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Isolation, Antimicrobial Activities, and Primary Structures of Hamster Neutrophil Defensins

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Hamster (*Mesocricetus auratus*) neutrophil granules contain at least four microbicidal peptides belonging to the defensin family. These compounds were purified from granule acid extracts by reverse-phase chromatography and termed HaNP-1 to -4 (hamster neutrophil peptide). HaNP-1 and HaNP-3 revealed the most bactericidal activity, with a 50% inhibitory concentration of 0.3 to 0.8 μg/ml for *Staphylococcus aureus* and *Streptococcus pyogenes* strains. The HaNP-4 was always isolated in concentrations exceeding about 10 times the concentrations of other hamster peptides, but its antibacterial activity as well as that of HaNP-2 was relatively lower, probably as a result of conserved Arg residue substitutions. Other microorganisms were also tested, and generally, hamster defensins exhibited less potency against gram-negative bacteria. The amino acid sequence of hamster defensins showed a high percentage of identity to the sequence of mouse enteric defensins, reaching about 60% identical residues in the case of HaNP-3 and cryptdin 3.

Nowadays, the attention of many investigators is focused on antimicrobial peptides. As an important part of the host defense system, this group of compounds is widely distributed in both the plant and animal kingdoms (4, 6, 27). In general, they have three features in common: small molecular masses, a cationic nature, and activity limited to the cell membrane. All the known antimicrobial peptides are differentiated into a few families with regard to either their structure or the anatomical sites of their origin or action (3, 28, 30, 33).

According to these criteria, mammalian defensins may be classified as cysteine-rich, β-sheet peptides which are found in all sites most involved in potential inflammation. Particularly, they consist of 29 to 35 amino acid residues with a characteristic, highly conserved motif of six cysteines. The cationic nature of defensin molecules enables their interaction with a negatively charged target cell membrane; in addition, their amphipathic character allows for incorporation into a phospholipid bilayer, resulting in membrane disruption. The disulfide bonds stabilize primarily the rigid β-sheet structure of the molecule, and the different order of these bonds as well as additional distinct consensus sequences distinguishes these defensins from the β-defensin family.

Defensins have been isolated from granules of neutrophils of humans (42), rabbits (36), rats (12), and guinea pigs (43) but have also been found in rabbit lung macrophages (32) as well as in human, rabbit, and mouse small-intestinal Paneth cells (19, 20, 31, 40). Defensin cDNAs from humans (10), mice (18), guinea pigs (29), rats (44), and rabbits (16) have been cloned and sequenced. Mouse neutrophils lack defensins (13), while horse, bovine, and porcine neutrophils contain another class of peptides like, for example, the previously mentioned β-defensins (9, 14, 24, 37).

Defensins exhibit a microbicidal activity against a variety of microorganisms, demonstrate cytotoxicity against many mammalian cells, and possess some other less physiologically significant features (reviewed in references 22, 26, and 38). Except for neutrophil phagolysosomes, defensins may reach an effective microbicidal or cytotoxic concentration in neutrophil-rich pus (15) and are the only components of granulocytes able to penetrate the blood-brain barrier (35). Paneth cells do not phagocytose, and the components of granules, including defensins, are delivered into the crypt lumen where they probably limit microbial proliferation (11).

Unfortunately, even though the Syrian hamster belongs to the group of experimental animals used most often, its neutrophil peptide antibacterial system has not yet been characterized. In this article, we report that granules of hamster peritoneal exudate neutrophils contain at least four classical defensins, highly homologous to the mouse cryptdin family. The presence of defensins in hamster neutrophils, the sequence data we present, and the similarity of hamster defensins to cryptdins may help in examining phylogenetic considerations. We can speculate that the universality of the defensin-like antimicrobial mechanisms among a variety of species (the mice are here an interesting exception) reflects its importance for the defense system.

