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Research Article

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Primary Structures of Three Human Neutrophil Defensins

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Abstract

The primary structures of three human neutrophil antimicrobial peptides (HNP) were determined. The peptides, HNP-1, HNP-2, and HNP-3, which we have termed defensins, were rich in cystine, arginine, and aromatic residues, but were devoid of free sulfhydryl groups and carbohydrate moieties. They were 29–30 residues in length and identical in sequence in all but their amino terminal residues. The defensins were homologous in sequence to peptides of similar size and biological activity previously purified from rabbit polymorphonuclear leukocytes, but unrelated to other neutrophil proteins of known sequence. 11 amino acid residues of the human defensins, including all six cysteinyl residues, were invariantly conserved in the six rabbit members of this multigene peptide family. That similarly structured antimicrobial peptides are present in both rabbit and human leukocytes supports their purported role as cidal agents in phagocyte-mediated host defense.

Introduction

Prominent among the “oxygen-independent” antimicrobial substances isolated from mammalian neutrophils, are a family of low molecular weight cationic peptides exemplified by those recently characterized in rabbit polymorphonuclear leukocytes (PMN).¹ Six rabbit peptides have been purified to homogeneity and have been found to be active against gram-positive and gram-negative bacteria (1), fungi (2), and herpes simplex virus (3). The peptides were cysteine-rich and arginine-rich, 33–34 residues in length, and within their primary structures, each contained a conserved core of 11 residues (4). The unique structural and functional features of the rabbit peptide family led us to search for peptides of similar size and biological activity in human PMN. Three human neutrophil peptides (HNP), individually named HNP-1, HNP-2, and HNP-3, and collectively termed “defensins,” were purified to homogeneity as described (5). The human

defensins exerted prominent antibacterial, antifungal, and antiviral effects *in vitro*, and were localized immunohistochemically to azurophil granules.

We now report the amino acid sequences of the three human neutrophil defensins. The structures of these peptides were highly conserved and were homologous in sequence to the rabbit neutrophil peptide family.

Methods

Purification of human defensins. HNP-1, HNP-2, and HNP-3 were purified as recently described (5). Briefly, human blood neutrophils were purified from leukopheresis packs to >90% PMN. A granule-rich subcellular fraction was obtained by homogenization and differential centrifugation steps. Granule protein, obtained by acetic acid extraction, was fractionated by gel filtration on a Bio-Gel P-10 column. Homogeneously pure HNP-1, HNP-2, and HNP-3 were obtained by subsequent ion-exchange, gel filtration, and reverse-phase high performance liquid chromatography (RP-HPLC) steps.

S-Alkylation of HNP 1–3. Each peptide was reduced and alkylated with iodoacetic acid or iodoacetamide in 6.0 M guanidine hydrochloride, 0.5 M Tris-HCl, pH 8.5, 0.2 mM EDTA as previously described (6). The carboxymethyl or carboxamidomethyl peptide derivatives were then desalted by RP-HPLC purification on a 10 × 250 mm C-18 column (Vydac;

Table I. Amino Acid Analysis of Human Defensins

	HNP-1	HNP-2	HNP-3
Arg	4.14 (4)	4.05 (4)	4.05 (4)
Asp	0 (0)	0 (0)	1.07 (1)
Thr	1.12 (1)	1.06 (1)	1.07 (1)
Glu	2.04 (1)	1.90 (1)	2.08 (1)
Gln	0 (1)	0 (1)	0 (1)
Pro	1.02 (1)	1.05 (1)	1.11 (1)
Gly	3.02 (3)	3.23 (3)	2.94 (3)
Ala	4.02 (4)	3.06 (3)	3.07 (3)
Cys	6.01 (6)	4.93 (6)	5.94 (6)
Ile	2.67 (3)	2.61 (3)	2.61 (3)
Leu	1.14 (1)	1.09 (1)	1.08 (1)
Tyr	2.88 (3)	2.88 (3)	2.87 (3)
Phe	1.16 (1)	1.16 (1)	1.10 (1)
Trp	1.13 (1)	1.08 (1)	1.06 (1)
Total	(30)	(29)	(30)

Representative amino acid analyses of HNP-1, HNP-2, and HNP-3. Compositions were determined after 24 h hydrolysis of 50–75 pmol of each carboxymethylated peptide. Hydrolysates were analyzed as PTC derivatives. Tryptophan content was determined spectrophotometrically in 6.0 M guanidine hydrochloride, 20 mM sodium phosphate, pH 6.55. Cysteine was determined as *S*-carboxymethylcysteine. Values in parentheses are residue values obtained by sequence analysis.

