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Human Antimicrobial Peptides: Analysis and Application

Alexander M. Cole and Tomas Ganz
UCLA School of Medicine
Los Angeles, CA, USA

ABSTRACT

Antimicrobial peptides are innate host defense molecules that have a direct effect on bacteria, fungi and enveloped viruses. They are found in evolutionarily diverse species ranging from prokaryotes and plants to invertebrate and vertebrate animals. Humans express several families of antimicrobial peptides in myeloid cells and on various epithelial surfaces where they are poised to defend against pathogens. Recently, antimicrobial peptides from animals and plants have served as templates for the design of new therapeutic antibiotics. This review provides an introduction to the biology of human antimicrobial peptides, followed by a more detailed discussion of their isolation from tissues and biological fluids, their purification by gel electrophoresis and chromatography and assays of their antimicrobial activities.

INTRODUCTION

In evolutionary terms, antimicrobial peptides are among the earliest molecular effectors of innate immunity and are found across diverse species (3). Multiple families of molecules throughout the animal and plant kingdoms display similar modes of action against a wide range of bacteria, fungi and enveloped viruses. These families share several common properties including broad-spectrum antimicrobial activity and cationic charge at physiological pH. The activity of most antimicrobial peptides is a direct result of electrostatic binding with anionic moieties on the microbial membrane and ensuing membrane disruption, although other mechanisms have been proposed (23).

Defensins are the most widely studied family of antimicrobial peptides. To date, there are over 80 different α-, β- and θ-defensins spanning numerous phyla (17,33,36). Humans possess at least six α-defensins and two β-defensins, as well as many other antimicrobial peptides (Table 1). The α or classical defensins (19) are β-sheet structures that possess in vitro microbicidal activity at micromolar concentrations against gram-positive and gram-negative bacteria, fungi, yeast and enveloped viruses (13,19,32,39,41). Two populations of α-defensins occur in humans: (*i*) human neutrophil peptides (HNPs) located in the azurophilic granules of the neutrophil where they constitute approximately 30% of the granule's total protein (18) and (*ii*) human defensins (HD-5 and HD-6) that have been localized in the secretory granules of the Paneth cells that are situated in the base of the crypts of the small intestine (29,30). More recently,

human β-defensin-1 and -2 (HBD-1 and HBD-2), antimicrobial peptides with similar structure and function to the α-defensins, have been identified as effector molecules of the epithelial host defense system (27,33).

PURIFICATION TECHNIQUES

Several biochemical properties of antimicrobial peptides facilitate their isolation. They are readily and often selectively soluble in acidic solvents that denature many larger proteins. Aside from a small group of anionic antimicrobial peptides (4–6), most known antimicrobial peptides are cationic at neutral pH, which affords separation from the anionic molecules by ion-exchange matrices with positive-charge selectivity. By definition, antimicrobial peptides are less than 10 kDa, so they can be separated from larger proteins by molecular filtration or size exclusion chromatography. In addition, most peptides confer an amphipathic "sidedness" in solution by exhibiting a conformation that regionalizes hydrophobic side chains. This feature enables separation using organic (reverse-phase) solvents. The following sections present a variety of protocols specifically adapted to one or more of these attributes.

Batch Separation

Batch purification can efficiently concentrate peptides of interest and is an effective means to separate antimicrobial peptides from other molecules. Thus, batch methods that require minimal prehandling of human fluids, cells or tissues are frequently used as primary modes of separation. Bodily fluids

