INFECTION AND IMMUNITY, Dec. 1992, p. 5042–5047
0019-9567/92/125042-06$02.00/0

Vol. 60, No. 12

eNAP-2, a Novel Cysteine-Rich Bactericidal Peptide from Equine Leukocytes

MARCELO A. COUTO,[1]†* SYLVIA S. L. HARWIG,[2] JAMES S. CULLOR,[3] JOHN P. HUGHES,[1] AND ROBERT I. LEHRER[2]

Departments of Reproduction[1] and Clinical Pathology,[3] School of Veterinary Medicine, University of California, Davis, California 95616, and Department of Medicine, Division of Hematology/Oncology, University of California Los Angeles, Center for the Health Sciences, Los Angeles, California 90024[2]

Received 22 June 1992/Accepted 21 September 1992

We purified a novel cysteine-rich antibiotic peptide, eNAP-2 (M_r, ~6,500), from acid extracts of equine neutrophils by sequential gel filtration and reversed-phase high-performance liquid chromatography and determined its partial N-terminal amino acid sequence. Although its cysteine motif distinguished eNAP-2 from all other currently known endogenous antibiotic peptides, including defensins and granulins, it showed substantial sequence similarity to *WDNM1*, a putative member of the four-disulfide-core protein family that also includes animal and human antiproteases, snake venom neurotoxins, and rat and mouse whey proteins. The antibacterial properties of eNAP-2 were tested against several equine uterine pathogens, namely, *Streptococcus zooepidemicus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. Killing of *S. zooepidemicus* was very efficient, as evidenced by a 94% decrease in numbers of CFU per milliliter after exposure to 100 μg of eNAP-2 per ml (~15 μM) for 2 h. Exposure of *E. coli* and *P. aeruginosa* to 200 μg of eNAP-2 per ml for 2 h resulted in 90.2 and 77.6% reduction, respectively, in the numbers of CFU per milliliter. Bacteriostasis, without bactericidal activity, occurred after *K. pneumoniae* was incubated with 200 μg of eNAP-2 per ml. Additional studies will be required in other species and cell types to determine whether eNAP-2 is restricted to equine neutrophils or is the index member of a larger family of endogenous antibiotics.

Polymorphonuclear leukocytes (PMN) play a crucial defensive role in the innate immune system by ingesting and killing invading microorganisms. The microbicidal arsenal of reactive oxygen intermediates and antibiotic proteins and peptides of the neutrophil effects postphagocytic killing of ingested bacteria and fungi by oxidative and/or nonoxidative mechanisms (3, 35). The granule-associated antimicrobial peptides and proteins of human PMN include azurocidin, bacterial permeability-increasing protein, cathepsin G, lysozyme, lactoferrin, and defensins (19). We recently isolated a novel cysteine-rich antimicrobial peptide, eNAP-1 (equine neutrophil antibiotic peptide 1), from acid extracts of equine neutrophil granules (5). Although this peptide was structurally distinct from defensins, its sequence exhibited >78% identity with one of the human granulins, a family of peptides recently reported to exist in human and rat neutrophils (1). We now report the identification and purification from equine PMN granules of an additional cysteine-rich antibiotic peptide that is structurally unrelated to either defensins or eNAP-1 (granulin). This peptide, eNAP-2, exerted appreciable antibacterial activity against selected pathogens that cause clinical endometritis in mares.

MATERIALS AND METHODS

Equine neutrophil isolation. Horses were housed at the University of California Davis Equine Research Laboratory, and a protocol for animal use and care was obtained. Jugular blood was collected from 24 normal mares, and neutrophils were isolated on a discontinuous Percoll density gradient (Pharmacia LKB, Piscataway, N.J.) as described previously (5). Percoll-purified neutrophils were suspended in 20 ml of 0.34 M sucrose (Fisher Scientific, San Francisco, Calif.) (pH 7.4), and aliquots were removed for PMN count and purity and viability analyses. Neutrophils were also collected from sterile uterine exudates after the induction of an acute, sterile endometritis by the intrauterine infusion of a *Streptococcus zooepidemicus* filtrate, as reported earlier (4, 6).