MATERIALS AND METHODS

Reagents. Heparin sodium salt grade I-A and MeO-Suc-Ala-Ala-Pro-Val-*p*NA were from Sigma Chemical Co., St. Louis, Mo. (*p*NA is *p*-nitroanilide). All media for microorganism culture were from Difco Laboratories, Detroit, Mich. Casein from bovine milk was purchased from Fluka Chemica, Buchs, Switzerland. Gradient-grade acetonitrile and sequencing-grade trifluoroacetic acid (TFA) were from Merck, Darmstadt, Germany. Constant-boiling HCl and phenylisothiocyanate were from Pierce, Rockford, Ill. All reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were from Bio-Rad Laboratories, Hercules, Calif. Deionized water was produced with the MilliQ system (Waters, Millipore Corp., Milford, Mass.). Cathepsin G was purified from human neutrophil granules by the protocol presented previously (41). All protein-sequencing reagents were from Applied Biosystems, Inc., Foster City, Calif.

Animals and neutrophil collection. Male Syrian hamsters (*Mesocricetus auratus*; CAMURA, Kraków, Poland), aged 4 months and weighing about 100 g, were kept under a 12-h night-day photoperiod on standard laboratory pelleted food and water ad libitum. With the hamsters under a weak ether narcosis, each animal was injected intraperitoneally with 1.5 ml of an 8% (wt/vol) casein suspension in 0.9% NaCl. After 24 h, the animals were etherized again, and the peritoneal exudates were collected and washed twice in 0.9% NaCl. In the case of erythrocyte contamination, additional hypotonic lysis was carried out. The cells were counted in a hemocytometer, and their population was determined microscopically after standard May-Grünwald–Giemsa staining.

Isolation of granule-rich fraction and extraction of its contents. The neutro-

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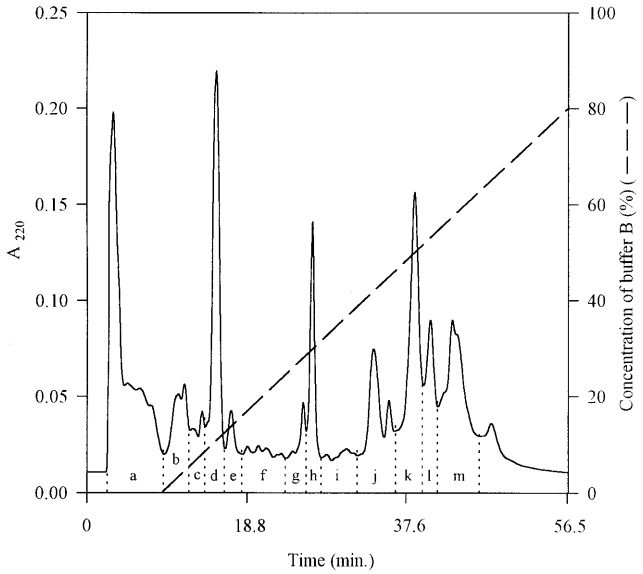

FIG. 1. Fractionation of hamster neutrophil granule extract on a C-8 column. The extract from granules of 4.4×10^8 cells was separated under conditions described in Materials and Methods. After lyophilization, all fractions were further subjected to SDS-PAGE and tested for their antimicrobial activity (see Fig. 2 and 3).

phils were washed twice and suspended in 0.2 M sucrose at a concentration of 10^9 cells per 50 ml. After the addition of 150 U of solid heparin sodium salt per ml, the suspension was vigorously shaken for 2 min and the undisrupted cells as well as the membranes were centrifuged for 15 min at 4°C and $500 \times g$ (23). The granules remaining in the supernatant were deposited and washed twice in 0.2 M sucrose with centrifugation at $15,000 \times g$ for 30 min at 4°C. The greenish pellet of granules was subjected to four cycles of fast freezing and thawing in 0.2 M ammonium acetate (pH 4.5; 1 ml per granule from 10^9 cells), extracted overnight through stirring on ice, and centrifuged at $15,000 \times g$ for 30 min at 4°C. The supernatant was collected, the pellet was extracted twice (for 2 h only), and both pooled supernatants as a final extract were checked for elastase activity by using a 0.3 mM final concentration of MeO-Suc-Ala-Ala-Pro-Val-*p*NA as the substrate in 0.1 M Tris-HCl (pH 8.0). The amount of released *p*NA was measured at 405 nm.

