Identification of eNAP-1, an Antimicrobial Peptide from Equine Neutrophils

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Endogenous, cysteine-rich antimicrobial peptides known as defensins are prominent components of human, rabbit, and rat neutrophils, yet little is known about their occurrence in other mammalian species. Although we did not detect mature (i.e., processed) defensins in equine neutrophil granules, we found that these granules contained small amounts of other cysteine-rich peptides with antimicrobial activity. One of these, eNAP-1, was purified by a combination of gel permeation and reversed-phase high-performance liquid chromatography from acid extracts prepared from the cytoplasmic granules of equine neutrophils. The molecular mass of eNAP-1 was approximately 7.2 kDa, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. Amino acid analysis revealed that eNAP-1 had an unusually high cysteine content and that it was relatively enriched in alanine, glycine, lysine, and proline residues. The partial (N-terminal) amino acid sequence of eNAP-1 was DVQCGEGHFCHDXQTCCRASQGGXACCPYSQGVCCADQRHCCPVGF. Thirty-six of these residues (78.3%) were identical to those of a recently cloned human neutrophil peptide of unknown function and belonging to the granulin family. Homologous peptides have also been noted in rat bone marrow cells and rat kidney epithelins. We tested the ability of eNAP-1 to kill several equine uterine pathogens. *Streptococcus zooepidemicus* was killed most effectively, sustaining a >99.8% decrease in CFU per milliliter after a 2-h exposure to 100 μg of eNAP-1 per ml (~15 μM). *Escherichia coli* and *Pseudomonas aeruginosa* were somewhat less susceptible, manifesting 87.0 and 87.1% mean decreases in CFU per milliliter, respectively, after incubation for 2 h with 200 μg of eNAP-1 per ml. *Klebsiella pneumoniae* numbers were not significantly reduced after exposure to eNAP-1. These antimicrobial properties suggest that eNAP-1 may contribute to phagocyte-mediated host defense against equine infections.

The prompt elimination of potentially pathogenic microorganisms is effected by the phagocytic arm of the innate immune system. Microbial agents, mainly bacteria and fungi, are largely destroyed by the combined efforts of phagocytes and antibody-producing cells. Neutrophils (polymorphonuclear leukocytes [PMNs]) are key effector cells in the defense against microbial invasion (25). Following phagocytosis by PMNs, most microorganisms are killed intracellularly by oxidative and/or nonoxidative mechanisms (19). The former rely on the oxygen-dependent "respiratory burst" to generate strong microbicidal oxidants, whereas the latter utilize an array of granule-associated enzymatic and nonenzymatic proteins, such as lysozyme, cathepsin G, lactoferrin, and bacterial permeability-increasing protein, as well as antibiotic peptides, such as defensins and related molecules (17). The extent to which neutrophils from diverse species vary in their content of endogenous antimicrobial polypeptides has received little attention. In this report, we describe both the absence of mature defensins and the presence of a novel, cysteine-rich antimicrobial peptide, eNAP-1, in equine neutrophils. eNAP-1 proved to be homologous to several recently isolated peptides (epithelins) that can regulate epithelial cell proliferation in vitro (30).

MATERIALS AND METHODS

Experimental animals and neutrophil isolation. A protocol for animal use and care was filed with the Environmental Health and Safety, Animal Health and Welfare Veterinarian's Office at the University of California, Davis, Campus. Neutrophils were obtained from the venous blood of 24 normal young mares by collecting 500 ml of blood from the jugular vein of each mare into evacuated bottles that contained 70 ml of acid-citrate-glucose anticoagulant. After being gently mixed by repeated inversion, the blood samples were stored on ice until 250-ml aliquots were centrifuged at 950 × *g* for 20 min at 20°C. After the plasma supernatant was aspirated and discarded, the top 1 cm containing the PMN-rich buffy coat was carefully aspirated and subjected to three cycles of cold hypotonic lysis, 15 s each, and centrifugation at 150 × *g* for 10 min at 20°C (4). The concentrated leukocyte layer was suspended and washed three times by centrifugation (150 × *g* for 10 min at 20°C) in Hanks balanced salt solution (pH 7.4) (HBSS; Sigma Chemical Co., St. Louis, Mo.) without calcium or magnesium but with 2% EDTA. This leukocyte preparation was separated on a discontinuous density gradient consisting of 60% Percoll (Sigma) underlaid with 75% Percoll (8). Sixty percent Percoll was prepared by mixing 6.0 ml of stock Percoll with 1.0 ml of 10× HBSS and 3.0 ml of deionized water; 75% Percoll consisted of 7.5 ml of stock Percoll, 1.0 ml of 10× HBSS, and 1.5 ml of deionized water. After centrifugation in a swinging-bucket rotor at 200 × *g* for 20 min at 20°C, the PMN-enriched layer was carefully aspirated with a Pasteur pipette and suspended in 20 ml of 0.34 M sucrose (pH 7.4). Cells were counted