Purification of eNAP-2. A preparation rich in neutrophil cytoplasmic granules was prepared by differential centrifugation of nitrogen cavitation-disrupted equine PMN, as described previously (4, 5). Briefly, the granule-rich pellets were extracted for 18 h in 10% acetic acid, on ice, and cleared by centrifugation at 27,000 × *g* for 20 min at 2°C. The extract was subsequently concentrated (SpeedVac; Savant, Hicksville, N.Y.), fractionated by gel permeation on a Bio-Gel P-10 chromatography column (1.0 by 50 cm) (Bio-Rad Laboratories, Richmond, Calif.), and the eluate was monitored for A_{280}. Every third P-10 fraction was analyzed by acid-urea-polyacrylamide gel electrophoresis (AU-PAGE) (24) for protein content and complexity. On the basis of their antimicrobial activity against *S. zooepidemicus* (see below), selected P-10 fractions were further purified by reversed-phase high-performance liquid chromatography (RP-HPLC) with a C_{18} column (0.46 by 25 cm) (Vydac, The Separations Group, Hesperia, Calif.) on an M45/680 binary solvent delivery system (Millipore-Waters, Bedford, Mass.) (5). A linear gradient of 0 to 60% acetonitrile (Fisher) in water, containing 0.1% trifluoroacetic acid (Fisher), over 60 min, was employed to elute the proteins from the C_{18} column. The eluting proteins were monitored spectrophotometrically by A_{220} or A_{225} and A_{280} and were analyzed by AU-PAGE. To estimate the molecular mass of eNAP-2, aliquots of the purified peptide were analyzed by sodium dodecyl sulfate-tricine polyacrylamide gel electrophoresis

* Corresponding author.
† Present address: Department of Medicine, University of California Los Angeles, Center for the Health Sciences, Los Angeles, CA 90024.

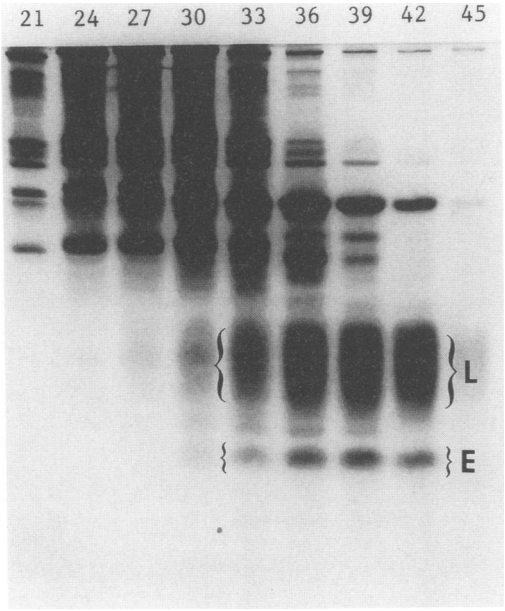

FIG. 1. Initial purification of eNAP-2; AU-PAGE analysis of P-10 column fractions. An acetic acid extract of equine neutrophil granules was chromatographed as described in the text, and every third fraction was analyzed by AU-PAGE. Lysozyme (L) and eNAPs (E) coeluted between fractions 30 and 45.

(SDS-TPAGE) on 0.75-mm-thick minigels (Hoefer Scientific Instruments, San Francisco, Calif.) containing 16.5% polyacrylamide in the resolving layer and 4% polyacrylamide in the stacking layer (30).

Amino acid composition analysis. Purified eNAP-2 was hydrolyzed in vacuo in 5.6 N HCl for 40 h at 115°C. Cysteine was quantified as cysteic acid after performic acid oxidation of a sample aliquot (22). After hydrolysis, amino acid residues were derivatized with phenylisothiocyanate (Pierce Laboratories, Rockford, Ill.) (2, 15), and separated and quantified by RP-HPLC as described earlier (14, 33).

Sequence determination and analysis. Purified eNAP-2 was reduced and alkylated with 4-vinylpyridine (Aldrich Chemicals, Milwaukee, Wis.) or iodoacetamide (Sigma Chemical Co., St. Louis, Mo.) in 6.0 M guanidine HCl–0.5 M Tris HCl (pH 8.1)–0.2 mM EDTA (14). Modified eNAP-2 was desalted by RP-HPLC and subjected to automated gas-phase Edman degradation on a model 475A Applied Biosystems sequencer (34). Although our initial attempts at sequencing eNAP-2 indicated that it was N-terminally blocked, the peptide was deblocked by treating approximately 20 μg of purified carboxyamidomethylated eNAP-2 with pyroglutamyl aminopeptidase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) in a 0.1 M sodium phosphate buffer (pH 8.5) containing 5 mM dithiothreitol, 10 mM EDTA, 5% glycerol, and 1 μg of the enzyme (27). Samples were flushed with nitrogen for 2 h at room temperature and stored overnight at 4°C. The reaction was terminated by the addition of 50 μl of acetic acid and lyophilization. The efficiency of the deblocking reaction was monitored by AU-PAGE. Unblocked peptide samples were subsequently desalted by RP-HPLC and sequenced as described above. Amino acid sequences were analyzed for similarity to those of known proteins, by using the FASTA algorithm (25) and multiple computer data bases, e.g., GenBank 70.0, EMBL 29.0, Swiss-Prot 19, and GenPept 70.0.

