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Polymorphic Expression of Defensins in Neutrophils from Outbred Rats

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We isolated and characterized a rat neutrophil defensin, RatNP-2, that differs from the previously described defensin RatNP-1 by containing Ser-7 in place of Arg-7. Although the resulting charge difference rendered RatNP-2 easily distinguishable from RatNP-1 on polyacrylamide gel electrophoresis gels, the two defensins exhibited very similar antimicrobial efficacies against *Salmonella typhimurium*, *Staphylococcus aureus*, and *Candida albicans*. The polymorphonuclear leukocytes of Sprague-Dawley rats obtained from one of two breeders also showed a marked polymorphism for defensin RatNP-4. This defensin was absent in two of seven animals and present in 1× or 2× relative amounts in the others. These observations indicate that a striking degree of defensin polymorphism exists in the polymorphonuclear leukocytes of outbred rodents.

Defensins are small, cysteine-rich microbicidal peptides that are found in neutrophils (polymorphonuclear leukocytes [PMN]) and certain macrophages of many mammals (reviewed in reference 6). Homologous antimicrobial peptides (insect defensins) have also been described recently in some invertebrates (9, 13). Given their abundance in PMN granules, their apparent evolutionary conservation, and their broad-spectrum in vitro antimicrobial activity, defensins are likely to contribute significantly to oxygen-independent antimicrobial systems of phagocytes. In addition, some defensins exhibit cytotoxic (10–12, 17, 19), chemotactic (20), or opsonic (4) properties, while others can inhibit corticosterone production by adrenocorticotropin-stimulated adrenal cells (18, 22, 23) or release histamine from mast cells (21).

We recently reported the biochemical and antimicrobial properties of three defensins that were regularly expressed in the PMN of outbred Sprague-Dawley rats and mentioned the existence of additional minor defensins (1). In this paper we describe the results of recent experiments on PMN from Sprague-Dawley rats obtained from a different breeding colony that clarified the nature of one of these minor defensins and indicated that defensin expression in rat PMN exhibits marked polymorphism.

MATERIALS AND METHODS

Purification of neutrophils and peptides. Sprague-Dawley female rats that were 8 to 12 weeks old were obtained from Charles River Breeding Laboratories, Inc., Wilmington, Mass., and Harlan Sprague Dawley, Inc., Indianapolis, Ind. Elicited rat neutrophils were prepared as previously described (1) and contained >90% viable PMN as determined by trypan blue exclusion and Giemsa staining. Neutrophils from 18 rats were pooled and suspended in ice-cold calcium- and magnesium-free Hanks balanced salt solution (pH 7.4) containing 2.5 mM $MgCl_2$, placed in a cell disruption bomb (Parr Instrument Co., Moline, Ill.) at 4°C, and equilibrated with nitrogen at 750 lb/in^2 for 20 min. Following its release from the bomb, the suspension was collected into Hanks balanced salt solution containing 5 mM disodium EDTA and centrifuged at 450 × *g* for 10 min at 4°C to sediment nuclei and undisrupted cells. The supernatant was removed and centrifuged at 27,000 × *g* in a Sorvall model RC-5B centrifuge for 20 min at 4°C. The granule-rich pellet was recovered, suspended in 40 ml of ice-cold 0.05 M sodium phosphate buffer (pH 6.6) containing protease inhibitors, sonicated, and extracted twice with 5% acetic acid as previously described for whole cells (1). This preparation was stirred for 1 h at 4°C, and the acid-insoluble residue was removed by centrifugation at 27,000 × *g* for 20 min at 4°C. The supernatants were dialyzed against 5% acetic acid, concentrated, and then fractionated by gel filtration on a Bio-Gel P-10 column previously equilibrated with 5% acetic acid. Defensin-containing fractions, which were identified by their electrophoretic migration on acid-urea and sodium dodecyl sulfate-polyacrylamide gels, were pooled and concentrated by vacuum centrifugation. RatNP-2 was obtained by reverse-phase high-performance liquid chromatography (RP-HPLC) on a Vydac C_{18} column (Separations Group, Hesperia, Calif.), using a water-acetonitrile gradient that contained 0.1% trifluoracetic acid as previously described (16). Peptide purity was assessed by acid-urea polyacrylamide gel electrophoresis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and RP-HPLC.