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electronically (Coulter Counter; Coulter Electronics, Hialeah, Fla.) and assessed for purity (Romanowsky-stained cytospins) and viability (trypan blue exclusion). Neutrophils were also obtained from the uterine exudate of young mares after induction of acute, sterile endometritis by intrauterine infusion of 50 ml of a sterilized streptococcal filtrate as previously described (7).

For collection of the uterine exudate, the uterine cavity was washed with 250 to 500 ml of sterile Dulbecco's phosphate-buffered saline (pH 7.3) (Sigma) without calcium or magnesium but with 5 mM glucose and 5 U of heparin per ml (lavage buffer). The exudate was collected through a sterile silastic catheter and stored on ice. Within 2 h of harvest, the recovered cells were washed with lavage buffer by low-speed centrifugation (330 × g for 10 min at 2°C) and suspended in Dulbecco's phosphate-buffered saline. Contaminating endometrial cells and erythrocytes were simultaneously removed by 15 s of cold hypotonic lysis followed by centrifugation at 150 × g for 10 min at 2°C. The resulting pellet was suspended in 20 ml of 0.34 M sucrose (pH 7.4). Differential cell counts and viability were determined as described above.

Granule extracts from human and rabbit neutrophils. Neutrophils were obtained and their granules were extracted as described previously (12, 29).

Purification of eNAP-1. A granule-rich fraction was prepared as previously described (4). In brief, PMNs were disrupted by nitrogen cavitation in a cell disruption chamber (Parr Instruments, Moline, Ill.) at 950 lb/in² for 30 min on ice and then centrifuged at 330 × g for 10 min at 2°C, a process that substantially removed contaminating membrane and nuclear debris and intact cells. The pellet from the low-speed centrifugation was washed several times with 0.34 M sucrose, until few additional granules, as determined by phase-contrast light microscopy, were present in the supernatants. The granule-rich supernatants were combined and centrifuged at 27,000 × g for 20 min at 2°C. The purity of the granule preparation was assessed by phase-contrast light microscopy and, in the initial stages, by transmission electron microscopy in accordance with published protocols (20). In brief, a Percoll-purified blood PMN suspension (3 × 10^7 cells in 200 μl) and a granule preparation from the centrifugation at 27,000 × g were fixed in 200 and 35 μl of Karnovsky solution (1.33 M paraformaldehyde, 0.5 M glutaraldehyde, 0.08 M Na cacodylate) (16), respectively, and stored at 4°C for 48 h. The fixed samples were placed in 2% agarose, centrifuged, sliced, embedded, and processed as described previously (20). The granule-enriched pellets from the centrifugation at 27,000 × g were pooled, and their granules were extracted overnight on ice with gentle stirring in 10% acetic acid. The extract was cleared by centrifugation at 27,000 × g for 20 min at 2°C and concentrated in a vacuum centrifuge (SpeedVac; Savant Industries, Hicksville, N.Y.). The concentrate was gel filtered on a Bio-Gel P-10 column (1 by 50 cm; Bio-Rad Laboratories, Richmond, Calif.) previously equilibrated in 5% acetic acid. The eluate was monitored spectrophotometrically at A_{280}, and every third chromatographic fraction was analyzed by acid-urea-polyacrylamide gel electrophoresis (AU-PAGE) (22). Fractions of interest were selected on the basis of their antimicrobial activity against *Streptococcus zooepidemicus* (vide infra), concentrated, and further purified by reversed-phase high-performance liquid chromatography (RP-HPLC), as described previously (4), on a Vydac C_{18} column (0.46 by 25 cm; The Separations Group, Hesperia, Calif.) with an M45/680 binary-solvent delivery system (Millipore-Waters, Mil-