Table 1. Distribution of Human Antimicrobial Peptides

Antimicrobial Peptide	Size (kDa)	Distribution	References
α-defensins			
HNP1-4	4	neutrophils	13,19
HD-5 and HD-6	4	Paneth cells of the small intestine	29,30
β-defensins			
HBD-1	5	urogenital tract, pancreas, oral mucosa, plasma, respiratory tract	2,21,37,38,48,60,62
HBD-2	5	epidermis, lung, respiratory secretions, oral mucosa	24,25,37,49
cathelicidin (LL-37)	5	neutrophils, epithelia	1,12,53
NK lysin	9	cytotoxic T cells	44
Lipophilins	6–8	tears	35
Histatins	3–5	saliva	16,28,42

can be acidified to a 5%–10% acetic acid concentration that selectively denatures and precipitates many proteins but leaves cationic peptides in solution. Cells and tissues are homogenized by a hand-held blender or microtip sonicator in 1%–10% acetic acid. Selective precipitation of unwanted proteins can be further enhanced by adding organic solvents such as methanol (56). To collect cationic peptides, clarified extracts can be adsorbed at neutral or mildly acidic pH onto weak cation-exchange matrices such as carboxymethyl (CM) resin (Macroprep® support; Bio-Rad Laboratories, Hercules, CA, USA) or Sep-Pak® CM cartridges (Waters, Milford, MA, USA) (60). Elution with 5%–10% acetic acid sufficiently protonates the matrix and dissociates the bound peptides. Alternatively, hydrophobic supports with straight-chain hydrocarbons ranging from C4 to C18 can retain amphipathic antimicrobial peptides from aqueous solutions (SepPak C4-C18). The retained peptides can be selectively batch-eluted with appropriately chosen mixtures of aqueous and organic solvents, for example, 40% acetonitrile in water (9,14,46,47). Recovered peptides from either technique can be further purified by gel chromatography.

Gel Chromatography and Electrophoresis

Gel filtration. Since antimicrobial peptides are small compared to most proteins, gel filtration (permeation) chromatography can be used as the first purification step for antimicrobial peptides, as diverse as those of frog (40), fish (9), horse (11) and human (19). Polyacrylamide matrices that separate molecules with an M_r less than 10 000 (e.g., BioGel® P-10; Bio-Rad Laboratories) have been most useful in antimicrobial peptide purification, and in some cases their selectivity is enhanced by additional interactions of antimicrobial peptides with the matrix, which delays their elution beyond that expected from size considerations alone. Many running buffers may be used, including sodium phosphate, sodium chloride, ammonium formate, acetic acid and urea-acetic acid. If the antimicrobial peptides lack tyrosine and tryptophan, monitoring of absorbance at less than 230 nm is necessary, and elution buffers that do not absorb at these wavelengths must be selected.

Analytical acid-urea polyacrylamide gel electrophoresis. Acid-urea polyacrylamide gel electrophoresis (AU-PAGE) resolves peptides and proteins based on their cationic charge density and is highly suited for the analysis of antimicrobial peptides (43). Small peptides with a high concentration of basic residues migrate more cathodally than larger or less positively charged peptides and proteins. Electrophoresed samples are stained with Coomassie® Brilliant Blue® or silver stains and compared to known peptide standards (Figure 1a). The same system can be used to transfer peptides onto PVDF membranes in an acetic acid/methanol buffer for Western blots or amino acid sequencing (60).

Preparative AU-PAGE. Although a number of antimicrobial peptides are abundant or can be isolated from abundant source material, many others are scarce or come from less readily obtainable tissue. Preparative AU-PAGE was devised to efficiently separate antimicrobial peptides from scant sources (26,51). Acid-extracted samples are concentrated in a centrifugal evaporator and loaded onto a 12.5% polyacrylamide water-cooled preparative tube gel (Bio-Rad Laboratories) containing 5% acetic acid and 5 M urea. Samples are electrophoresed and eluted from the cathodal end of the tube gel with 5% acetic acid. Fractions are lyophilized, resuspended in 0.01% acetic acid and directly subjected to antimicrobial assays to determine which fractions contain active peptides. Such fractions can be pooled for further purification.