Antibacterial assays. Logarithmic-phase metabolically active *S. zooepidemicus*, *Escherichia coli*, *Pseudomonas*

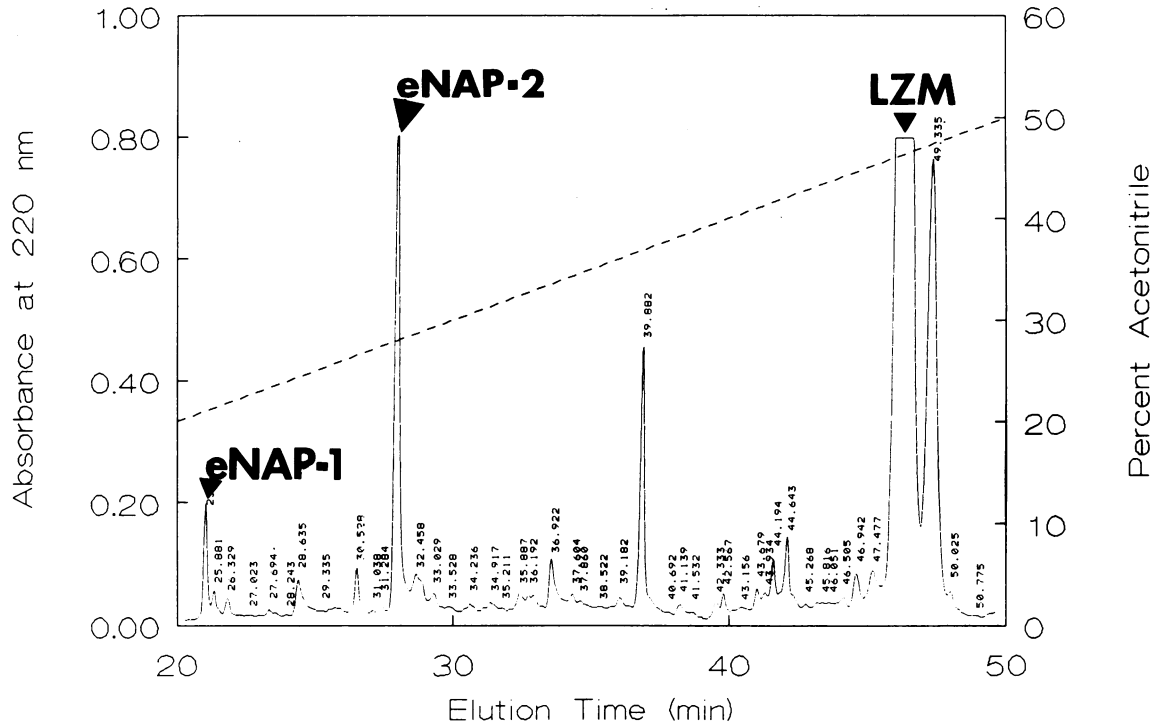

FIG. 2. Purification of eNAP-2. Selected fractions were subjected to RP-HPLC with an acetonitrile gradient (dotted line). This was fraction 42 shown in Fig. 1. The positions of eNAP-2 and lysozyme (LZM) are indicated.

aeruginosa, and *Klebsiella pneumoniae*, each originally obtained from the uteri of mares with clinical endometritis, were used as target organisms in antimicrobial assays of eNAP-2 (4, 6). Assays were performed as described previously (5, 13). Briefly, frozen isolates, maintained in nutrient broth containing 10% glycerol, were streaked on appropriate agar plates and incubated at 37°C for 24 to 48 h. A single colony was cultured for 18 h in broth at 37°C, after which 1 ml of this stationary-phase culture was transferred to 40 ml of fresh broth and incubated for an additional 4 h. This mid-log-phase culture was washed by centrifugation in 10 mM sodium phosphate buffer (pH 7.4), and the bacterial concentration was adjusted spectrophotometrically by A_{600} by using previously determined standard values. The incubation mixtures contained 5×10^4 bacterial CFU/ml in 10 mM sodium phosphate buffer (pH 7.4) and 100 or 200 μg of eNAP-2 per ml in a 0.01% sterile acetic acid vehicle. Control mixtures, lacking eNAP-2, contained a comparable volume of 0.01% sterile acetic acid. After the mixtures were incubated for 2 h in a 37°C shaking water bath, aliquots were removed, diluted 10- and 100-fold, and plated in duplicate on nutrient agar plates, by using a spiral plater (Spiral System Instruments, Bethesda, Md.). Inoculated plates were incubated at 37°C for 24 to 48 h to allow for any colony development. Assays were performed in duplicate, and the data were analyzed by Student's *t* test. Ninety-five and 99% confidence intervals were built for each time point for both control and treated samples. Sample values were deemed to differ significantly when nonoverlapping 95% confidence intervals existed for a given time point between control and treated samples. The coefficient of variation of values was <5%. Antibacterial activity was expressed both as the percentage of input CFU per milliliter remaining after incubation, and as the $\log_{10}$ reduction of input CFU per milliliter after exposure to eNAP-2 (5).