ford, Mass.). Proteins were eluted by an increasing gradient (0 to 60% in 60 min) of acetonitrile that contained 0.1% trifluoroacetic acid as an ion-pairing agent. The eluate was monitored by use of dual variable-wavelength detectors that were set at 280 nm and at 220, 225, or 230 nm. The purity of the peaks was ascertained by AU-PAGE and analytical RP-HPLC. Several repurification steps with various acetonitrile gradients were needed to achieve baseline-separated peaks. The molecular masses of the antimicrobial molecules were estimated by sodium dodecyl sulfate-tricine polyacrylamide gel electrophoresis (SDS-TPAGE) (24) and by classical Laemmli sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (18) with 0.75-mm-thick minigels (Hoefer Scientific Instruments, San Francisco, Calif.) containing 16.5% (SDS-TPAGE) or 18% (SDS-PAGE) polyacrylamide in the resolving layer and 4.5% polyacrylamide in the stacking layer.

Amino acid composition. Native peptides were hydrolyzed in 5.6 N HCl for 40 h at 115°C. Cysteine was measured as cysteic acid following performic acid oxidation prior to hydrolysis (21). Residual vapors were eliminated by lyophilization, and the amino acids were derivatized with phenylisothiocyanate (Pierce Laboratories, Rockford, Ill.) (3). The corresponding phenylthiocarbamyl residues were subsequently separated and quantified by RP-HPLC with a 510 binary-solvent delivery system (Millipore-Waters) on a Nova-Pak C_{18} column (Millipore-Waters) (13, 26).

Sequence determination and analysis. Purified eNAP-1 was reduced and alkylated with 4-vinylpyridine (Aldrich Chemicals) or iodoacetamide (Sigma) in 6.0 M guanidine HCl–0.5 M Tris HCl (pH 8.1)–0.2 mM EDTA (13), desalted by RP-HPLC on a Vydac C_{18} column, and subjected to gas-phase Edman degradation on a model 475A automated sequencing system (Applied Biosystems, Inc., Foster City, Calif.) (26). For carboxyamidomethylated (CAM) eNAP-1, we loaded 1.4 nmol of sample and recovered 751 and 775 pmol of aspartic acid and valine, respectively, in cycles 1 and 2. The repetitive yield for all amino acids was 90.99% ± 0.617% (mean ± standard error of the mean). For pyridylethylated (PE) eNAP-1, we loaded approximately 1.0 nmol of sample and recovered 436 and 385 pmol of aspartic acid and valine, respectively, in cycles 1 and 2. The overall repetitive yield was 90.17% ± 0.63%. As PE cysteine could not be distinguished from proline, cysteine residues were assigned from analysis of CAM eNAP-1. The amino acid sequences were analyzed for similarity to those of known proteins by use of the FASTA algorithm (23) and the following computer data bases: GenBank 70.0; EMBL 29.0; Swiss-Prot 19; and GenPept 70.0.

Antibacterial assays. The antimicrobial activity of eNAP-1 was assessed with clinical uterine isolates of *S. zooepidemicus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* that had been obtained from mares with spontaneous endometritis. Bactericidal activity assays were performed essentially as described previously (12). eNAP-1 stock solutions were prepared by dissolving lyophilized preparations in 0.01% sterile acetic acid. Gram-negative organisms were maintained frozen in Trypticase soy broth containing 10% glycerol. The surface of the frozen cultures was lightly scraped with a sterile loop, and the material obtained was plated on Trypticase soy agar plates. Plates were incubated at 37°C for 24 h or until full colony development occurred. A single colony was picked from the Trypticase soy agar plates. After it had grown for 18 h in Trypticase soy broth at 37°C, 1 ml of this intermediate culture was subcultured into 40 ml of fresh broth and

incubated for an additional 4 h. The resulting cultures were washed three times by centrifugation in 10 mM sodium phosphate buffer (pH 7.4). *S. zooepidemicus* was processed essentially in the same way, except that brain heart infusion broth and brain heart infusion agar were used as nutrient media. After the bacteria were washed in buffer, the number of CFU per milliliter was quantified spectrophotometrically at 600 nm. Incubation mixtures contained 5×10^4 CFU/ml in 10 mM sodium phosphate buffer (pH 7.4) and 100 or 200 µg of eNAP-1 per ml. Control mixtures lacked eNAP-1 but contained comparable amounts of 0.01% sterile acetic acid. All tubes were incubated at 37°C in a shaking water bath. At predetermined intervals, aliquots were removed, serially diluted (i.e., 1:10 and 1:100), plated in duplicate on nutrient agar plates by use of a spiral plater instrument (Spiral Technologies, Rockville, Md.), and incubated at 37°C for 24 to 48 h to allow full colony development. All experiments were performed in duplicate. The spiral plater provides accurate bacterial counts over a 4-$\log_{10}$ range of CFU per milliliter (10). Analysis of data was performed by use of Student's *t* test. Confidence intervals (95 and 99%) were calculated for each time point for control and treated samples. Differences between means were considered significant when no overlapping 95% confidence intervals occurred at a given time point for control and treated samples. The coefficient of variation was <5%. Antibacterial activity was expressed as the $\log_{10}$ reduction in CFU per milliliter after exposure to eNAP-1, relative to the levels of control, unexposed microorganisms (12).