Tricine-SDS-PAGE. Schagger and von Jagow (45) devised a discontinuous SDS-PAGE system for the separation of proteins from 1 to 100 kDa. Superior resolution of proteins and peptides, especially between 3 and 20 kDa, is achieved by the use of tricine (instead of the standard glycine) as the trailing ion. Figure 1b shows reduced (with 50 mM DTT) and nonreduced fractions containing defensin that have been partially purified by BioGel P-10 gel permeation chromatography. Tricine-SDS-PAGE is also highly suited for electroblotting to

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PVDF membranes for Western blots and amino acid sequencing.

HPLC

A high point in peptide purification technology, open-column, low-performance gel purification techniques were supplemented and sometimes replaced with HPLC. Two commonly used HPLC methods of particular value in the purification of antimicrobial peptides are cation-exchange and reverse-phase (RP) chromatography (50). Peptides subjected to cation-exchange HPLC are bound under aqueous conditions and eluted on an increasing salt (e.g., NaCl) gradient. The disadvantages of cation-exchange HPLC are twofold: it has a lower resolving power than RP-HPLC, and fractionated samples require desalting before analyzing antimicrobial activity. The most commonly used RP-HPLC matrix uses supports to which C4–C18 hydrocarbons are bound and gradients of water-miscible organic solvents to elute in the reverse phase. RP-HPLC is often used to desalt and further purify heterogeneous fractions from cation-exchange HPLC. By monitoring absorbance at 205–220 nm and 280 nm, peak protein fractions can be collected, lyophilized and subjected to antimicrobial assays.

Mass Spectral Analysis

Besides the conventional mass spectral analysis of purified peptides, typically using electrospray mass spectroscopy or MALDI-TOF mass spectroscopy, several groups have devised methods to analyze unfractionated extracts and secretions. Kuwata and colleagues (31) have evaluated the in vivo generation of lactoferricin derived from ingested bovine lactoferrin by surface-enhanced laser desorption/ionization (SELDI). SELDI was used in the affinity mass spectrometry operational mode to detect and quantify lactoferricin directly from unfractionated gastric contents using a chemically defined ligand with a terminal n-butyl group to affinity-capture lactoferricin. By this method, they were able to detect and quantify lactoferricin directly after collection of unfractionated gastric contents recovered

10 min after ingestion of bovine lactoferrin. The captured lactoferricin was then confirmed by MALDI-TOF. Another group directly applied MALDI-TOF to total hemolymph from individual *Drosophila* before and after bacterial challenge (59). They were able to detect 24 induced molecules, four of which were known antimicrobial peptides. These techniques will soon be adopted by researchers of higher eukaryotes to study the antimicrobial components of whole cells, tissues and fluids.

Optimal Storage Conditions and Working Solutions

Many antimicrobial peptides are stable under extreme conditions such as high heat and low pH. To aid in the initial purification, some researchers have boiled crude samples and extracts in 5%–10% acetic acid to denature most other peptides and proteins (15). Therefore, the storage conditions are rarely a concern, and many peptides are stored at 4°C for extended periods of time without losing potency. All of the known human antimicrobial peptides can be stored and diluted in a weakly acidic solution such as 0.01% acetic acid (pH 3.5–4.0) that effectively dissolves the cationic proteins at high concentrations (1–10 mg/mL) and reduces the chance of proteolytic digestion.

ANTIMICROBIAL ASSAYS

Many robust techniques have been specifically developed or modified to analyze the antimicrobial activity of peptides. Each method clearly has advantages and disadvantages that should be carefully considered, many of which depend on the abundance and relative purity of the sample.

Radial Diffusion Assay

Peak fractions are tested for antimicrobial activity throughout the process of purification. The most sensitive and reproducible technique is the radial diffusion assay (RDA) that tests the effect of peptides on immobilized microorganisms (34,54). Target microbes are grown to mid log phase and suspended in low electroendoosmotic (EEO) agarose (Sigma, St. Louis, MO, USA) that is minimally supplemented with trypticase soy broth (typically at a 1:100 dilution). The use of low-EEO agarose is crucial in these assays to prevent cationic peptides from electrostatically interacting with the agarose matrix.

