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Purification, Primary Structure, and Biological Activity of Guinea Pig Neutrophil Cationic Peptides

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The guinea pig neutrophil cationic peptides GNCP-1 and GNCP-2 were purified from a granule-rich subcellular fraction of peritoneal exudate neutrophils by acid-gel electrophoresis and reversed-phase high-performance liquid chromatography. Both peptides not only released histamine from rat mast cells to the same extent but also were equally active against *Staphylococcus aureus* and *Escherichia coli*. The peptides were rich in arginine and cystine and lacked free sulfhydryl groups. Composition and sequence determinations revealed that GNCP-1 and GNCP-2 are each single polypeptides containing 31 amino acid residues and three intramolecular disulfide bonds. The complete amino acid sequence of GNCP-1 is Arg-Arg-Cys-Ile-Cys-Thr-Thr-Arg-Thr-Cys-Arg-Phe-Pro-Tyr-Arg-Arg-Leu-Gly-Thr-Cys-Ile-Phe-Gln-Asn-Arg-Val-Tyr-Thr-Phe-Cys-Cys. The sequence of GNCP-2 is identical except for the substitution of isoleucine for leucine at residue 21.

The release of histamine from rat peritoneal mast cells can be induced by a variety of secretagogues such as antigen, anti-immunoglobulin E, concanavalin A, compound 48/80, and calcium ionophore. Cationic proteins from rabbit and guinea pig neutrophilic granules have been reported to be a potent releaser of histamine (8, 11, 12). However, the purification and characterization of such a cationic protein with histamine-releasing activity have not been achieved yet. On the other hand, cationic peptides with potent antimicrobial activity have been purified from rabbit, human, and guinea pig neutrophils and have been charaterized (2, 13, 15–17).

Therefore, the present study was undertaken to purify a cationic protein with histamine-releasing activity from guinea pig neutrophils and to examine the relationship between histamine-releasing and antimicrobial cationic peptide.

MATERIALS AND METHODS

Preparation of neutrophils. Guinea pig neutrophils were isolated from peritoneal exudate 13 to 15 h after intraperitoneal injection of 0.17% glycogen in saline, as described previously (21). Neutrophil suspensions were washed twice with buffer A (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) after removal of contaminating erythrocytes by a hypotonic treatment with 0.2% NaCl. Then the washed cells were resuspended in buffer A. Differential cell counts with Wright-Giemsa stain showed that more than 96% of the cells were neutrophils.

Preparation of rat mast cells. Mast cells were obtained by lavage similar to the method of Sullivan et al. (19), using Sprague-Dawley rats. Toluidine blue staining was used as a purity criterion (80 to 95%).

Preparation of granule fraction. A granule-rich fraction was obtained by differential centrifugation of the cell homogenate as described previously (7). The cells were suspended at a concentration of 8×10^7 cells per ml in ice-cold 0.34 M sucrose and homogenized in a glass Teflon-pestle homogenizer at 0°C until most cells appeared broken under phase-contrast microscopy. The homogenate was centrifuged at 420 × *g* for 12 min at 4°C. The supernatant was further centrifuged at 8,200 × *g* for 15 min at 4°C. The resultant pellet was washed once with 0.34 M sucrose and then suspended in 0.34 M sucrose at a concentration equivalent to 2.5×10^9 cells per ml. The granule-rich fraction obtained was sonicated for 1 min in ice at a setting of 168 W (Supersonic vibrator, model UR-150 P, Tominaga Works Ltd.) for solubilization.

Preparative PAGE. For preparative polyacrylamide gel electrophoresis (PAGE), granule-rich samples (0.7 to 1.0 ml) were electrophoresed on 15% polyacrylamide gel slabs (2 by 120 by 160 mm) in β-alanine buffer (pH 4.5) at 4°C for approximately 4 h at 150 V according to the method described by Reisfeld et al. (10) with certain modifications. Methylgrün was used as a tracking dye. Thereafter, the cationic polypeptide was visualized as a transverse magenta band by immersing the slab gel in a solution of 0.25% (wt/vol) eosin Y in 0.1 N NaOH for 30 s according to the method described by Selsted et al. (14). The band containing cationic peptide was excised, ground, and subjected to electrophoretic elution in 0.16% acetic acid. The recovered peptide was lyophilized, dialyzed exhaustively against 0.16% acetic acid, and lyophilized again.