RESULTS

Neutrophil granule preparation. Approximately 2.0×10^9 blood-derived cells or 1.0×10^{10} uterus-derived cells were processed at a time. Both types of samples typically consisted of >99% neutrophils determined to be >90% viable by trypan blue exclusion. Figure 1 shows the appearance, in transmission electron microscopy, of equine PMNs and of the granule-enriched sediment (from centrifugation at 27,000 $\times$ *g*) that served as the starting material for our peptide purifications.

Identification and purification of eNAP-1. After the neutrophil granule extract was fractionated by Bio-Gel P-10 gel filtration, our attention centered on two peaks of antimicrobial activity; the second peak contained eNAP-1, the peptide described in this report. The first peak contained a combination of lysozyme and eNAP-2, a structurally unrelated antimicrobial peptide that will be described elsewhere. Both peaks, which were equally effective against *S. zooepidemicus* (>3 $\log_{10}$ reduction in CFU per milliliter; vide infra) were eluted from the Bio-Gel P-10 column within 70% of its total bed volume. While equine neutrophil lysozyme and eNAP-2 coeluted, eNAP-1 had a slightly longer retention time. Granules prepared from 10^9 PMNs yielded approximately 0.5 µg of eNAP-1 and 2.5 to 5.0 µg of eNAP-2, indicating that these peptides are less than 1% as abundant as human neutrophil defensins (~1.25 mg/10^9 PMNs) (14). eNAP-1 was further purified by RP-HPLC, where it emerged at an acetonitrile concentration of 22% (Fig. 2). It was relatively pure after the first run, and minor adjustments of the acetonitrile gradient sufficed to allow its complete baseline separation. Figure 3 shows the appearance of eNAP-1 after SDS-PAGE and AU-PAGE. By SDS-PAGE, we estimated the molecular mass of eNAP-1 to be approximately 7.2 kDa. SDS-PAGE also revealed the absence of any detectable defensin bands in acid-extracted equine PMN
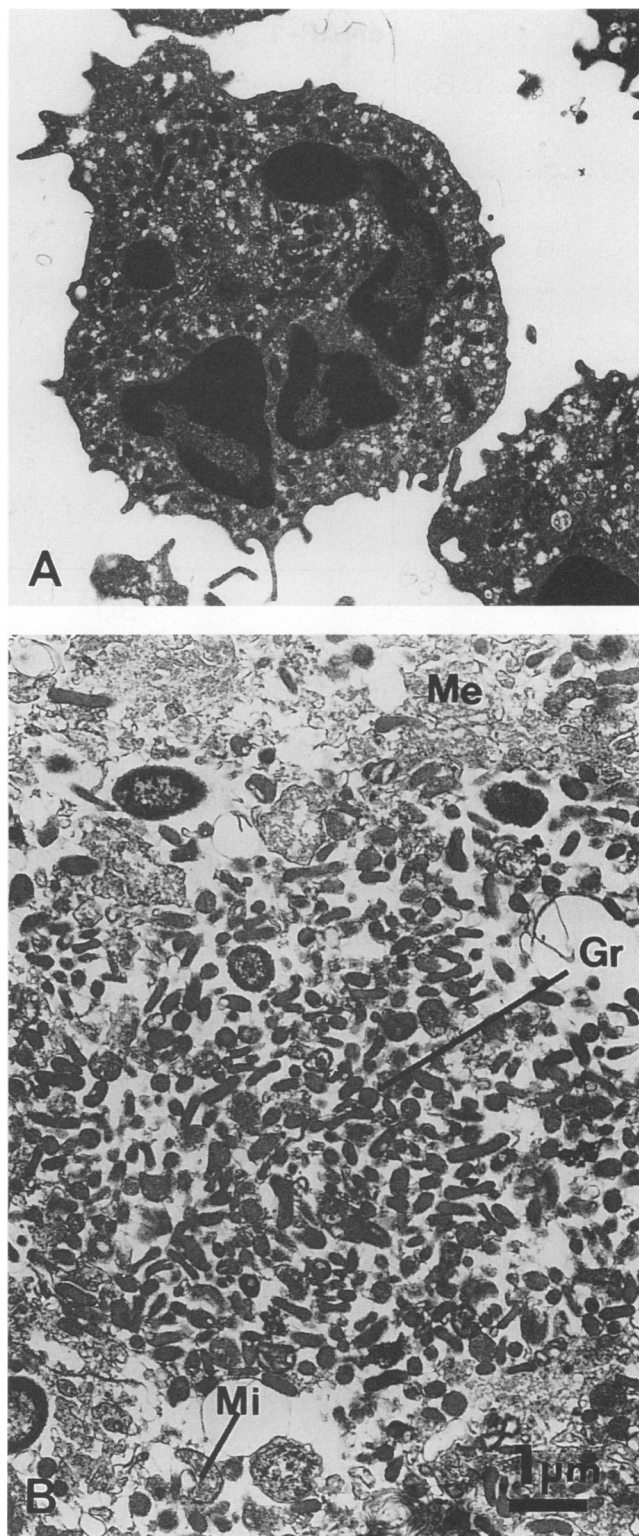