Using a modified Ouchterlony punch, 3-mm wells are cut in the agarose, and 5 µL test material are deposited into each well. Subsequent incubation at 37°C for 3 h allows the peptides and proteins to diffuse outward
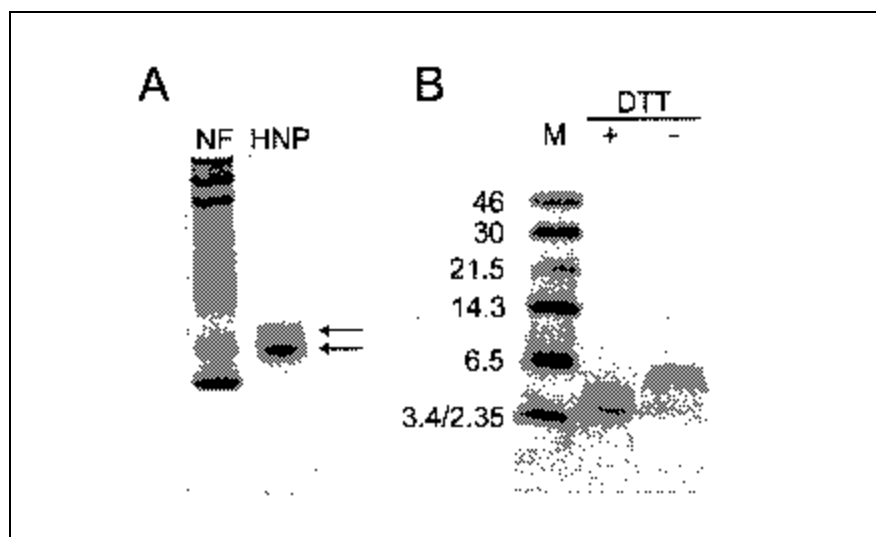
Figure 1. Gel electrophoresis of human neutrophil peptides (HNP). HNP were purified from nasal secretions by BioGel P-10 gel filtration. (A) Coomassie-strained AU polyacrylamide gel of whole nasal fluid (NF) and purified defensins (HNP) (arrows). (B) Coomassie-stained tricine-SDS polyacrylamide gel of HNP incubated with + and without - 50 mM DTT for 10 min before electrophoresis. Note that only the reduced defensin exhibited the correct size (about 4 kDa). Marker "M" is in kDa.

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from the well and interact with the entrapped microbes. Since the limited nutrients within the underlay will only allow 6–8 microbial doublings (54), a nutrient-rich molten agarose layer is then poured on top of the plate, allowed to harden and incubated overnight to allow growth of surviving microbes. Zones of clearance can be observed the following day and represent areas in which microbes have lost their ability to grow into visible colonies, despite the eventual decrease in the concentration of test substance as it diffuses away from the well (Figure 2). By measuring the diameter of each zone under 10× magnification and comparing it to known antimicrobial standards, the RDA is readily quantifiable in conventional units: radial diffusion units (RDU) = [zone of inhibition (in mm) - well diameter (in mm)] × 10.

Immobilization of the target organism prevents microbial aggregates that may influence colony-counting assays. However, the major disadvantage of the RDA is that it can only measure the activity of readily diffusible molecules. This generally does not pose a problem when analyzing fractions with one to several peptides. However, the activity of complex samples and whole biological fluids may not be accurately represented because of the differential diffusion rates of the components.