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1. *Abbreviations used in this paper:* HNP, human neutrophil peptides; PTC, phenylisothiocyanate; PMN, polymorphonuclear leukocytes; PTH, phenylthiocarbonyl; PTH, phenylthiohydantoin; RP-HPLC, reverse-phase high performance liquid chromatography.

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The Separations Group, Hesperia, CA), using a water-acetonitrile gradient in which 0.1% trifluoroacetic acid was present as the ion-pairing agent. Salt-free, reduced and alkylated peptide, obtained by lyophilization in an evaporator (Speed-Vac; Savant Instruments, Inc., Hicksville, NY), was used for all subsequent chemical analyses.

Composition analysis. 50–75 μg of carboxymethylated HNP was hydrolyzed in vacuo in 5.7 N HCl employing the micro method described by Bidlingmeyer et al. (7). Amino acids were quantitated as phenylthiocarbonyl (PTC) derivatives (8) on a 510 binary HPLC system (Millipore-Waters, Milford, MA) using a Nova-Pak C-18 column (Millipore-Waters) (7). Tryptophan was quantitated spectrophotometrically by the method of Edelhoeh (9). For detection of free sulfhydryls, native peptide was titrated with 5,5'-dithiobis (2-nitrobenzoate) (10). Peptide carbohydrate content was estimated by the phenol-sulfuric acid assay (11).

Proteolytic digestion. Reduced and alkylated peptide was digested in 0.1 M *N*-ethylmorpholine acetate, pH 8.20, with 1.0% (wt/wt) trypsin, chymotrypsin (Worthington Biochemical Corp., Freehold, NJ), or *Staphylococcus aureus* V protease (Pierce Chemical Co., Rockford, IL). Digestion was carried out for 2.0 h (chymotrypsin), 5.0 h (trypsin), or

18 h (*S. aureus* V protease) at 37°C. Digestion products were purified by RP-HPLC on a 4.6 \times 250 mm Vydac 300 Å C-18 column as previously described (6). Purified fragments were hydrolyzed and subjected to amino acid analysis as described above.

Sequence determination. 10–20 nmol of *S*-carboxamidomethylated HNP-1, HNP-2, and HNP-3 were subjected to gas-phase Edman degradation on an Applied Biosystems, Inc. (Foster City, CA) instrument, essentially as described in reference 12. Phenylthiohydantoin (PTH) amino acids were identified by RP-HPLC on a Zorbax cyanopropyl column (5 μm , 4.5 \times 250 mm) as previously described (6).

The carboxyl terminal segment of each defensin was identified and characterized by the tritium-label method of Matsuo and Narita (13). Briefly, each defensin was labeled with tritium at its carboxyl terminus and then digested with trypsin. The digestion fragments were purified by RP-HPLC, and the tritium-containing fragments were characterized by amino acid analysis (6). Carboxypeptidase Y digestion of each peptide was performed as described in reference 14. Briefly, samples of each carboxymethylated HNP were digested with 1.0% (wt/wt) carboxypeptidase Y in 0.1 M pyridine-acetate, pH 6.35, for 1, 2, 4, 6, or 10 min at 37°C. Samples were snap-frozen, lyophilized, and derivatized with PITC for amino acid analysis. Samples of the derivatized digest were injected onto the C-18 amino acid column without prior fractionation.

Table II. Gas Phase Edman Degradation of Human Defensins

Cycle no.	HNP-1		HNP-2		HNP-3	
	PTH-AA	Yield*	PTH-AA	Yield	PTH-AA	Yield
1	Ala	2,100	Cys	1,800	Asp	1,070
2	Cys	1,650	Tyr	1,620	Cys	940
3	Tyr	1,260	Cys	1,420	Tyr	530
4	Cys	1,200	Arg	1,290	Cys	860
5	Arg	360	Ile	1,850	Arg	660
6	Ile	1,340	Pro	1,590	Ile	1,680
7	Pro	640	Ala	2,030	Pro	1,300
8	Ala	1,320	Cys	1,220	Ala	1,400
9	Cys	840	Ile	1,550	Cys	1,070
10	Ile	480	Ala	1,470	Ile	950
11	Ala	1,070	Gly	1,870	Ala	840
12	Gly	850	Glu	1,040	Gly	890
13	Glu	170	Arg	920	Glu	430
14	Arg	250	Arg	670	Arg	370
15	Arg	240	Tyr	990	Arg	280
16	Tyr	190	Gly	1,090	Tyr	530
17	Gly	300	Thr	560	Gly	460
18	Thr	590	Cys	450	Thr	130
19	Cys	360	Ile	620	Cys	280
20	Ile	530	Tyr	620	Ile	400
21	Tyr	100	Gln	700	Tyr	350
22	Gln	90	Gly	680	Gln	350
23	Gly	100	Arg	420	Gly	380
24	Arg	110	Leu	540	Arg	170
25	Leu	110	Trp	40	Leu	270
26	Trp	10	Ala	420	Trp	40
27	Ala	160	Phe	410	Ala	250
28	Phe	310	Cys	180	Phe	210
29	Cys	120	Cys	70	Cys	110
30	Cys	80	—	0	Cys	70
31	—	0	—	0	—	0