FIG. 1. Electron micrographs depicting an equine blood neutrophil (A) and its subcellular constituents (B) after nitrogen cavitation and centrifugation at 27,000 $\times$ *g*. Gr, cytoplasmic granules; Mi, mitochondrion; Me, plasma membrane.
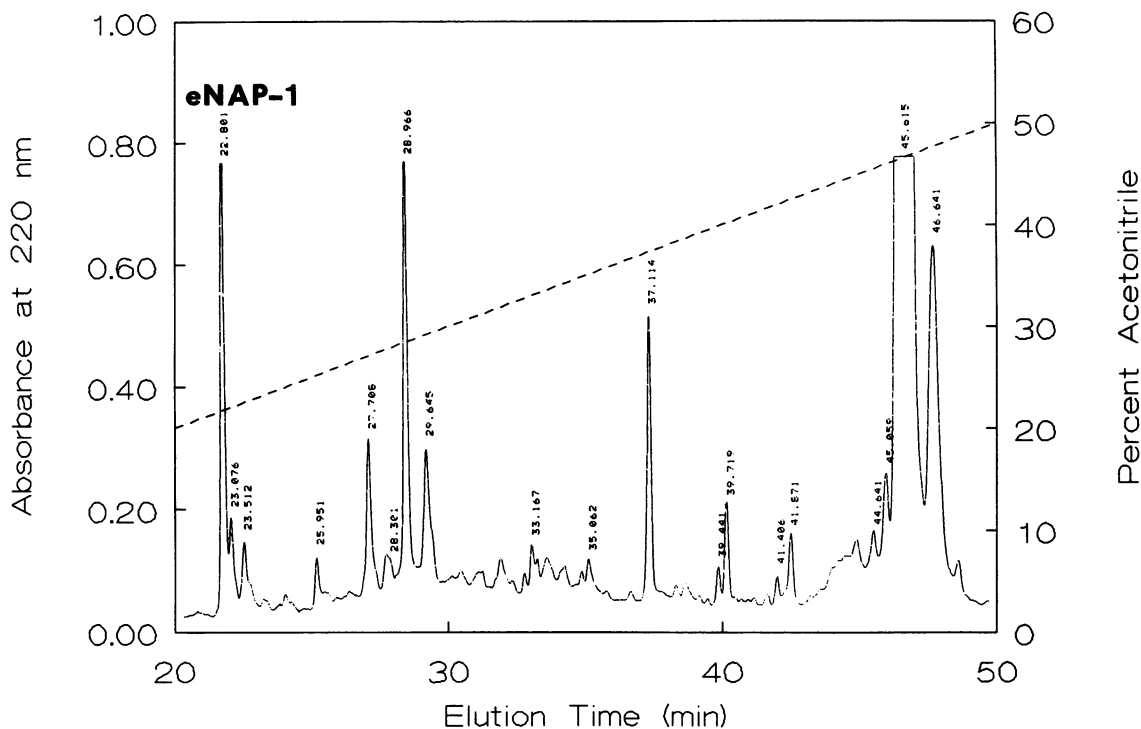
FIG. 2. Initial RP-HPLC purification of eNAP-1 (peak 2) from the Bio-Gel P-10 column (see the text).

granules (Fig. 3B). In AU-PAGE, eNAP-1 manifested a prominent cathodal migration, suggesting that it had a net positive charge.