Purification of peptides. The granule extract was acidified with TFA to a final concentration of 0.1% (vol/vol) and then centrifuged for 15 min at 4°C and $15,000 \times g$. The supernatant (extract from 0.5×10^9 cells per one run, up to 1 ml per sample) was applied to a Waters (Millipore Corp.) RadialPak C-8 cartridge (8 by 100 mm) installed on a Waters high-performance liquid chromatography (HPLC) system with a model 410E multiwavelength detector. The separations were maintained at a flow rate of 1.5 ml/min with the following buffers: A, 0.1% (vol/vol) TFA in H_2O; B, 80% acetonitrile–0.07% (vol/vol) TFA in H_2O. A gradient of 0 to 100% buffer B over 60 min was formed. The two fractions containing microbicidal peptides were lyophilized, dissolved in 0.1% (vol/vol) TFA, and applied to a μBondapak C-18 or a μBondapak phenyl column (3.9 by 300 mm; both from Waters, Millipore Corp.). The same HPLC and buffer systems were used as described above, but with a 1-ml/min flow rate and a 50-min gradient of 0 to 50% buffer B for the C-18 column or a 60-min gradient of 0 to 55% buffer B for the phenyl column applied. The sample size for both columns did not exceed a 100-μl volume and an equivalent of 0.5×10^9 cells per one run. The fractions containing peptides were lyophilized and dissolved in water.

Microbial strains and growth conditions. *Staphylococcus aureus* ATCC 8325-4, *Escherichia coli* ATCC 33694, *Enterococcus faecalis* ATCC 29212, and *Pseudomonas aeruginosa* from clinical isolation were maintained in Lennox L broth medium, *Salmonella* serotype krefeld KOS 1497 (a gift from the National Center for Salmonella, Gdynia, Poland) and *Klebsiella oxytoca* CBM21 (from the collection of the Center of Microbiological Research and Autovaccines, Kraków, Poland) were grown in Trypticase soy broth, and *Candida albicans*, *Cryptococcus neoformans*, and *Streptococcus pyogenes*, all from clinical isolation, were maintained on brain heart infusion medium supplemented with 0.1% maltose.

Microbicidal assays. Overnight bacterial or fungal stationary-phase cultures were transferred to fresh 1:100 (vol/vol) diluted medium appropriate for each microorganism and grown to the mid-logarithmic phase. The cultures were subsequently suspended in 10% (wt/vol) Hanks balanced salt solution to give approximately 10^5 CFU/ml. Such microorganism suspensions were incubated at 37°C for 60 min with several concentrations of hamster peptides (or human cathepsin G as a reference) and dissolved in water in a total volume of 10, 25, or 50 μl. Controls contained appropriate amounts of water instead of the peptide solution. After incubation, the samples were diluted 10 times in 10% (wt/vol) Hanks balanced salt solution, and immediately, 20- or 50-μl amounts were plated on Lennox broth, Trypticase soy broth, or brain heart infusion agar for each microorganism. The agar plates were incubated overnight (or 72 h for *Cryptococcus neoformans*) at 37°C, and resulting colonies were counted. All assays were repeated at least three times. The IC_{50} (concentration giving half-maximal inhibition of growth) was calculated as the micrograms of peptide needed to reduce the number of colonies by 50% per 1 ml of the incubation mixture.

Protein chemistry techniques. The amino acid composition was determined after acid gas-phase hydrolysis. Before hydrolysis, the samples for cysteine determinations were oxidized with performic acid. The determinations were performed with a Waters (Millipore Corp.), HPLC system a Waters PicoTag column (3.9 by 150 mm), and amino acids converted to phenylthiocarbamyl derivatives by standard protocols (8).

Automated Edman degradation was performed with an Applied Biosystems model 477A liquid-phase sequencer with on-line phenylthiohydantoin derivative analysis using an Applied Biosystems 120A HPLC system operated as described in the manufacturer's recommendations.

The mass spectra of peptides (50 pmol) were recorded by using nitrocellulose targets (21) in an Applied Biosystems Bio-Ion 20 instrument. The spectrum was accumulated for 10^6 fission events corresponding to approximately 10 min. The details of the instrumentation and spectral analysis have been described elsewhere (39).