RP-HPLC. Final purification of the cationic protein was achieved by reversed-phase high-performance liquid chromatography (RP-HPLC) on a Jasco TRI ROTAR-VI HPLC system (Japan Spectroscopic Co., Ltd.) equipped with a Rheodyne 7125 sample injector (1.0-ml loop), a Jasco UVIDEC-100-VI detector, a Jasco RC-250 desktop recorder, and a Wakosil 5C8 column (4.6 by 250 mm; pore size, 12.0 nm; Wako Pure Chemical Industries, Ltd.). Water-acetonitrile gradients containing 0.1% trifluoroacetic acid were employed for elution. This final purification step resolved the electrophoretically single band into two components.

Analytical PAGE. Sodium dodecyl sulfate (SDS)-PAGE in the presence of urea was carried out for the homogeneity and molecular weight estimation of the cationic peptide, using 0.1% SDS–0.1 M H_3PO_4 buffer adjusted to pH 6.8 with Tris, as described by Swank and Munkres (20). Bromophenol blue (0.02%) was used as a tracking dye. Samples were heated at 100°C for 5 min in 0.01 M H_3PO_4 at pH 6.8 in the presence of 8 M urea and 2.5% SDS and applied to a 12.5% polyacryl-

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amide slab gel (1 by 60 by 85 mm) in the presence of 8 M urea and 0.1% SDS. Electrophoresis was carried out for about 6 h at 40 V.

Sulfhydryl analysis. Native cationic peptides were each assayed for free sulfhydryl groups by reaction with 5,5'-dithiobis-(2-nitrobenzoate) in 50 mM potassium phosphate buffer (pH 8.0) containing 6.0 M guanidine hydrochloride and 1.5 mM EDTA for 5 min at room temperature (1).

***S*-Alkylation of cationic peptide.** *S*-β-4-Pyridylethylation was performed essentially as described by Hermodson et al. (4). An 885-μg sample of cationic peptide was dissolved by stirring in 0.5 ml of 0.13 M Tris hydrochloride buffer (pH 7.6) containing 6 M guanidine hydrochloride and 0.01% EDTA and reduced by addition of 21.6 μl of 94.5-mg/ml dithioerythritol (about 20 molar excess over total disulfides) for 3 h at room temperature. The free sulfhydryl groups were then exposed to 4.23 μl of 4-vinylpyridine (3:1 molar ratio with respect to reducing agents used), and the solution was stirred for 90 min. The *S*-pyridylethylated peptide solution then was adjusted to pH 3 with 90 μl of glacial acetic acid, dialyzed at 4°C for 48 h against 0.01 N acetic acid by using a Micro-Ultrafiltration system model 8 MC (Amicon Corp.) with Ultra Filter UH-1 membranes of molecular weight cutoff of 1,000 (Toyo Roshi Kaisha, Ltd., Tokyo, Japan), and lyophilized.

Amino acid analysis. Ten micrograms of the reduced-alkylated peptides was hydrolyzed by 6 N hydrochloric acid in an evacuated, sealed tube at 110°C for 24 h. The acid was then removed on a rotary evaporator at 40°C under reduced pressure, and the residue was dissolved in 250 μl of 0.01 N HCl. Portions (220 μl) of these samples were analyzed on a Hitachi 835 high-speed amino acid analyzer.

Sequence determination. The amino acid sequences of *S*-pyridylethylated peptides were determined by pulsed-liquid Edman degradation, using an Applied Biosystems 477 A protein sequencer. Phenylthiohydantoin derivatives of amino acids were identified by an ABI model 120 A RP-μHPLC.