Amino acid composition. Amino acid analysis of performic acid-oxidized eNAP-1 (Table 1) indicated that cysteine (recovered as cysteic acid) represented approximately 14.54 mol% of the amino acids. Lysine accounted for 7.28 mol% of

the amino acids recovered after hydrolysis of the performic acid-oxidized samples. Arginine was somewhat less abundant, representing only 4.94 mol% of the total amino acids recovered in native and oxidized eNAP-1 hydrolysates. On the basis of an estimated M_r of 7,200, the amino acid composition analysis correctly predicted that eNAP-1 con-
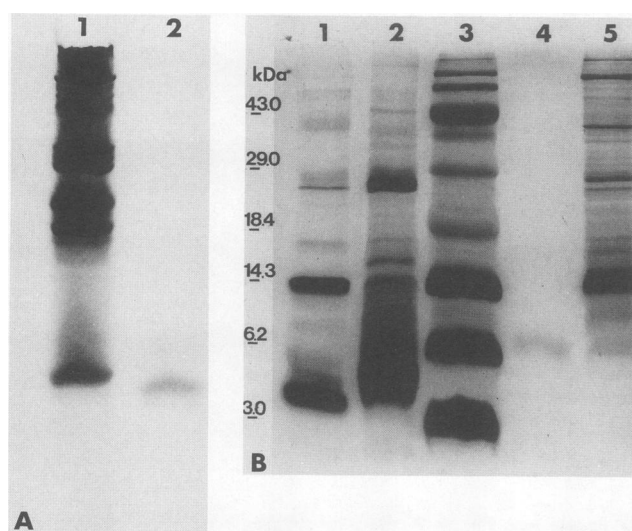

FIG. 3. (A) AU-PAGE. Lanes: 1, equine PMN crude granule extract; 2, highly purified eNAP-1 (~500 ng). (B) SDS-PAGE (silver-stained gel). Lanes: 1, human PMN crude granule extract; 2, rabbit PMN crude granule extract; 3, molecular size standards; 4, highly purified eNAP-1 (~200 ng); 5, equine PMN crude granule extract.

TABLE 1. Amino acid analysis of eNAP-1

Amino acid	nmol	Mol%
Alanine	0.4965	12.15
Arginine	0.2016	4.94
Asp(x)[a]	0.2996	7.33
Cysteine[b]	0.5939 (calculated)	14.54 (calculated)
Glu(x)[c]	0.4221	10.33
Glycine	0.5517	13.50
Histidine	0.1697	4.15
Isoleucine	0.00	0.00
Leucine	0.0899	2.20
Lysine	0.2975	7.28
Methionine	TU[d]	TU
Phenylalanine	0.0661	1.62
Proline	0.3599	8.81
Serine	0.1615	3.65
Threonine	0.1459	3.57
Tryptophan	ND[e]	ND
Tyrosine	0.0301	0.74
Valine	0.1987	4.86
Total	3.9984	99.67

[a] Asp(x), aspartic acid plus asparagine.
[b] Cysteine was measured as cysteic acid in a separate, performic acid-oxidized sample.
[c] Glu(x), glutamic acid plus glutamine.
[d] TU, technically unsatisfactory.
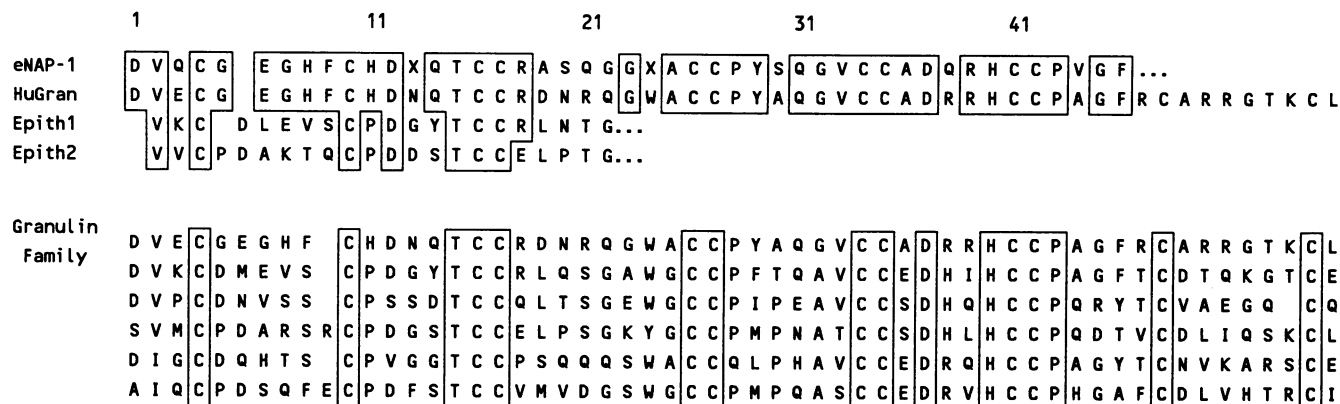[e] ND, not determined.