RESULTS

The intraperitoneal instillation of casein gave, on average, 3.3×10^8 cells per hamster (range, 1.0×10^9 to 5.6×10^9, population pooled from at least three animals and seven independent isolations). The cells identified after May-Grünwald–Giemsa staining consisted, on average, of 76.9% polymorphonuclear granulocytes (range, 69.3 to 84.6%), 21.2% macrophages (range, 15.4 to 30.4%), and 1.8% lymphocytes (range, 0 to 5.4%). The membranes of collected cells were selectively disrupted by shaking in the presence of heparin (23). The preliminary experiments showed that 2 min of shaking disrupted about 95% of the cells. The membranes and unbroken cells were sedimented, and the remaining intact granular fraction was then deprived of heparin through several washing and centrifuging steps, resulting in a greenish pellet of granule-rich fraction. The procedure of homogenization by repetitive freeze-thawing steps followed by overnight extrac-

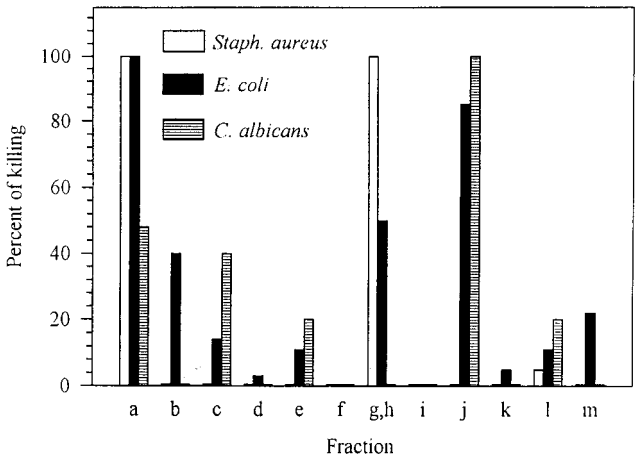

FIG. 2. Antimicrobial activities of hamster neutrophil granule extract fractionated on a C-8 column. Fifty microliters of microbe suspensions at a concentration of about 10^4 cells per ml were incubated at 37°C for 60 min with 10 μl of a water solution of fractions obtained by HPLC separation on a C-8 column (an amount equivalent to that obtained from 1.2×10^7 cells; see Fig. 1). Following incubation, the mixtures were diluted 10 times, 50-μl amounts were plated on agar plates, and after overnight incubation at 37°C, the colonies were counted. The percentage of killed microorganisms was calculated in relation to colonies grown after treatment with 10 μl of water. The details of microorganism maintenance are presented in Materials and Methods.

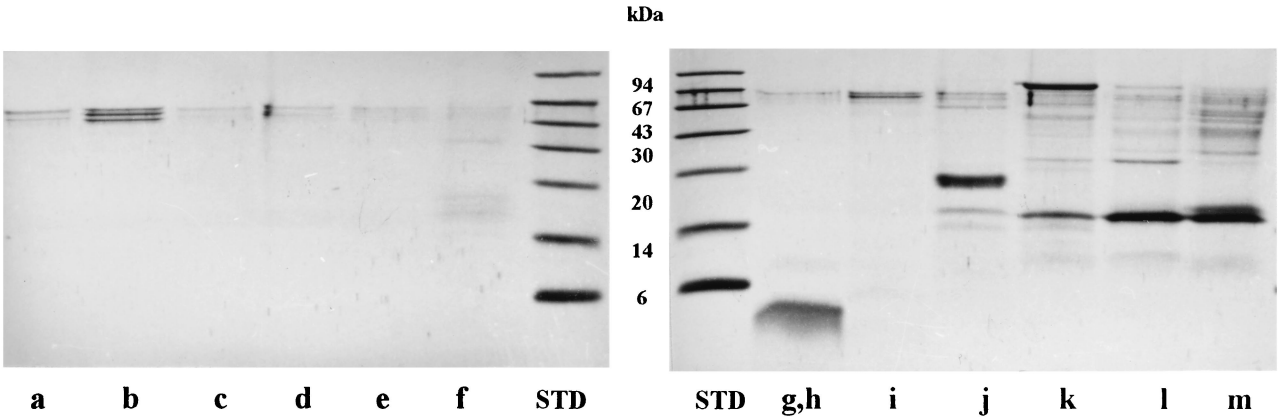

FIG. 3. SDS-PAGE of hamster neutrophil granule extract fractionated on a C-8 column. The amounts of a water solution of fractions (see Fig. 1) equivalent to that obtained from 2.4×10^7 cells were mixed in 1:1 proportions with reducing sample buffer, boiled for 2 min, and loaded on 16.5% SDS-PAGE gels (34). The reducing sample buffer with 50 mg of dithiothreitol per ml was applied to all samples. The gels were stained with Coomassie blue G-250.

tion gave a crude granule extract exhibiting elastinolytic activity checked by using a synthetic substrate, MeO-Suc-Ala-Ala-Pro-Val-*p*NA. The extract equivalent of 10^6 cells releases, on average, 3.0 nmol of *p*-nitroaniline during 10 min of incubation at room temperature.