Carboxypeptidase Y was used for the COOH-terminal residue and sequence determinations of cationic peptides (3). *S*-Pyridylethylated peptides (50 μg) were hydrolyzed with 7.62 μg of carboxypeptidase Y (molar ratio of peptide to enzyme, 100:1) at 25°C in a total volume of 250 μl of 50 mM sodium phosphate buffer (pH 6.5). At appropriate time intervals, samples (60 μl) were withdrawn from the mixture and mixed with an equal volume of 60 mg of sulfosalicylic acid per ml in deionized distilled water to stop the reaction. The precipitate was then removed by centrifugation at 7,600 × *g* for 10 min at 4°C. The supernatant (100 μl) was applied to an amino acid analyzer.

Histamine release from rat mast cells. Mast cells at 2 × 10^4/ml were incubated with cationic peptide at 37°C for 30 min in a total volume of 1.50 ml of Tris-ACM buffer (119 mM NaCl, 5 mM KCl, 0.6 mM $CaCl_2$, 1.2 mM $MgCl_2$, 0.03% bovine serum albumin, and 31 mM Tris hydrochloride, pH 7.4) as described previously (22). Histamine release was initiated by the addition of 0.05 ml of cationic peptide in 0.01% acetic acid and terminated by placing test tubes in an ice-water bath, followed by centrifugation at 220 × *g* for 10 min at 4°C. Supernatant histamine was determined by the *o*-phthaldialdehyde spectrophotofluorometric procedure of Shore et al. (18), as modified by May et al. (6). Total histamine was determined on cell samples which had been incubated at 37°C with cationic peptide for 30 min and then sonicated for 30 s. Histamine release is expressed as the percent of total cellular histamine.

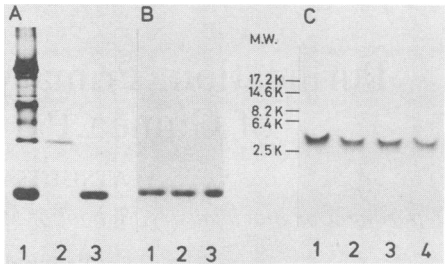

FIG. 1. PAGE of GNCP-1 and GNCP-2. (A) Acid PAGE. Lane 1, 43.5 μg of granule fraction; lane 2, 2.7 μg of egg white lysozyme; lane 3, 5.7 μg of acid PAGE-isolated peptide. (B) Acid PAGE. Lane 1, 4.7 μg of GNCP-1; lane 2, 4.7 μg of GNCP-2; lane 3, 2.4 μg each of GNCP-1 and GNCP-2. (C) Urea-SDS-PAGE. Lane 1, 1 μg of acid PAGE-isolated peptide; lane 2, 1 μg of GNCP-1; lane 3, 1 μg of GNCP-2; lane 4, 0.5 μg each of GNCP-1 and GNCP-2. After electrophoresis, gels were stained with Coomassie brilliant blue R-250.

Antibacterial assay. The bactericidal activity of a cationic peptide was tested against *Escherichia coli* NIHJ JC-2 and *Staphylococcus aureus* NIHJ JC-1 as described previously (5). The organisms were maintained on MacConkey (for *E. coli*) or heart infusion (for *S. aureus*) agar plates for 18 to 24 h at 37°C, and organisms from a single colony were inoculated into 5 ml of nutrient broth (Eiken Chemical Co., Ltd.) and cultured for 16 to 18 h at 37°C. Each species of bacteria was then sedimented by centrifugation at 6,000 × *g* for 10 min, washed twice, and suspended in sterile 10 mM phosphate buffer (pH 7.4). The concentration of CFU per milliliter was quantitated by measuring the A_{650} of each preparation with reference to previously determined standards. To 0.1-ml volumes of bacterial suspension (2 × 10^6 CFU/ml) were added various concentrations of the cationic peptide in a total volume of 0.2 ml of 10 mM phosphate buffer, and the mixtures were incubated for 20 min at 37°C. In control experiments, organisms were exposed to cationic peptide-free buffer for 20 min. After 1,000-fold dilution of incubation mixtures with phosphate buffer, 0.1-ml samples were spread on nutrient agar plates and incubated for 18 to 20 h to allow full colony development. The resulting colonies were counted. Killing of microorganisms was expressed as a percentage of control colony count.