FIG. 4. Amino-terminal sequence of eNAP-1. The eNAP-1 sequence is aligned with those of a human leukocyte granulin (HuGran) and rat kidney epithelins (Epith1 and Epith2) (30). The conserved residues are boxed. Shown below are the translated sequences of six human granulins. The human granulin sequences were derived from cDNA data deposited in GenBank (accession M75161) by Bhandari et al. (2) on 26 August 1991. 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; X, unassigned residue; Y, tyrosine.

tained at least nine cysteine residues. This value was in agreement with the amino acid sequence data (vide infra). The low tyrosine content (0.74 mol%) was consistent with the negligible A_{280} of eNAP-1, which was <1% of its A_{225}. eNAP-1 hydrolysates also contained relatively large amounts of glycine (13.5 mol%), alanine (12.15 mol%), and proline (8.81 mol%). This analysis was performed on eNAP-1 purified by a single cycle of RP-HPLC (Fig. 2) after Bio-Gel column P-10 chromatography.

Amino acid sequence. A partial N-terminal sequence of more highly purified eNAP-1 was obtained by automated Edman degradation of its CAM and PE derivatives and is shown in Fig. 4. No significant sequence similarity between eNAP-1 and eNAP-2 was observed (data not shown). However, 36 of the first 46 residues of eNAP-1 (78.3%) were identical to those in one of the six human granulins, a family of peptides that was recently isolated from human leukocytes (1) and cloned from a human bone marrow cDNA library (2). There was complete conservation of all 10 comparable cysteinyl residues between eNAP-1 and a cloned human granulin. The primary structure of eNAP-1 was also closely related to those of two recently described peptides, epithelins, isolated from rat kidneys (30). Thus, when aligned and compared with partial N-terminal sequences of epithelins 1 and 2, the N-terminal sequence of eNAP-1 showed the conservation of nine and eight residues, respectively, including the four comparable half-cystines (Fig. 4).

Antibacterial activity. The antimicrobial activity of 100 or 200 μg of eNAP-1 per ml (~15 or 30 μM) was tested against four clinical isolates of common equine uterine pathogens. As shown in Table 2, S. zooepidemicus, E. coli, and P. aeruginosa were killed, as evidenced by the decreased CFU per milliliter relative to the initial input (5 × 10⁴ CFU/ml) and the CFU per milliliter in the incubated controls. After 2 h of incubation in the presence of 100 or 200 μg of eNAP-1 per ml, the mean reductions in colony counts relative to the input CFU per milliliter were as follows: S. zooepidemicus, >99.8%; E. coli, 87.0%; and P. aeruginosa, 87.1%. In addition, eNAP-1 exerted a mild, although not statistically significant, bacteriostatic activity against K. pneumoniae, as indicated by the reduced growth (~0.46 $\log_{10}$ decrease) of K.

pneumoniae exposed to eNAP-1 with respect to the growth of the untreated control.