During fractionation on a C-8 column, the crude extract was initially divided into 12 fractions designated a to m, including fractions g and h treated together in initial experiments (Fig. 1). Each fraction was then subjected to a microbicidal activity assay with standard strains representative of gram-negative and gram-positive bacteria, i.e., *E. coli* and *Staphylococcus aureus* as well as the common pathogenic fungus *Candida albicans*. As shown in Fig. 2, the majority of fractions express microbicidal activity with a very different specificity. Only fractions with a molecular mass below 6 kDa (fractions g and h) (Fig. 3) were chosen for further purification. Fraction g resolves on a μBondapak phenyl column, giving two major peaks, g1 and g2 (Fig. 4), but in the case of fraction h, only a μBondapak C-18 column allows for sufficient separation of major peak h2 from the considerably minor fraction h1 (Fig. 5).

SDS-PAGE of each of four finally obtained fractions gave bands localizing in the range of insulin B-chain peptide, a standard with a molecular mass 3.49 kDa (data not shown). These results were confirmed by mass spectroscopy. Recorded spectra (data not shown) present very homogeneous compounds with the following molecular masses: g1, 4,005.0 Da; g2, 3,618.2 Da; h1, 3,885.7 Da; h2, 3,819.7 Da. The amino acid analysis of purified peptides performed (data not shown) allowed the estimation of their real concentration, which was particularly important for further determination of the IC_{50} in a microbicidal assay. Our sample procedure gave 1.9×10^9 neutrophils from 10 hamsters, which resulted finally in 15.4 μg of peptide g1, 15.2 μg of peptide g2, 9.2 μg of peptide h1, and 163.4 μg peptide of h2.

Because of the small amounts of peptides obtained during purification, the cysteine residues were not modified prior to Edman degradation, although clear proof of the peptides' presence was obtained from amino acid analysis. For that reason, each peptide gave six blanks in positions where the cysteine residues were expected. The theoretical molecular masses of the peptides, calculated from the sequence including the mass of the six cysteine residues, were as follows: 4,002.5

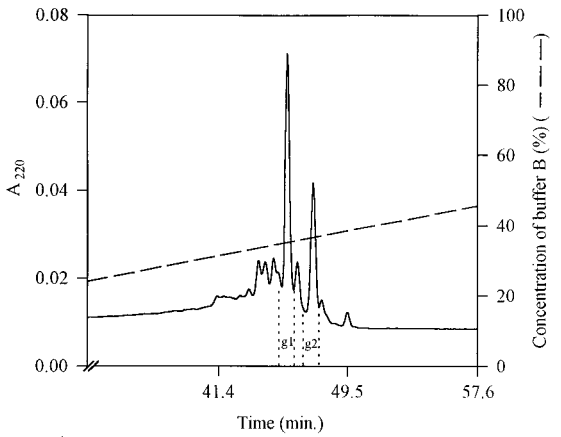

FIG. 4. Final purification of HaNP-1 and HaNP-2 (fractions g1 and g2) on a μBondapak phenyl column. Fraction g from the C-8 column (see Fig. 1), equivalent to that obtained from 9.7×10^8 cells, was resolved under conditions described in Materials and Methods.