RESULTS

Peptide purification. When examined by acid PAGE, a granule-rich fraction appeared to contain only one cationic peptide whose cathodal migration greatly exceeded that of lysozyme (Fig. 1A). The peptide was recovered from the polyacrylamide gel by reversibly staining the gel with an alkaline eosin solution, followed by electroelution. When the electroeluted peptide was further analyzed by RP-HPLC, this peptide was found to consist of two components, designated GNCP-1 and GNCP-2 (for guinea pig neutrophil cationic peptide) (the peak area ratio of both was 56.6 ± 1.5 to 43.4 ± 1.5; mean ± standard deviation; n = 6) (Fig. 2). Two peptides were more clearly resolved when the electroeluted peptide was alkylated with 4-vinylpyridine (not shown). Figure 1B shows that two HPLC-purified peptides comigrated in acid PAGE. The results in Fig. 1C, which shows the comigration of two peptides on a urea-SDS-PAGE gel, suggest that the peptides have a molecular weight of 3,500 (mean, n = 8).

Composition analysis. The amino acid compositions of the

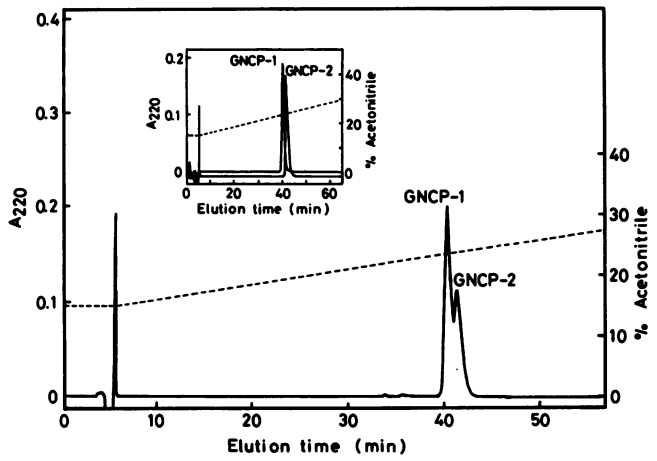

FIG. 2. RP-HPLC of GNCPs. Acid PAGE-isolated peptide (100 μg) was dissolved in 0.2 ml of 0.1% trifluoroacetic acid and loaded onto a C-8 column (4.6 by 250 mm) equilibrated in 0.1% trifluoroacetic acid–15% acetonitrile in water. A linear gradient (– – – –) of acetonitrile (15 to 30%) was developed for 60 min at 1.0 ml/min. The effluent was monitored at 220 nm. Inset, Analytical HPLC of purified GNCP-1 and GNCP-2; 50 μg of GNCP-1 or GNCP-2 was applied to a C-8 column under the same conditions as above.

two peptides were virtually identical (Table 1). Each contained six half-cystine and seven arginine residues, as well as an abundance of A_{280}-absorbing aromatic residues. Three phenylalanine and two tyrosine residues were contained in each peptide. From the composition data in Table 1, the minimum molecular weights of GNCP-1 and GNCP-2 were calculated to be 3,828 each. This value agrees well with the urea-SDS-PAGE estimate of 3,500. Neither of the peptides reacted with 5,5′-dithiobis-(2-nitrobenzoate) under denaturing conditions, indicating the absence of free sulfhydryl groups and suggesting that the six half-cystine residues of each peptide exist as three intramolecular disulfides.

