Purification, primary structure, and antimicrobial activities of a guinea pig neutrophil defensin. Infect. Immun. **55**:2281–2286.
39a. **Selsted, M. E., and S. S. L. Harwig.** 1989. Determination of the disulfide array in the human defensin HNP-2. J. Biol. Chem. **264**:4003–4007.
40. **Selsted, M. E., S. S. L. Harwig, T. Ganz, J. W. Schilling, and R. I. Lehrer.** 1985. Primary structures of three human neutrophil defensins. J. Clin. Invest. **76**:1436–1439.
41. **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.
42. **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.