RESULTS

Neutrophil preparation. Typical neutrophil preparations exhibited high purity (>99% neutrophils) and viability (>90% viable neutrophils). Transmission electron microscopic appearance of the 27,000 × *g* sediment (starting material for our peptide purification) revealed an abundance of cytoplasmic granules, with only minor contamination by other subcellular components (5).

Identification and purification of eNAP-2. Gel permeation analysis of the equine neutrophil granule extract, followed by gel electrophoresis and antimicrobial testing (see below), identified a P-10 peak with bactericidal activity against *S. zooepidemicus*. This peak, spanning 15 fractions, contained several antimicrobial polypeptides, including eNAP-1 (5), eNAP-2 (described herein) and equine neutrophil lysozyme. eNAP-2 eluted with lysozyme from the Bio-Gel P-10 column within 70% of its total bed volume (Fig. 1). This P-10 peak was further resolved by RP-HPLC. eNAP-2 eluted from the C_{18} column at an acetonitrile concentration of 28%, while eNAP-1 eluted at 22% and lysozyme eluted at 45% (Fig. 2). Figure 3 shows the appearance of HPLC-purified eNAP-2 by SDS-TPAGE (Fig. 3a) and AU-PAGE (Fig. 3b). eNAP-2 revealed itself as a relatively abundant gene product of equine PMN. From Coomassie-stained AU-polyacrylamide gels (e.g., Fig. 1), we estimated that it was approximately 5 to 10% as abundant as lysozyme, whose content was reported to be 3.2 ± 0.2 mg (mean ± standard error of the mean) per 10^9 equine PMN (28). By SDS-TPAGE, the molecular mass of eNAP-2 was ca. 6.5 kDa. eNAP-2 exhibited a markedly cathodal migration on acid-urea gels. This was consistent with its relatively small size and suggestive of a polycationic structure.

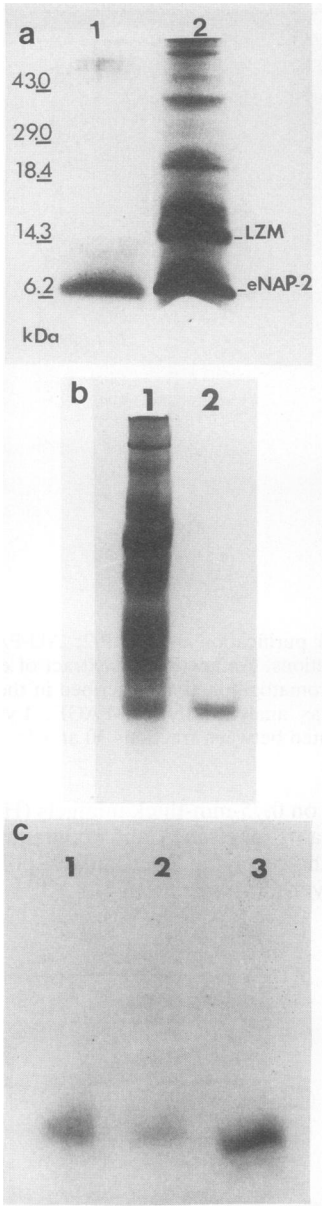

FIG. 3. Purified eNAP-2. (a) Silver-stained 16.5% SDS-tricine polyacrylamide gel. Lanes: 1, eNAP-2 (~0.3 μg); 2, equine neutrophil granule extract (~10^6 cell equivalents). (b) Coomassie brilliant blue-stained acid-urea–12.5% polyacrylamide gel. Lanes: 1, equine neutrophil granule extract (~10^7 cell equivalents); 2, eNAP-2 (~0.6 μg). (c) Coomassie brilliant blue-stained acid-urea–12.5% polyacrylamide gel. Lanes: 1, N-terminally blocked carboxyamidomethylated eNAP-2 (CAM eNAP-2); 2, CAM eNAP-2 after treatment with deblocking buffer (control); 3, CAM eNAP-2 after deblocking with pyroglutamyl aminopeptidase (see text).