Amino acid sequences. The primary structures of the peptides were determined by automated Edman degradation. Phenylthiohydantoin-amino acid residues from 31 sequencing cycles were identified (Table 2). Digestion of each *S*-pyridylethylated peptide with carboxypeptidase Y resulted in the sequential release of *S*-pyridylethylcysteine, phenylalanine, threonine, and tyrosine, with kinetics and stoichiometry results consistent with the sequence of the carboxyl terminus (Fig. 3). Carboxypeptidase Y hydrolysis also released isoleucine from GNCP-1, but not from GNCP-2.

TABLE 1. Amino acid compositions of GNCPs[a]

Amino acid	No. of residues per molecule[b] of:		
	GNCP-1	GNCP-2	Acid PAGE-isolated peptide
Aspartic acid	1.12 (0)	1.13 (0)	1.05
Asparagine	0 (1)	0 (1)	0
Threonine	4.79 (5)	4.76 (5)	4.56
Glutamic acid	1.13 (0)	1.20 (0)	1.05
Glutamine	0 (1)	0 (1)	0
Proline	1.18 (1)	1.02 (1)	0.98
Glycine	1.32 (1)	1.43 (1)	1.26
Valine	0.96 (1)	1.11 (1)	0.99
Isoleucine	1.96 (2)	1.06 (1)	1.51
Leucine	1.12 (1)	1.99 (2)	1.42
Tyrosine	1.90 (2)	1.96 (2)	1.92
Phenylalanine	3.10 (3)	2.97 (3)	2.94
Arginine	6.85 (7)	6.84 (7)	6.97
Half-cystine[c]	5.58 (6)	5.50 (6)	6.26
Total	31	31	

[a] Values determined from analysis of 24-h hydrolysates.
[b] Numbers in parentheses indicate residues as determined by sequence analysis. Molecular weight of GNCP-1 and GNCP-2 was calculated from sequence analysis data to be 3,828 each.
[c] Determined as *S*-pyridylethylcysteine.

TABLE 2. Pulsed-liquid Edman degradation of GNCP-1 and GNCP-2[a]

Cycle no.	GNCP-1		GNCP-2	
	PTH-AA[b]	Yield (pmol)	PTH-AA[b]	Yield (pmol)
1	Arg	49.8	Arg	39.3
2	Arg	80.7	Arg	28.5
3	Cys	281.4	Cys	138.9
4	Ile	464.4	Ile	440.4
5	Cys	176.4	Cys	242.1
6	Thr	24.0	Thr	55.8
7	Thr	51.0	Thr	48.3
8	Arg	26.4	Arg	51.0
9	Thr	57.0	Thr	51.6
10	Cys	69.3	Cys	92.4
11	Arg	20.7	Arg	72.9
12	Phe	247.5	Phe	246.3
13	Pro	130.5	Pro	166.2
14	Tyr	114.0	Tyr	41.4
15	Arg	38.4	Arg	31.5
16	Arg	135.0	Arg	35.1
17	Leu	166.5	Leu	193.8
18	Gly	57.6	Gly	123.3
19	Thr	45.0	Thr	39.6
20	Cys	78.9	Cys	69.9
21	Ile	115.5	Leu	142.5
22	Phe	125.4	Phe	141.3
23	Gln	58.5	Gln	109.8
24	Asn	44.1	Asn	31.2
25	Arg	15.9	Arg	6.0
26	Val	84.3	Val	62.7
27	Tyr	35.4	Tyr	37.2
28	Thr	34.5	Thr	6.6
29	Phe	87.3	Phe	66.3
30	Cys	42.3	Cys	50.4
31	Cys	24.3	Cys	32.1

[a] Approximately 500 pmol each of *S*-pyridylethylated GNCP-1 and GNCP-2 were used.
[b] PTH-AA, Phenylthiohydantoin-amino acid residue.

Biological activity. The two purified peptides were approximately equipotent in histamine-releasing and antibacterial activities. The cationic peptides equally increased histamine release from mast cells in a dose-dependent fashion (Fig. 4). The 50% effective dose of the peptide was 0.5 μg/ml under our conditions. Figure 5 demonstrates the potency of GNCP-1 and GNCP-2 against gram-positive and gram-negative organisms. When the bacterial inoculum was exposed to increasing concentrations of peptide for 20 min at 37°C, ≥95% of the initial culture was killed by GNCPs at concentrations as low as 20 μg/ml.