Gel Overlay Assay

The gel overlay can analyze complex extracts or secretions by first separating the sample by AU-PAGE and subsequently imprinting the gel on top of a microbe-laden agarose layer (34,54).
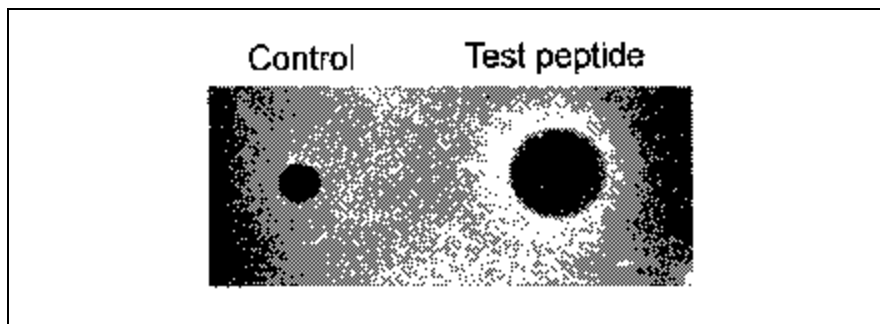
Figure 2. Radial diffusion assay. Five microliters each of 0.01% acetic acid solvent control and 200 µg/mL HNP test peptide were deposited into wells cut into an *E. coli* agarose underlay in 10 mM sodium phosphate, pH 7.4. Zones of clearing around the well reflected the absence of microbial growth. Bar = 3 mm.

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The concepts of identifying activity are quite similar to the RDA—peptides diffuse into an agarose layer, exert their microbicidal effect on the immobilized microbes and clear zones of inhibition are noted following an overnight incubation. Comparing the number and relative position of the microbe-free zones with Coomassie-stained duplicate gels can help identify the most active molecules (Figure 3). Single bands of interest can be excised, acid-extracted (acetic or formic acid) or electroblotted onto PVDF membranes and submitted for peptide microsequencing.

A minor drawback of the gel overlay technique is that substantially more sample is required than in the RDA. The latter problem is compounded by the loss of peptide caused by buffer washes that are necessary to leach acetic acid and urea (substances that in high concentrations are toxic to microbes) from the polyacrylamide gel before overlaying onto the microbial lawn. In addition, it may be difficult to attribute activity when several bands migrate in close proximity to one another in AU-PAGE. However, the ability of the gel overlay assay to identify active components directly from whole fluids significantly outweighs these shortcomings.

Both the RDA and the gel overlay permit analysis in a variety of buffer conditions. Under the most permissive conditions (e.g., 10 mM sodium phosphate, pH 7.4), many peptides express antimicrobial activity. The addition of physiological concentrations of salt (100–200 mM NaCl) and divalent cations (1 mM $MgSO_4$ and 1 mM $CaCl_2$) greatly reduces the observed number of microbicidal peptides and proteins. The investigator can thus optimize the conditions of both the RDA and gel overlay assay to answer specific biological questions.

CFU Assay

The colony-forming unit (CFU) assay has been regarded as the "gold-standard" antimicrobial assay. CFU assays may be used to test single peptides (60), combinations of peptides (20) and whole biological fluids (8,10). In short, microbes are grown to mid log phase, diluted to working concentrations and incubated with test peptide. Alterna-tively, high concentrations of microbes can be added directly to whole biological fluids to test the natural activity of the native secretions. In each instance, aliquots are removed at defined time-points (0–24 h) and spread onto nutrient-rich agar plates. After the plates are incubated long enough to permit visible colony growth, the colonies are counted to determine antimicrobial activity. Control incubations that substitute the peptide-free solvent for the peptide solution are used for comparison.

Although the CFU assay requires less material than the microbroth dilution assay, the conventional method still consumes 25–30 µL per reaction, an almost sixfold increase over the RDA. To minimize the consumption of scarce materials, a recent modification of the CFU assay uses 1 µL biological fluids per reaction (10). The 1-µL combined volume of bacteria and test substance are premixed and added underneath 1.5 µL liquid wax to prevent evaporation. The entire reaction is incubated at 37°C and is then spread onto nutrient agar at defined timepoints. The colonies are counted and analyzed as before. The results from the CFU microassays are comparable to the larger CFU assays (10).