Amino acid composition. Amino acid analysis of eNAP-2 revealed that glycine and proline constituted approximately 16 and 12 mol%, respectively, of the total residues and that the molecule was relatively rich in cysteine (Table 1). The amino acid composition analysis also revealed an absence of

TABLE 1. Amino acid analysis of eNAP-2

Amino acid	Amt (nmol)	Mol%
Alanine	0.4724	6.84
Arginine	0.3724	5.39
Asp(x)[a]	0.3371	4.88
Cysteine[b]	0.6893 (calc)[c]	9.98
Glu(x)[a]	0.4788	6.93
Glycine	1.1087	16.05
Histidine	0.4112	5.95
Isoleucine	0.041	0.59
Leucine	0.3203	4.64
Lysine	0.397	5.75
Methionine	TU[d]	TU
Phenylalanine	0.0852	1.23
Proline	0.801	11.60
Serine	0.4036	5.84
Threonine	0.469	6.79
Tryptophan	ND[e]	ND
Tyrosine	0.00	0.00
Valine	0.5169	7.48
Total	6.9039	99.94

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

tyrosine that was consistent with the low A_{280}/A_{225} ratio exhibited by eNAP-2 (data not shown).

Deblocking and sequencing of eNAP-2. As anticipated, unblocked eNAP-2 exhibited a more-cathodal migration, by AU-PAGE, than its blocked counterpart (Fig. 3c). Approximately 400 pmol of unblocked eNAP-2 that had been carboxyamidomethylated was subjected to partial N-terminal sequence analysis by automated Edman degradation. The first 10 residues, VERKHPLGGS, were recovered in the following amounts (in picomoles): 481, 223, 25, 236, 20, 159, 186, 57, 101, and 74, respectively. Overall, the average repetitive yield was 88.39% ± 0.62 (mean ± standard error of the mean). Substantial sequence similarity existed between eNAP-2 and *WDNM1*, a putative member of the previously described four-disulfide-core family of proteins (9) (see below), and a significant gene product of nonmetastatic rat mammary adenocarcinoma cells (7). The degree of sequence identity is illustrated in Fig. 4.

Antibacterial assays. The antimicrobial activity of eNAP-2 was assessed on several equine pathogens (Table 2). *S. zooepidemicus* was killed at a concentration of 100 μg/ml within 2 h. While the input numbers of CFU of *E. coli* and *P. aeruginosa* per milliliter were significantly reduced within 2 h, the strain of *K. pneumoniae* tested was unaffected by exposure to 200 μg of eNAP-2 per ml. Overall, eNAP-2 appeared to be as active as eNAP-1 (5) against these gram-negative bacteria but somewhat less active against the gram-positive *S. zooepidemicus*.

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

Organism	Mean CFU/ml[b] in:		Log_{10} reduction of input CFU/ml[c] (%)[d] after incubation
	Control samples	Treated samples	
S. zooepidemicus[e]	1.5×10^5	2.99×10^3	1.70 ± 0.22 (94.0)
E. coli	1.3×10^5	4.89×10^3	1.42 ± 0.23 (90.2)
P. aeruginosa	1.0×10^5	1.12×10^4	0.95 ± 0.10 (77.6)
K. pneumoniae	5.0×10^5	1.94×10^5	NR[f]

[a] After 2 h of incubation at 37°C in the presence of 200 μg of eNAP-2 per ml.
[b] The input was 5×10^4 CFU/ml.
[c] Mean ± standard error of the mean.
[d] Mean.
[e] Tested with 100 μg of eNAP-2 per ml.
[f] NR, no reduction.