DISCUSSION

In early studies of mammalian polymorphonuclear cationic proteins, antibacterial activity (23, 24) and histamine-releasing activity (8, 9, 11, 12) were independently found to be present in lysosomal fractions and also in a basic protein

GNCP-1

1 20 25 30

H_2N-Arg—Arg—- - - - - - - - -Leu—Gly—Thr—Cys—Ile—Phe—Gln—Asn—Arg—Val—Tyr—Thr—Phe—Cys—Cys-COOH

Carboxypeptidase Y digestion

25°C, 2 min	0	0	0.07	0.07	0	0.21	0	0.22	0.27	0.31	0.63
25°C, 5 min	0.03	0.03	0.20	0.17	0	0.26	0.26	0.42	0.46	0.59	1.00
25°C, 10 min	0.03	0.05	0.37	0.38	0.37	0.47	0.49	0.59	0.62	0.97	1.54
25°C, 90 min	0.47	0.90	0.90	0.94	1.07	1.67	0.99	1.23	1.93	1.96	2.75

GNCP-2

1 20 25 30

H_2N-Arg—Arg—- - - - - - - -Leu—Gly—Thr—Cys—Leu—Phe—Gln—Asn—Arg—Val—Tyr—Thr—Phe—Cys—Cys-COOH

Carboxypeptidase Y digestion

25°C, 2 min	0	0.17	0.17	0	0.18	0	0.28	0.28	0.42	0.77
25°C, 5 min	0.08	0.35	0.36	0.38	0.35	0.16	0.38	0.45	0.69	1.19
25°C, 10 min	0.29	0.49	0.50	0.52	0.50	0.43	0.49	0.78	0.93	1.59
25°C, 90 min	0.76	1.41	0.98	1.15	2.22	0.87	1.41	2.04	2.13	3.75

FIG. 3. Determination of COOH-terminal sequences of GNCP-1 and GNCP-2. *S*-Pyridylethylated peptides were hydrolyzed with carboxypeptidase Y at 25°C for the indicated time in 50 mM sodium phosphate buffer (pH 6.5). Values represent moles of amino acids released per mole of peptide.

fraction obtained from neutrophil granules by acid extraction and precipitation with 20% (vol/vol) ethanol (ET20 fraction). The purification and characterization of cationic proteins with such a biological activity have been attempted by many investigators. Recently, cationic peptides with potent antimicrobial activity have been purified from rabbit, human, and guinea pig neutrophils and characterized (2, 13, 15–17). Cationic peptides with histamine-releasing activity, however, have not been purified yet. In this study, therefore, we tried to purify cationic peptides to cause the degranulation of rat mast cells from guinea pig neutrophil granules and to determine their primary structure.

The two cationic peptides obtained proved to be remarkably alike. Both were single-chain peptides, 31 amino acids long, differing only by the substitution of an isoleucine (GNCP-1) for a leucine (GNCP-2) at position 21. This difference is consistent with a single-base-pair change between the respective genomic DNAs.

Recently Selsted and Harwig (15) purified a single guinea pig peritoneal neutrophil peptide with antimicrobial activity and determined its primary structure. The amino acid se-