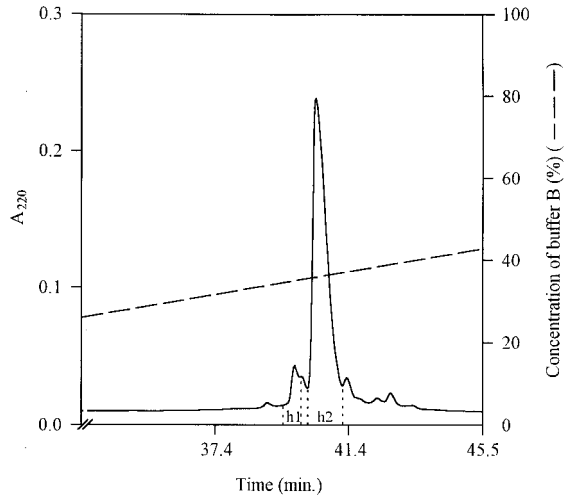

FIG. 5. Final purification of HaNP-3 and HaNP-4 (fractions h1 and h2) on a μBondapak C-18 column. The fraction h from a C-8 column (see Fig. 1) equivalent to that obtained from 9.7×10^8 cells was resolved under conditions described in Materials and Methods.

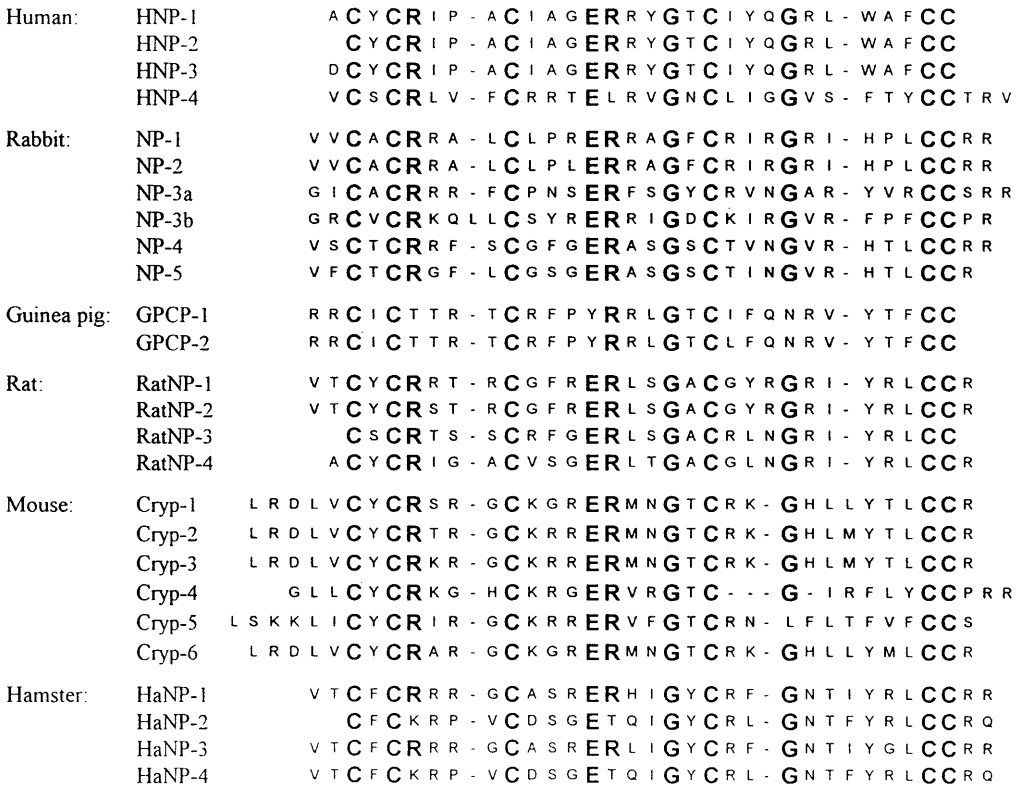

FIG. 6. Alignment of amino acid sequences of isolated mammalian defensin. To maximize the alignment, the gaps are shown and the identical as well as conservative residues are indicated by bold type. The sequences were obtained from the following sources: human (38) (comparison does not include enteric defensin 5 and 6 known only from mRNA sequences [19, 20]), rabbit (36), guinea pig (43), rat (38) (not including other defensins purified from bone marrow [2]), mouse (18) (not comprising defensin 7 to 17 known only from mRNA sequences [31]), hamster (this paper). Hamster peptides HaNP-1 to -4 are identical to fractions g1, g2, h1, and h2 appearing in the text, respectively.