Biochemical analysis. The amino acid composition of the purified peptide was determined by RP-HPLC on a Novapak C_{18} column (Millipore Waters, Milford, Mass.), using phenylthiocarbamyl derivatives (15). Native or carboxamidomethylated (8) RatNP-2 was hydrolyzed in vacuo in 5.7 N HCl for 40 h. For quantification of half-cystine residues, hydrolysis was performed on performic acid-oxidized peptide. For the primary sequence determination, 1 to 2 nmol of *S*-carboxamidomethylated RatNP-2 was subjected to gas phase Edman degradation in an instrument obtained from Applied Biosys-

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TABLE 1. Amino acid sequences of RatNP-1 and RatNP-2

Defensin	Sequence[a]
RatNP-1	VTCYCRRTRCGFRERLSGACGYRGRIYRLCCR
RatNP-2	VTCYCRSTRCGFRERLSGACGYRGRIYRLCCR

[a] Abbreviations: A, alanine; C, cysteine; E, glutamic acid; F, phenylalanine; G, glycine; I, isoleucine; L, leucine; P, proline; R, arginine; S, serine; T, threonine; V, valine; Y, tyrosine.

tems, Inc., Foster City, Calif., as previously described (14). Fast atom bombardment mass spectrometry analysis was performed by workers in Terry Lee's laboratory at the Beckman Research Institute of the City of Hope, Duarte, Calif., using a JEOL model HX100HF double-focusing magnetic sector mass spectrometer. Chymotrypsin digestion (Worthington Biochemical Corp., Freehold, N.J.) was carried out on performic acid-oxidized RatNP-1 and RatNP-2 as previously described (15). RatNP-1 and RatNP-2 digestion products were purified by RP-HPLC on a Vydac 300 Angstrom C_{18} column (4.6 by 250 mm), using a water-acetonitrile gradient containing 0.1% trifluoroacetic acid. Purified fragments were hydrolyzed and subjected to amino acid analysis as described above. The corresponding C-terminal fragments were identified, and the amino acid compositions were compared.

Antimicrobial assays. *Candida albicans* UC820 was used to test for fungicidal activity (1). Bactericidal assays were performed by using *Staphylococcus aureus* 502A and *Salmonella typhimurium* 14028S and 7953S (2, 3), which were provided by Eduardo Groismann and Fred Heffron, Scripps Clinic Research Institute, La Jolla, Calif. *Salmonella typhimurium* strains were cultured in Trypticase soy broth and harvested in the stationary growth phase after 18 h of incubation. Mid-logarithmic-phase *Staphylococcus aureus* cultures were prepared as previously described (1).

RESULTS

Characterization of RatNP-2. Tables 1 and 2 show the primary amino acid sequences and amino acid compositions of RatNP-1 and RatNP-2. Gas phase sequencing demonstrated that these peptides differed in only a single residue; the arginine-7 in RatNP-1 was replaced by serine-7 in RatNP-2. Neither the amino acid analysis nor the sequence data clearly resolved whether the carboxy terminus of RatNP-2 ended with a single or double arginine residue. However, the C-terminal fragments of chymotrypsin-digested performic acid-oxidized RatNP-1 and RatNP-2 were eluted at the same acetonitrile concentration during RP-HPLC, and during subsequent amino acid analysis they yielded almost identical ratios of cysteic acid to arginine to leucine (2:2:0.9 for RatNP-1 and 2:2:0.8 for RatNP-2). This indication that only a single arginine residue was present at the C-terminal end of both peptides was confirmed by fast atom bombardment mass spectrometry analysis of RatNP-2, which revealed a mass of 3,761.2 a.m.u. The calculated average mass for the protonated molecular ion of RatNP-2 with a single arginine at its C terminus was 3,761.48 atomic mass units.

TABLE 2. Amino acid compositions of RatNP-1 and RatNP-2

Amino acid	Composition[a]	
	RatNP-1	RatNP-2
Cys[b]	5.9 (6)[c]	5.9 (6)
Glu	1.2 (1)	1.2 (1)
Ser	1.2 (1)	1.8 (2)
Gly	4.1 (4)	3.9 (4)
Arg[d]	8.5 (9)	7.5 (8)
Thr	2.1 (2)	1.9 (2)
Ala	1.1 (1)	1.1 (1)
Tyr	2.4 (3)	2.6 (3)
Val	0.9 (1)	0.9 (1)
Ile	0.8 (1)	0.7 (1)
Leu	1.9 (2)	1.7 (2)
Phe	0.9 (1)	0.9 (1)

[a] Values determined from an analysis of 40-h hydrolysates. The following molecular weights were determined from a sequence analysis: RatNP-1, 3,829.5; RatNP-2, 3,760.4.

[b] Cysteine was determined as cysteic acid.

[c] The numbers in parentheses are the numbers of residues as determined by a sequence analysis.

[d] Chymotrypsin digestion was used to analyze the carboxy-terminal residues.