DISCUSSION

Although several nonoxidative endogenous bactericides have been defined in neutrophils of humans (13, 19) and other animal species (10, 33, 34), remarkably little is known about comparable components from equine PMN. We previously reported that although equine PMN lack defensins, other microbicidal polypeptides are present in their granules, including eNAP-1 (5) and lysozyme (4). Both eNAP-2, the subject of this report, and eNAP-1 were cysteine rich, relatively small (~6.5 and ~7.2 kDa, respectively), and represented the most cathodally migrating protein species in acid-extracted equine PMN granules. Although eNAP-2 resembled eNAP-1 in its size, charge, and cysteine-rich nature, no structural homology existed between the two peptides. Whereas eNAP-1 is present in equine neutrophils in very low quantities, eNAP-2 is a major gene product of equine neutrophils (or their myeloid precursors). Although eNAP-2 showed no amino acid sequence similarity to other known antimicrobial species (e.g., eNAP-1, defensins), it exhibited a remarkable similarity to *WDNM1*, a gene product of nonmetastatic rat mammary carcinoma cells (7). It has been proposed that *WDNM1* is a member of the four-disulfide core family of proteins (9). This group consists of several cysteine-rich proteins containing a common core of eight similarly positioned cysteines. This highly conserved cysteine motif is presumed to be important in the overall folding pattern of the protein and in stabilizing small hydrophobic domains (9). Members of this family include proteins of diverse, or unknown, functions such as human antileukoproteases (32), a basic protease inhibitor of Red Sea turtles (17), whey acidic proteins of rats and mice (16), wheat germ agglutinin (31), and snake venom neurotoxin (20).

eNAP-2 exhibited the greatest sequence identity to *WDNM1*, as illustrated in Fig. 4. Note, however, that there

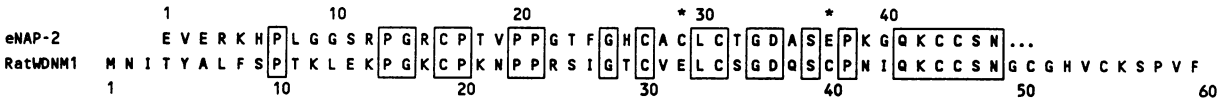

FIG. 4. N-terminal sequence of eNAP-2. Note that eNAP-2 is homologous to the *WDNM1* gene product, in that 21 of 46 (45.6%) of the sequenced residues (boxed) are identical. The two nonaligning cysteines are indicated by asterisks. Abbreviations: A, alanine; C, cysteine; D, aspartic acid; E, glutamic 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.

was one nonaligning cysteine residue (eNAP-2 Cys-29; *WDNM1* Cys-40; Fig. 4). Curiously, the corresponding replacement amino acid residue was in both instances glutamic acid. Although the homology between eNAP-2 and *WDNM1* is strong, it is imperfect, and studies at the DNA level will be required to clarify the relationship between eNAP-2 and the four-disulfide core family of proteins. The mRNA of *WDNM1* from nonmetastatic cells was found to be expressed at a level 20-fold higher than in their metastatic counterparts (7). Dear et al. (7) hypothesized that the modulation in the levels of the putative proteinase inhibitor *WDNM1* could influence the invasiveness and metastatic potential of adenocarcinomatous cells. Considering the sequence similarity between eNAP-2 and several four-disulfide core antiproteases, we reasoned that, in addition to its direct antibacterial function, eNAP-2 might also have antiproteinase activity. Indeed, in recent experiments, we have shown that eNAP-2 is a potent inhibitor of both subtilisin and proteinase K (5a), suggesting that it is likely to be one of the three equine neutrophil cationic peptides (molecular masses of ~6.3, 7.1, and 7.4 kDa) exhibiting selective antiprotease activity against subtilisin and proteinase K (communicated by Pellegrini et al. [26]). However, since no amino acid composition or sequence data were presented in that report, the relationship of these antiprotease peptides with eNAP-1 and -2 remains unclear. We have not yet tested eNAP-1 for antiprotease properties.

On the basis of its antibacterial activity at concentrations of 100 to 200 μg/ml, we believe that eNAP-2 is likely to contribute to the overall oxygen-independent intracellular killing by equine neutrophils. This conclusion is based on the following assumptions: (i) the volume containing 10^9 equine PMN, like that of their human counterparts, is ~0.35 ml; (ii) the granules of equine PMN occupy no more than 10% of the cytoplasmic compartment; and (iii) 10^9 equine PMN contain ~160 to 320 μg of eNAP-2 (i.e., 5 to 10% of their lysozyme content, assumed to be 3.2 mg as described by Rausch and Moore [28]). These assumptions lead to the conclusion that the concentration of eNAP-2 within equine PMN granules approximates 4.5 to 9.0 mg/ml.

Further studies will be required to determine if eNAP-2 homologs occur in cells other than equine neutrophils. Given the widespread distribution of cysteine-rich antimicrobial peptides in diverse host defense cells of animals (8, 10–13, 18, 21, 23, 29, 33, 34), we expect that eNAP-2 will prove to be the index member of a larger family of antimicrobial peptides that will contain its unique cysteine motif.