Da for g1, 3,610.0 Da for g2, 3,879.4 Da for h1, and 3,810.2 Da for h2. These values agree very well with the data obtained from mass spectroscopy. We recognized this agreement as sufficient proof for placing cysteine residues in our sequence data. The final results of sequencing are presented in Fig. 6, where the fractions g1, g2, h1, and h2 were designated HaNP (hamster neutrophil peptide) −1, −2, −3, and −4, respectively.

The antimicrobial activities of purified peptides expressed as IC_{50} calculated for each microorganism are presented in Table 1. The published data of microbicidal activity of identical compounds are often distinct or difficult to compare with regard to different experimental conditions, additives to buffers, or different units of activity. This fact obliged us to perform an additional experiment combining the antimicrobial activity of our peptides with that of a common antimicrobial compound of neutrophil granules, cathepsin G. The two selected bacteria and *Candida albicans* were incubated with agents in previously determined concentrations equal for each tested compound, which in the case of cathepsin G resulted in 50% inhibition of microorganism growth. The results obtained are shown in Fig. 7.

TABLE 1. IC_{50} values of hamster neutrophil peptides against various microorganisms

Microorganism	IC_{50} (μg/ml)			
	HaNP-1	HaNP-2	HaNP-3	HaNP-4
Staphylococcus aureus	0.8	18.2	0.55	6.5
Streptococcus pyogenes	0.6	6.1	0.3	5.9
Enterococcus faecalis	0.4	12.0	0.7	9.2
Escherichia coli	1.5	30.4	11.0	11.8
Salmonella serotype krefeld	1.2	24.3	1.8	9.8
Klebsiella oxytoca	4.3	20.2	2.6	13.1
Pseudomonas aeruginosa	46.2	25.8	15.6	32.7
Candida albicans	4.6	38.0	12.9	81.7
Cryptococcus neoformans	1.8	ND[a]	0.9	35.9

[a] ND, not determined.

DISCUSSION

The population of cells we used for isolation contained about 20% macrophages, and this quantity is similar to that observed by other authors (13). The hamster leukocyte elastase has been described previously (5), and our preparation exhibiting its activity confirms this report. The elastase may also be used as a simple and useful granule marker. The granules of neutrophils contain a variety of antimicrobial compounds (25), and therefore, the antimicrobial activity observed in almost all fractions from granule extract (Fig. 2) is understandable.

The protocols of isolation of defensins from neutrophil granules (12, 36, 42, 43) are usually a combination of size exclusion, reverse-phase, and sometimes ion-exchange chromatographies, but good results are also obtained by preparative continuous acid-urea PAGE (17). Our procedure seems to have one major advantage, i.e., all separations could be carried out quickly on the same HPLC apparatus and with the same set of

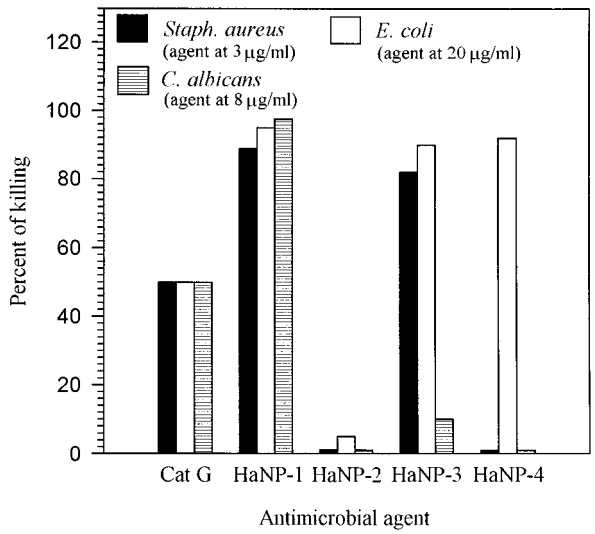

FIG. 7. Comparison of antimicrobial activities of hamster defensins and human cathepsin G. The water solutions of agents were incubated for 60 min at 37°C in 50 μl of suspension containing about 10^5 microorganisms per ml. The mixtures were further diluted 10 times and plated on agar in 20-μl amounts. After overnight incubation, the number of colonies was counted and compared with the number of colonies grown from control incubations with water. For each microorganism, the tested agents were used in the same concentration which caused 50% inhibition of growth in the case of cathepsin G.