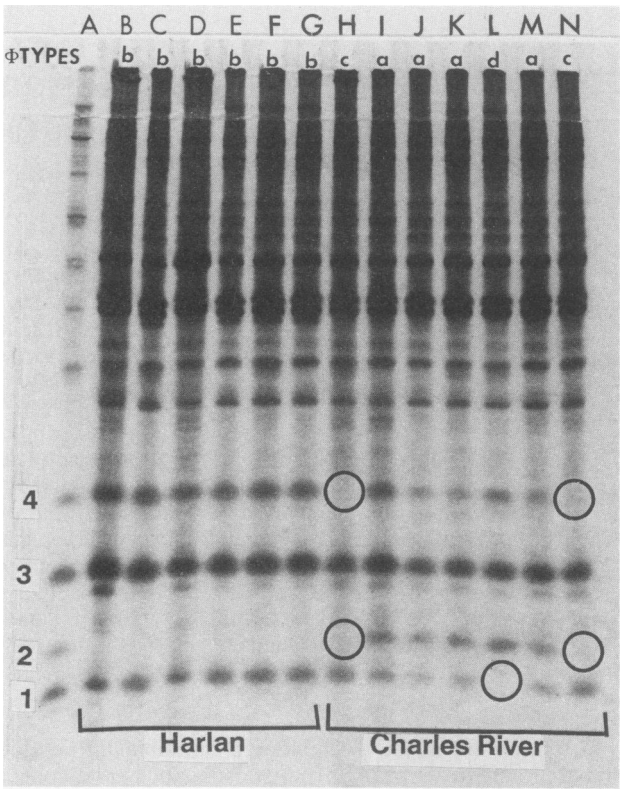

FIG. 1. Acid-urea polyacrylamide gel electrophoresis of PMN extracts from individual rats obtained from different breeding colonies. The 12.5% acrylamide gel contained 5 M urea buffered with 0.9 M acetic acid and was stained with Coomassie brilliant blue. Lane A contained crude PMN granule extract corresponding to 10^7 cell equivalents. Band 1, RatNP-1; band 2, RatNP-2; band 3, RatNP-3; band 4, RatNP-4. The rat defensins were designated in order of their relative cathodal migration, as previously described (1). Lanes B through G contained crude whole-cell PMN extracts from 10^7 neutrophils from Harlan Sprague Dawley rats, and lanes H through N contained crude whole-cell PMN extracts from 10^7 neutrophils from Charles River Breeding Laboratories rats. The circles indicate specific defensins that were absent. Overall the following defensin phenotypes (ϕ types) were included: phenotype a [1(+), 2(+), 3(+), 4(+)], four animals; phenotype b [1(+), 2(−), 3(+), 4(+)], six animals; phenotype c [1(+), 2(−), 3(+), 4(−)], two animals; phenotype d [1(−), 2(+), 3(+), 4(+)], one animal.

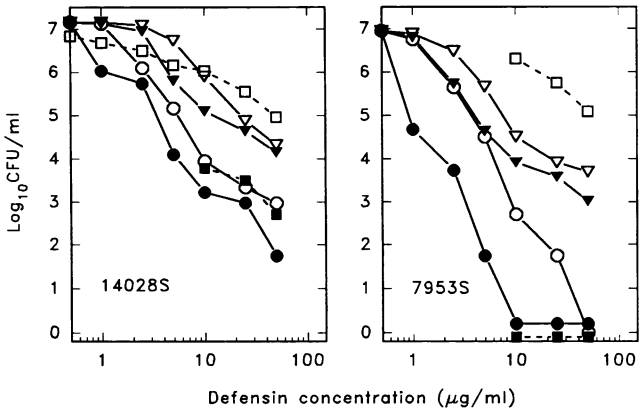

FIG. 2. Antibacterial activity of rat defensins against *Salmonella typhimurium* 14028S and 7953S. Stationary-phase (18-h) organisms (10^6 CFU/ml) were incubated with different concentrations of RatNP-1 (●), RatNP-2 (○), RatNP-3 (▼), RatNP-4 (▽), human HNP-1 (□), and rabbit NP-1 (■) in 10 mM sodium phosphate buffer supplemented with Trypticase soy broth (pH 7.4) for 2 h at 37°C. Homogeneously pure RatNP-1, RatNP-3, RatNP-4, rabbit NP-1, and human HNP-1 were prepared as previously described (1, 7, 16). Purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and acid-urea polyacrylamide gel electrophoresis, as well as by RP-HPLC.