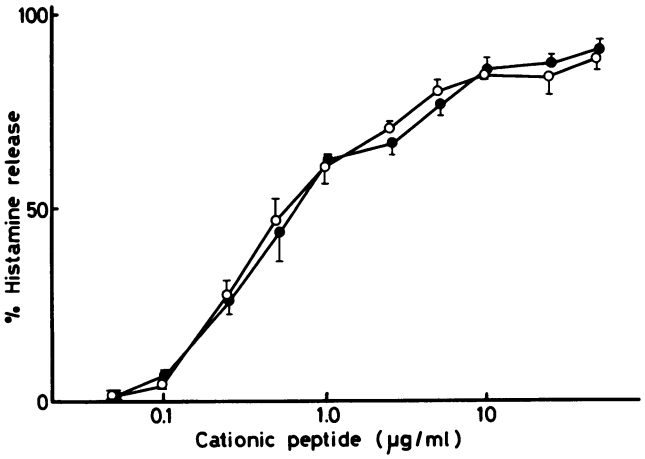

FIG. 4. Histamine-releasing activity of GNCP-1 and GNCP-2. Mast cells were incubated with various concentrations of GNCP-1 (○) or GNCP-2 (●) in Tris-ACM (pH 7.4) for 30 min at 37°C. Histamine release is expressed as the percent of total histamine and is corrected for spontaneous release (4.4 ± 0.2%; mean ± standard error; n = 3). Values plotted are the means of three separate experiments. Bars represent standard error.

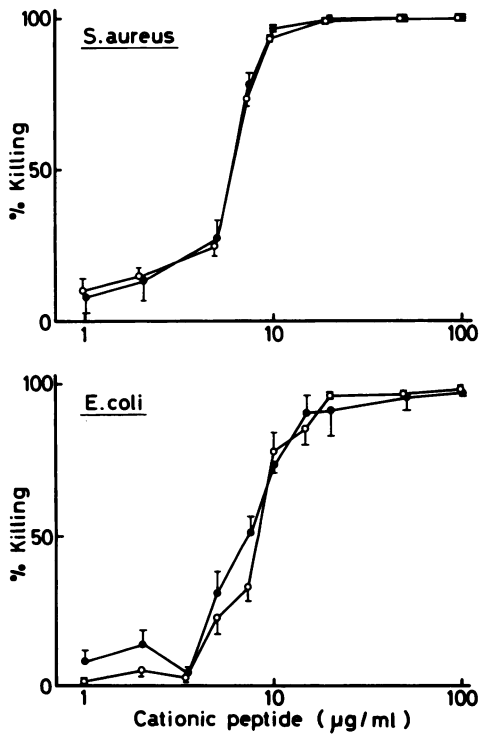

FIG. 5. Antibacterial activity of GNCP-1 and GNCP-2. *S. aureus* or *E. coli* was incubated with various concentrations of GNCP-1 (○) or GNCP-2 (●) in 10 mM sodium phosphate buffer (pH 7.4) for 20 min at 37°C. Antibacterial activity is expressed as the percentage of killed bacteria to control bacteria. Values plotted are the means of three separate experiments. Bars represent standard error.

quence of this peptide was the same as that of GNCP-1, one of the cationic peptides obtained by us. Its amino acid composition, however, especially the amounts of Leu and Ile, was not consistent with that of GNCP-1 and rather similar to that of the cationic peptide mixture isolated as a single band by acid PAGE (Table 1). Therefore, we chromatographed the acid PAGE-isolated peptide on a C-18 column by using trifluoroacetic or heptafluorobutyric acid as an ion-pairing agent under the conditions of Selsted and Harwig, but the two peptides were not separated. Taking into consideration the comigration of GNCP-1 and GNCP-2 on acid PAGE and SDS-PAGE, therefore, it might well be that the guinea pig peritoneal neutrophil peptide is a mixture of GNCP-1 and GNCP-2, although the difference in the number of cationic peptides obtained between these two laboratories is not explained at this stage.

The cationic peptides we purified have not only histamine-releasing activity but also antibacterial activity, although the details of their mechanism of action remain obscure. Neutrophils serve to protect mammalian hosts against microbial infections by intracellular killing of ingested organisms with two principal mechanisms, either oxygen dependent or oxygen independent. Cationic peptides present in neutrophil granules may contribute to the oxygen-independent antimicrobial system of phagocytes. On the other hand, extracellular cationic peptides released from neutrophils might act to recruit mast cells into participating in acute inflammatory reactions.

ACKNOWLEDGMENTS

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