The CFU assay has the advantage that it measures the total activity of whole fluids without the dilution or separation that is associated with the RDA and gel overlay. However, since the bacteria are free to interact with one another, both single microbes and aggregates are measured as one colony. Thus, when microbes are aggregated, the peptide's bactericidal and bacteriostatic activities could be overestimated as with any liquid media-based analysis.

Modified NCCLS Microbroth Dilution Assay

The National Committee for Clinical Laboratory Standards' (NCCLS) conventional microbroth dilution method was devised to accommodate large numbers of samples through the use of 96-well microplates (approved standard M7-A3; NCCLS, Villanova, PA, USA). The microbroth method was adapted to antimicrobial peptide testing by modifying the medium so it does not interfere with the activity of the peptide (54). After twofold serial dilutions of test peptide in media and the addition of microbial inoculum to each well, the assay is incubated at 37°C overnight. Microbial growth is detected by measuring OD_{600} the following day, and the lowest peptide concentration that inhibited bacterial growth is designated as the minimal inhibitory concentration (MIC). If desired, the subsequent dilution and plating of each well onto nutrient agar allows quantitation of the number of CFUs. The peptide concentration at which no detectable microbial growth occurs when plated is the minimal bactericidal concentration (MBC). The sensitivity of the assay relies in part on the NCCLS-recommended Mueller Hinton Broth, which has low concentrations of the inhibitory divalent cations magnesium and calcium. The addition of 0.1% human serum albumin prevents peptide adherence to the plastic well, and preparing 10× concentrations of peptide in 0.01% acetic acid enables cationic peptides to remain in solution at final concentrations up to the NCCLS standard of 512 µg/mL. Additionally, polypropylene microplates have been used in conjunction with human
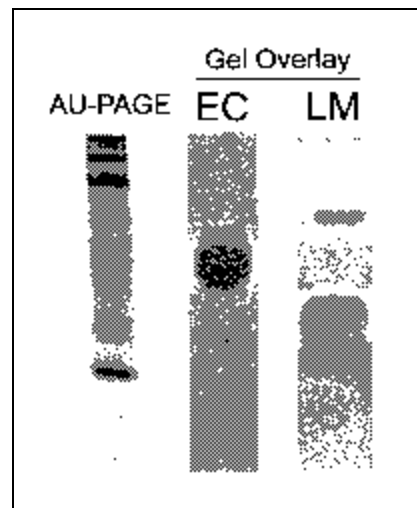
Figure 3. Gel overlay analysis. Multiple microbicidal components were identified when acetic acid-extracted nasal fluid was subjected to gel overlay analysis. Groups of (poly)peptides that confer antimicrobial activity are identified in a duplicate Coomassie-stained AU polyacrylamide gel when compared to the zones of clearing in *E. coli* (EC, middle panel) and *Listeria monocytogenes* (LM, right panel) overlays. Gel overlays were performed in 100 mM NaCl and 10 mM sodium phosphate, pH 7.4. Note that the detection of active antimicrobials within nasal fluid depended on the target microbe.

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serum albumin to reduce peptide adherence to the well (54).

Although microbroth dilution is the standard assay for growth inhibition, it can require up to 10 times more peptide than the RDA and does not allow analysis of whole biological fluids. Therefore, this assay is often reserved for testing abundant and pure peptides either alone or in combination with other peptides and antibiotics (7,20,61). A major disadvantage of microbroth dilution is that the MIC and MBC are end-point measurements. If an inoculum of 10^6 microbes/well were reduced to zero at any time during the incubation, then the final result would indicate no growth for that concentration. However, if just one microbe survives and multiplies during the remaining time of incubation, a falsely high MIC may be inferred. Measuring the MIC and MBC at intermediate timepoints would obviate this problem but would require additional sample and labor.