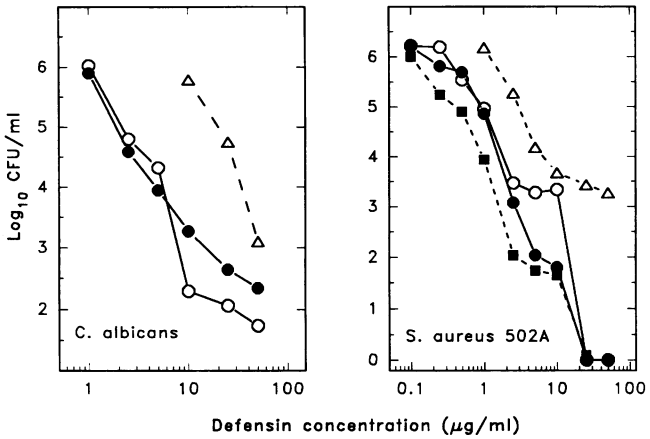

FIG. 3. Antimicrobial activity of RatNP-1 and RatNP-2 against *C. albicans* UC820 and *Staphylococcus aureus* 502A. Organisms (10^6 CFU/ml) were incubated for 2 h at 37°C with different concentrations of RatNP-1 (●), RatNP-2 (○), human HNP-1 (△), and rabbit NP-1 (■) in 10 mM sodium phosphate buffer (*C. albicans*) or in buffer that was supplemented with 1% (vol/vol) Trypticase soy broth (*Staphylococcus aureus*). The staphylococci were in the mid-logarithmic growth phase, and the *C. albicans* cells were from an overnight (18-h) stationary-phase culture.

Polymorphism of defensin expression. Figure 1 shows the results of the acid-urea polyacrylamide gel electrophoresis analysis of PMN from 13 individual Sprague-Dawley rats obtained from the two breeders. Whereas PMN from the Harlan Sprague Dawley colony regularly expressed only RatNP-1, RatNP-3, and RatNP-4, rats from the Charles River Breeding Laboratories colony expressed RatNP-2 in addition to or instead of RatNP-1. Judging from the staining intensity of RatNP-1 on Coomassie blue-stained gels, when only RatNP-1 was present, it was about twice as abundant as when both RatNP-1 and RatNP-2 were present. RatNP-3 expression was constant in the PMN of rats from both colonies, while RatNP-4 expression was very variable. PMN from some Charles River rats lacked RatNP-4, and PMN from others expressed only about one-half as much RatNP-4 as PMN from Harlan rats. The four distinct PMN defensin phenotypes found in this group of rats are summarized in the legend to Fig. 1.

Antimicrobial activity. RatNP-1 and RatNP-2 exhibited substantially the same antimicrobial activity against *Salmonella typhimurium* as rabbit defensin NP-1 did (Fig. 2). Like NP-1, these rat defensins were less effective against wild-type, mouse-virulent *Salmonella typhimurium* strain 14028S than against its *phoP*-negative, mouse-avirulent isogenic derivative, strain 7953S (2). RatNP-1 and RatNP-2 were considerably more effective against both of these *Salmonella* strains than the less cationic rat defensins RatNP-3 and RatNP-4 were. Human defensin HNP-1 was considerably less active against *Salmonella typhimurium* than any of rat or rabbit defensins shown in Fig. 2. RatNP-1 and RatNP-2 also displayed virtually identical microbicidal activities against *Candida albicans* and *Staphylococcus aureus* 502A (Fig. 3).

DISCUSSION

We purified a fourth defensin, RatNP-2, from the neutrophils of outbred Sprague-Dawley rats. Our chemical analyses revealed that RatNP-1 and RatNP-2 were identical in 31 of their 32 residues (Tables 1 and 2), and antimicrobial assays showed that these defensins have very similar potencies against *Staphylococcus aureus*, *Salmonella typhimurium*, and *C. albicans*. The single amino acid substitution that differentiated RatNP-1 and RatNP-2 was consistent with a single-base-pair alteration in the responsible gene. The observed patterns of expression of RatNP-1 and RatNP-2 were consistent with allelic behavior of the responsible genes (Fig. 1).

We have previously reported that outbred New Zealand White rabbit PMN also contain two defensins, NP-1 and NP-2, and that these differ from each other only by a single amino acid substitution (14). However, unlike rat PMN, rabbit PMN from individual animals express NP-1 and NP-2 in constant and approximately equal relative amounts, and our recent genetic studies have indicated that NP-1 and NP-2 arise from distinct, nonallelic genes that are closely linked on the chromosome, which is consistent with an origin by gene reduplication (5). Defensins are remarkably abundant in the neutrophils of many mammalian species. For example, they comprise more than 5% of the total protein in human neutrophils and more than 15% of the total protein in rabbit heterophils (6). The presence of multiple nonallelic defensin genes may facilitate the rapid synthesis of large amounts of these peptides during the brief period of neutrophil maturation in the bone marrow. In addition, since individual defensins can vary in their antibiotic spectra, in their relative potencies, and possibly in their modes of action, the presence of multiple defensin species might benefit the host for these other reasons as well. The existence of defensin polymorphisms in outbred rodents also raises the possibility that differential expression of defensins might underlie some of the subtle differences in host resistance to infection between certain inbred rodent strains.

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