DISCUSSION

Whereas cationic, cysteine-rich natural antibiotic peptides (e.g., defensins) have been identified in the neutrophils of several species, including humans, rats, rabbits, and guinea pigs (11, 12, 27, 29), the antimicrobial peptides of equine PMNs have not been previously defined. In contrast to the remarkable abundance of defensins in the neutrophils of the aforementioned species, our SDS-PAGE analysis of equine PMN granule extracts revealed that these cells had little, if any, defensin content. Although defensins were lacking, other peptides with antimicrobial properties were clearly present, including eNAP-1, the subject of this report. eNAP-1 differed from defensins in several important respects. The defensins, of which 16 are currently known, contain six invariantly positioned cysteines, contain 29 to 35 amino acid residues, and have molecular masses of <4.5 kDa. Tracheal antimicrobial peptide, a recently described microbicide peptide derived from bovine tracheal epithelial cells (9), is slightly larger (38 amino acid residues; molecular

TABLE 2. Antibacterial activity of eNAP-1 against test organisms[a]

Organism[b]	Mean CFU/ml[c] in:		Log₁₀ reduction of input CFU/ml[d] (%)[e] after incubation
	Control samples	Treated samples	
S. zooepidemicus[f]	1.5 × 10⁵	<10²	>2.7 (>99.8)
E. coli	1.3 × 10⁵	6.51 × 10³	0.88 ± 0.12 (87.0)
P. aeruginosa	1.0 × 10⁵	6.45 × 10³	0.89 ± 0.25 (87.1)
K. pneumoniae	5.0 × 10⁵	1.09 × 10⁵	NR[g]

[a] After 2 h of incubation at 37°C in the presence of 200μg of eNAP-1 per ml.
[b] Two isolates of each species were tested.
[c] The input was 5 × 10⁴ CFU/ml.
[d] Mean ± standard error of the mean.
[e] Mean.
[f] Tested with 100 μg of eNAP-1 per ml.
[g] NR, no reduction.

mass, 4.08 kDa), and its six cysteine residues do not align with those of the classic defensins.

eNAP-1 (M_r, 7,200) is approximately twice as large as the defensins or tracheal antimicrobial peptide and belongs to a family of peptides with 12 conserved cysteine residues, rather than 6. Eight of these cysteines occur as C-C pairs. The number and placement of cysteine residues in eNAP-1, granulins, and epithelins bear no obvious similarity to the cysteine motif of the defensins or other previously described endogenous antibacterial peptides (reviewed in reference 9).

Epithelins 1 and 2, isolated from rat kidneys, had apparent molecular masses of approximately 5.5 and 6.0 kDa, respectively, as judged by SDS-TPAGE (30). Epithelin 1, even when added at concentrations well below 5 ng/ml, stimulated the proliferation of murine keratinocytes. Its homolog, epithelin 2, not only lacked these stimulatory effects but also acted to inhibit the growth promotion induced by epithelin 1. Since epithelins 1 and 2 were obtained by processing of approximately 1 lb (ca. 454 g) of whole frozen rat kidneys (30), their cellular source is unknown, and an origin from intravascular blood neutrophils trapped within the confines of the kidneys has not been excluded. A family of homologous peptides, termed granulins, were recently isolated from human and rat leukocytes (1). The authors of this report acknowledged their considerable sequence similarity to epithelins. Although no functional studies of these peptides were conducted, the authors speculated that granulins might serve as cytokinelike regulatory molecules, with potential roles in inflammation, wound repair, and tissue remodeling. Since granulins (1) and epithelins (30) were discovered independently and virtually simultaneously, we designate the family granulothelins in this report.

Our antimicrobial studies with eNAP-1 to date have been limited by the small amounts of the peptide that are currently available and by our many competing needs for it. The low concentration of eNAP-1 in equine PMNs stands in contrast to the remarkable abundance of human neutrophil defensins (>1 mg/10^9 PMNs) (14). Thus, it remains to be determined whether the antimicrobial properties of eNAP-1 are ancillary to some other primary role in the biology of equine PMNs. The antimicrobial activity of eNAP-1 appeared to depend on the target microorganisms, since the sole gram-positive bacterium that we tested, *S. zooepidemicus*, was much more sensitive than any of the three gram-negative organisms that were included in our panel. Under our assay conditions, the potency of eNAP-1 was substantially lower than that of rabbit defensin NP-1 and somewhat lower than that of equine PMN lysozyme when both of the latter were tested against the same panel of microorganisms (5, 6). We have not yet tested the possibility that eNAP-1 operates synergistically with lysozyme or with other neutrophil-derived antimicrobial molecules and have not yet examined whether eNAP-1 has epithelinlike effects on cultured epithelial cells. These questions deserve further study. Currently, our efforts are directed towards identifying additional antimicrobial components from equine neutrophils, with the intention of elucidating their respective contributions to the microbicidal function of equine PMNs.

ACKNOWLEDGMENTS

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