ACKNOWLEDGMENTS

This work was supported in part by the University of California Davis Equine Research Laboratory (with funds provided by the Oak Tree Racing Association, the State of California satellite wagering fund, and private donors) and by NIH grant AI 22839. Protein sequencing, performed by Audree Fowler at the UCLA Protein Microsequencing Facility, was aided by a BRS Shared Instrumentation Grant (1 S10RR0555401) from the NIH.

We thank Tomas Ganz for critically reviewing the manuscript, Michael E. Selsted for useful suggestions, and Alane Park for expert technical assistance.

REFERENCES

1. **Bateman, A., D. Belcourt, H. Bennett, C. Lazure, and S. Solomon.** 1990. Granulins, a novel class of peptide from leukocytes. Biochem. Biophys. Res. Commun. **173:**1161–1168.
2. **Bidlingmeyer, B. A., S. A. Cohen, and T. L. Tarvin.** 1984. Rapid analysis of amino acid using precolumn derivatization. J. Chromatogr. **336:**93–104.
3. **Clark, R. A.** 1990. Perspective: the human respiratory burst oxidase. J. Infect. Dis. **161:**1140–1147.
4. **Couto, M. A.** 1990. Bactericidal polypeptides of equine neutrophil granules: purification and characterization. Ph.D. thesis. University of California, Davis.
5. **Couto, M. A., S. S. L. Harwig, J. S. Cullor, J. P. Hughes, and R. I. Lehrer.** Identification of eNAP-1, an antimicrobial peptide from equine neutrophils. Infect. Immun. **60:**3065–3071.

5a. **Couto, M. A., S. S. L. Harwig, and R. I. Lehrer.** Blood, in press.

6. **Couto, M. A., and J. P. Hughes.** 1985. Intrauterine inoculation of a bacteria-free filtrate of *Streptococcus zooepidemicus* in clinically normal and infected mares. J. Equine Vet. Sci. **5:**81–86.
7. **Dear, T. N., I. A. Ramshaw, and R. F. Kefford.** 1988. Differential expression of a novel gene, *WDNM1*, in nonmetastatic rat mammary adenocarcinoma cells. Cancer Res. **48:**5203–5209.
8. **Diamond, G., M. Zasloff, H. Eck, M. Brasseur, W. L. Maloy, and C. L. Bevins.** 1991. Tracheal antimicrobial peptide: a cysteine-rich peptide from mammalian tracheal mucosa: peptide isolation and cloning of a cDNA. Proc. Natl. Acad. Sci. USA **88:**3952–3956.
9. **Drenth, J., B. W. Low, J. S. Richardson, and C. S. Wright.** 1980. The toxin agglutinin fold. A new group of small protein structures organized around a four-disulfide core. J. Biol. Chem. **255:**2652–2655.
10. **Eisenhauer, P. B., S. S. L. Harwig, D. Szklarek, T. Ganz, M. E. Selsted, and R. I. Lehrer.** 1989. Purification and antimicrobial properties of three defensins from rat neutrophils. Infect. Immun. **57:**2021–2027.
11. **Frank, R. W., R. Gennaro, K. Schneider, M. Przybylski, and D. Romeo.** 1990. Amino acid sequences of two proline-rich bactenecins, antimicrobial peptides of bovine neutrophils. J. Biol. Chem. **265:**18871–18874.
12. **Fujiwara, S., J. Imai, M. Fujiwara, T. Yaeshima, T. Kawashima, and K. Kumpei.** 1990. A potent antimicrobial protein in royal jelly. Purification and determination of the primary structure of royalisin. J. Biol. Chem. **265:**11333–11337.
13. **Ganz, T., M. E. Selsted, D. Szklarek, S. S. L. Harwig, K. Daher, D. F. Bainton, and R. I. Lehrer.** 1985. Defensins: natural peptide antibiotics from human neutrophils. J. Clin. Invest. **76:**1427–1435.
14. **Glazer, A. M., R. J. DeLange, and D. S. Sigman.** 1975. Modification of protein side-chains: group-specific reagents, p. 103–104. *In* T. S. Work and E. Work (ed.), Chemical modification of proteins. Elsevier/North-Holland, Amsterdam.
15. **Heinrikson, R. L., and S. C. Meredith.** 1984. Amino acid analysis on reverse-phase high-performance liquid chromatography: precolumn derivatization with phenylisothiocyanate. Anal. Biochem. **136:**65–74.
16. **Henninghausen, L. G., and A. E. Sippel.** 1982. Mouse whey acidic protein is a novel member of "four-disulfide core" proteins. Nucleic Acids Res. **10:**2677–2684.
17. **Kato, I., and N. Tominaga.** 1979. Trypsin-subtilisin inhibitor from Red Sea turtle eggwhite consists of two tandem domains—one Kunitz—one of a new family. Fed. Proc. **38:**832.
18. **Lambert, J., E. Keppi, J. L. Dimarcq, C. Wicker, J. M. Reichhart, and B. Dunbar et al.** 1989. Insect immunity: isolation from the immune blood of the dipteran *Phormia terranovae* of two insect antibacterial peptides with sequence homology to rabbit lung bactericidal peptides. Proc. Natl. Acad. Sci. USA **86:**262–266.
19. **Lehrer, R. I., and T. Ganz.** 1990. Antimicrobial polypeptides of human neutrophils. Blood **76:**2169–2181.
20. **Low, B. W., H. S. Preston, A. Sato, L. S. Rosen, J. E. Searl, A. D. Rudko, and J. S. Richardson.** 1976. Three-dimensional structure of erabutoxin b neurotoxic protein: inhibitor of acetylcholine receptor. Proc. Natl. Acad. Sci. USA **73:**2991–2994.
21. **Matsuyama, K., and S. Natori.** 1988. Purification of three antibacterial proteins from the culture medium of NIH-Sape-4, an embryonic cell line of *Sarcophaga peregrina*. J. Biol. Chem. **263:**11712–11716.
22. **Moore, S.** 1963. On the determination of cystine as cysteic acid. J. Biol. Chem. **238:**235–237.