Gas phase Edman degradation of HNP 1–3. 20 nmol of each carboxamidomethylated sample was loaded on a 470 A sequencer (Applied Biosystems, Inc.).

* Yield is expressed as picomoles of identified PTH-amino acid per degradation step.

Results

The chemical compositions of the three defensins were nearly identical (Table I). Each contained six half-cystine and four arginine residues, as well as an abundance of A_{280} absorbing aromatic residues. The single tryptophan and three tyrosine residues contained in each peptide were consonant with the high extinction coefficients of the defensins: in 0.1% acetic acid, the $A_{280}^{1\%}$ for HNP-1 and HNP-3 was 30.3, and for HNP-2 was 31.3. None of the peptides contained detectable carbohydrate. 5,5'-dithiobis(2-nitrobenzoate) did not react with native HNP-1, HNP-2, or HNP-3, indicating the absence of free sulfhydryl

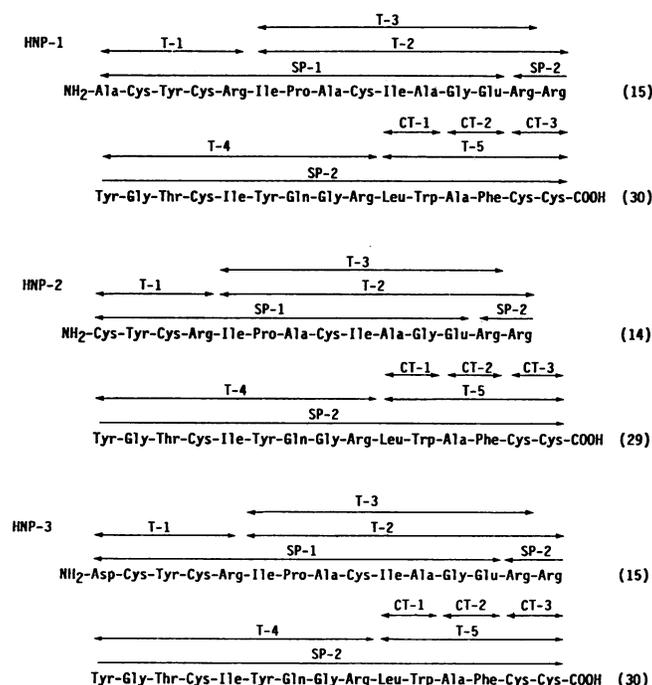


Figure 1. Sequences of human defensins. Arrows designate tryptic (T), chymotryptic (CT), or *S. aureus* V protease (SP) fragments. See text for details of residue assignment.

HUMAN	HNP-1	A	C	Y	C	R	I	P	A	C	I	A	G	E	R	R	Y	G	T	C	I	Y	Q	G	R	L	W	A	F	C	C				
	HNP-2		C	Y	C	R	I	P	A	C	I	A	G	E	R	R	Y	G	T	C	I	Y	Q	G	R	L	W	A	F	C	C				
	HNP-3	D	C	Y	C	R	I	P	A	C	I	A	G	E	R	R	Y	G	T	C	I	Y	Q	G	R	L	W	A	F	C	C				
RABBIT	NP-1	V	V	C	A	C	R	R	A	L	C	L	P	R	E	R	R	A	G	F	C	R	I	R	G	R	I	H	P	L	C	C	R	R	
	NP-2	V	V	C	A	C	R	R	A	L	C	L	P	L	E	R	R	A	G	F	C	R	I	R	G	R	I	H	P	L	C	C	R	R	
	NP-3a	G	I	C	A	C	R	R	R	F	C	P	N	S	E	R	F	S	G	Y	C	R	V	N	G	A	R	Y	V	R	C	C	S	R	R
	NP-3b	G	R	C	V	C	R	K	Q	L	L	C	S	Y	R	E	R	R	I	G	D	C	K	I	R	G	V	R	F	P	F	C	C	P	R
	NP-4	V	S	C	T	C	R	R	F	S	C	G	F	G	E	R	A	S	G	S	C	T	V	N	G	V	R	H	T	L	C	C	R	R	
NP-5	V	F	C	T	C	R	G	F	L	C	G	S	G	E	R	A	S	G	S	C	T	I	N	G	V	R	H	T	L	C	C	R	R		