buffers and only necessary column exchanges. On the other hand, the similar retention times of the hamster defensins we observed may require a high-quality column for sufficient separation. The SDS-PAGE and mass spectrometry results and the one amino acid residue released during each step of sequencing confirms the purity of the peptides obtained. The human neutrophils are reported to contain 4 to 5 μg of defensins per 10^6 cells (15), reaching 5 to 7% of total cellular protein (26), but unfortunately, we have not found any information regarding a yield from a specific purification procedure in the literature. In our case, HaNP-4, the most abundant peptide we have found, may be obtained from 10^6 cells in an amount of 0.1 μg. The remaining peptides are isolated in more than 10-fold-lower amounts, and the HPLC profiles (Fig. 4 and 5) suggest the probability that other defensins are present at concentrations too low for further characterization.

Generally, defensins exhibit greater potency against gram-positive bacteria and fungi than against gram-negative bacteria, and their effective concentrations are usually in range of 10 to 100 μg/ml (26) (other reports give a range of 1 to 50 μg [38]). In the case of hamster peptides, this is true for bacteria; however, the fungi we tested happened to be relatively poorly susceptible, with the exception of HaNP-1 and -3 for *Cryptococcus neoformans* (Table 1), but this result may be related only to the specific clinically isolated strains we used. The results obtained during repetitive isolations suggest that the peptide designated HaNP-4 is the most abundant among peptides in hamster neutrophil granules but reveals the lowest specific antimicrobial activity, similar to that of HaNP-2. Overall, the antimicrobial assays demonstrated that among members of the defensin family, hamster defensins were highly efficient in killing bacteria. We mentioned earlier that the antimicrobial activity data obtained in different papers are often difficult to compare. We tried to avoid this problem by comparing the activity of hamster defensins with the antimicrobial activity of cathepsin G (Fig. 7). Cathepsin G is a common granule component exhibiting an enzymatic as well as antimicrobial activity (1). The antimicrobial activity of cathepsin G is related to the presence of a few active domains, and therefore, using concentrations of micrograms per milliliter seems to be most reasonable. Good correlation between our results and literature values given for the whole cathepsin G activity (7) confirms simultaneously the validity of our results.

The Blitz sequence alignment searching algorithm (Biocomputing Research Unit, University of Edinburgh, Edinburgh, United Kingdom) performed on the SWISSPROT database showed that hamster defensins are most homologous to the mouse cryptdin family. The best alignments always refer to cryptdin 3 as showing levels of residue identity from 41.8% for HaNP-4 to 59.6% in the case of HaNP-3. In light of these results, it seems strange that the defensins are absent in phylogenetically close mouse neutrophils (13). Of special interest is that HaNP-2 and HaNP-4, lacking characteristic conservative arginine residues, are also relatively less microbicidal than HaNP-1 and HaNP-3, where these arginines are present. In a similar bactericidal assay system (31), the antibacterial potential of mouse cryptdins 1 and 3 to 6 is manifested only in a concentration range above 10 μg/ml.

The sequence data we have obtained (Fig. 6) suggest the possibility that HaNP-2 is a product of proteolytic N-terminal amino acid removal from HaNP-4, and this mechanism is discussed elsewhere (29). In our opinion, the fact of a generally lower microbicidal potency of HaNP-2 than of HaNP-4 (Table 1) and the abundance of HaNP-4 in granules as a potential substrate for proteinase strongly support the amino acid removal hypothesis. Additionally, our results might support future structural studies on the influence of N-terminal substitutions on the antimicrobial activity of defensins.

It may appear that the unhesitating inclusion of hamster peptides in the defensin family without determination of disulfide bond succession may be questionable. However, in our opinion, the high percentage of homology to defensin family members and small amounts of material we obtained make these determinations unnecessary.

In summary, the hamster neutrophil granules contain at least four peptides from the defensin family. One, HaNP-4, is always isolated in amounts about 10 times greater than the others. The defensins HaNP-1 and HaNP-3 are the most bactericidal, killing gram-positive bacteria with IC_{50}s even at concentrations below 1 μg/ml. The sequence homology search showed that hamster defensins are most similar to the mouse enteric defensin family but are more bactericidal. The serious phylogenetic considerations must be supported by further sequencing and the characterization of genes.

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