23. **Nakamura, T., H. Furunaka, T. Miyata, F. Tokunaga, T. Muta, and S. Iwanaga et al.** 1988. Tachyplesin, a class of antimicrobial peptide from the hemocytes of the horseshoe crab (*Tachypleus tridentatus*). Isolation and chemical structure. J. Biol. Chem. **263:**16709–16713.
24. **Panyim, S., and R. Chalkey.** 1969. High resolution acrylamide gel electrophoresis of histones. Arch. Biochem. Biophys. **130:** 337–346.
25. **Pearson, W. R., and D. J. Lipman.** 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA **85:**2444–2448.
26. **Pellegrini, A., G. Hägeli, and R. von Fellenberg.** 1988. Isolation and characterization of three protein proteinase isoinhibitors from the granular fraction of horse neutrophilic granulocytes. Biochem. Biophys. Res. Commun. **154:**1107–1113.
27. **Podell, D. N., and G. N. Abraham.** 1978. A technique for the removal of pyroglutamic acid from the amino terminus of proteins using calf liver pyroglutamate amino peptidase. Biochem. Biophys. Res. Commun. **81:**176–185.
28. **Rausch, P. G., and T. G. Moore.** 1975. Granule enzymes of polymorphonuclear neutrophils: a phylogenetic comparison. Blood **46:**913–919.
29. **Romeo, D., B. Skerlavaj, M. Bolognesi, and R. Gennaro.** 1988. Structure and bactericidal activity of an antibiotic dodecapeptide purified from bovine neutrophils. J. Biol. Chem. **263:**9573–9575.
30. **Schägger, H., and G. von Jagow.** 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. **166:**368–379.
31. **Schubert-Wright, C.** 1977. The crystal structure of wheat germ agglutinin at 2.2 Angstrom resolution. J. Mol. Biol. **111:**439–457.
32. **Seemüller, U., M. Arnhold, H. Fritz, K. Wiedenmann, W. Machleidt, R. Heinzel, H. Appelhans, H. Gunter Gassen, and F. Lottspeich.** 1986. The acid-stable proteinase inhibitor of human mucous secretions (HUSI-I, antileukoprotease). FEBS Lett. **199:**43–48.
33. **Selsted, M. E., D. M. Brown, R. J. DeLange, S. S. L. Harwig, and R. I. Lehrer.** 1985. Primary structures of six antimicrobial peptides of rabbit peritoneal neutrophils. J. Biol. Chem. **260:** 4579–4584.
34. **Selsted, M. E., and S. S. L. Harwig.** 1987. Purification, primary structure, and antimicrobial activities of a guinea pig neutrophil defensin. Infect. Immun. **55:**2281–2286.
35. **Spitznagel, J. K., and W. M. Shafer.** 1985. Neutrophil killing of bacteria by oxygen-independent mechanisms: a historical summary. Rev. Infect. Dis. **7:**398–403.