Figure 2. Sequence comparison of human and rabbit defensins. Each sequence is represented by the single-letter amino acid symbol. The sequences were maximally aligned to show the structural homology conferred by the 11 residues common to both the human and rabbit peptides (enclosed in boxes).

groups, suggesting that the six half-cystine residues of each peptide existed as three intramolecular disulfides.

The primary structures of the defensins were determined by automated Edman degradation and confirmed by characterization of purified proteolytic digestion fragments. As shown in Table II, phenylthiohydantoin (PTH)-amino acid residues from 29 (HNP-2) or 30 (HNP-1 and HNP-3) sequencing cycles were identified. The final residue obtained in each of the sequencing runs was determined to be the actual carboxyl terminal residue by (a) amino acid analysis of the tryptic fragments generated by digestion of carboxyl terminal tritium-labeled HNP, and (b) by the appearance of *S*-carboxymethylcysteine as the first residue released by carboxypeptidase Y degradation (data not shown). The sequences of the defensins, shown in Fig. 1, contained four trypsin sites (four arginines), and one *S. aureus* V protease site (glutamic acid). The single tryptic fragment not containing arginine (T-5) was necessarily the carboxyl terminal fragment and this fragment contained the great majority of tritium counts. The sequences of the carboxyl terminal regions were further confirmed by characterizing the purified chymotryptic peptides (CT-1, CT-2, and CT-3) generated by digestion of tryptic peptide T-5. As demonstrated in Fig. 1, the sequences of the three defensins were identical in all but their amino terminal residues.

The primary structures of the human defensins and the previously characterized rabbit PMN peptides were compared as shown in Fig. 2. Whereas the rabbit peptides each contain 33 or 34 amino acid residues, the human peptides contain 29 or 30. Both the human and rabbit peptides are uniformly rich in cysteine (6 residues/molecule). The rabbit peptides vary in their arginine content from 5 (NP-5)² to 10 (NP-1) residues/molecule; the human defensins each contain 4 arginine residues/molecule. With the exception of NP-3b, neither the rabbit nor human peptides contain lysine. At pH 7.0, the rabbit peptides vary in their net charge per molecule [(arginine + lysine) - (glutamic acid + aspartic acid)] from +4 (NP-5) to +9 (NP-1). The defensins have a net charge of +2 (HNP-3) or +3 (HNP-1 and HNP-2). The presence of three tyrosines and one tryptophan in each defensin molecule contrasts with the virtual absence of these residues from the rabbit peptides.

When aligned as in Fig. 2, the six rabbit and three human PMN peptides showed complete conservation of 11 residues, including all six cysteinyl residues. The absence of free-sulfhydryl

groups in both the rabbit and human peptides indicates that each peptide contains three structure-conferring disulfide bridges. Assignment of the cysteine pairs requires further study.

Discussion

HNP 1-3 and NP 1-5 are major gene products of human and rabbit PMNs. Whereas several larger (14-60 kD) bactericidal proteins have been isolated from human PMN granules (15), the existence of low molecular weight human antibiotic peptides homologous to those previously shown in rabbit PMN has not previously been described. Perhaps one reason that the human homologs have gone undetected is that attention has been focused on the cationicity of some of these peptides, as evidenced by the designation of the rabbit PMN peptides as "lysosomal cationic proteins" (15). While the defensins each possess a net positive charge at pH 7.0, their electrophoretic migration in low pH polyacrylamide gel electrophoresis is less cathodal than lysozyme (5), which possesses a computed net charge of +8 (16).

Our sequence data suggests that although certain arginine residues may be of primary consequence, it is the cystine infrastructure, and by inference the molecular conformation, that appears essential for biological activity. The expression and perpetuation of homologous antibiotic peptides in granulocytes of two evolutionarily distinct species is consistent with the possibility that such antimicrobial peptides play a significant role in granulocyte function.

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