Alternative Assays

Investigators have recently recognized that activity measurements with standard antimicrobial techniques should be supplemented by assays that test other endpoints in addition to colony growth. We have shown that lysozyme can induce spheroplast formation in *Pseudomonas aeruginosa* and *E. coli* (unpublished observations). If subjected to an assay that immobilizes the bacteria, preventing mechanical and osmotic stress, then the bacteria can recover and proliferate. However, if the damaged bacteria are spread onto agar plates in a CFU assay, they do not recover. To confirm these results, we used assays that determined bacterial viability through the analysis of metabolic function, either by measuring the color intensity of a tetrazolium salt reduced by bacterial metabolites (55) or by evaluating the incorporation of ^{14}C-labeled leucine into macromolecules. Alternatively, other groups have measured viability in live bacteria by optically determining the amount of resazurin, a redox-sensitive fluorescent indicator (52), or quantifying luminescence in bacteria expressing the *Vibrio fischeri* luminescence genes (57,58). These newer methods are readily automated and could greatly facilitate screening for new antimicrobial peptides.

FUTURE IMPLICATIONS: THERAPEUTIC DEVELOPMENT

Cationic antimicrobial peptides are recognized as an important component of innate host defense in humans and most other species tested. As potential therapeutics, they possess several desirable properties, including the ability to swiftly kill a broad spectrum of microorganisms while developing a low level of in vitro microbial resistance (22). In addition, strains of microbes that have developed resistance to conventional antibiotics remain sensitive to antimicrobial peptides. Many peptides can also neutralize the ability of lipopolysaccharides to elicit cytokine release from macrophages and other host defense cells and thus may protect against lipopolysaccharide-induced septic shock. In experimental animals, antimicrobial peptides protect them against both topical and systemic infections either alone or in synergy with conventional antibiotics. More recently, these peptides have entered clinical trials as agents for topical therapy of microbial infections. The ongoing discovery and development of potent peptide-based microbicides are promising strategies in the struggle against increasingly resistant pathogenic microbes.

REFERENCES

1. **Bals, R., X. Wang, M. Zasloff and J.M. Wilson.** 1998. The peptide antibiotic LL-37/hCAP-18 is expressed in epithelia of the human lung where it has broad antimicrobial activity at the airway surface. Proc. Natl. Acad. Sci. USA *95*:9541-9546.
2. **Bensch, K.W., M. Raida, H.J. Magert, P. Schulz-Knappe and W.G. Forssmann.** 1995. hBD-1: a novel β-defensin from human plasma. FEBS Lett. *368*:331-335.
3. **Boman, H.G.** 1995. Peptide antibiotics and their role in innate immunity. Annu. Rev. Immunol. *13*:61-92.
4. **Brogden, K.A., M. Ackermann and K.M. Huttner.** 1998. Detection of anionic antimicrobial peptides in ovine bronchoalveolar lavage fluid and respiratory epithelium. Infect. Immun. *66*:5948-5954.
5. **Brogden, K.A., M.R. Ackermann, P.B. McCray, Jr. and K.M. Huttner.** 1999. Differences in the concentrations of small, anionic, antimicrobial peptides in bronchoalveolar lavage fluid and in respiratory epithelia of patients with and without cystic fibrosis. Infect. Immun. *67*:4256-4259.
6. **Brogden, K.A., A.J. De Lucca, J. Bland and S. Elliott.** 1996. Isolation of an ovine pulmonary surfactant-associated anionic peptide bactericidal for *Pasteurella haemolytica*. Proc. Natl. Acad. Sci. USA *93*:412-416.
7. **Cole, A.M., R.O. Darouiche, D. Legarda, N. Connell and G. Diamond.** Characterization of a fish antimicrobial peptide: gene expression, subcellular localization and spectrum of activity. Antimicrob. Agents Chemother. In Press.
8. **Cole, A.M., P. Dewan and T. Ganz.** 1999. Innate antimicrobial activity of nasal secretions. Infect. Immun. *67*:3267-3275.
9. **Cole, A.M., P. Weis and G. Diamond.** 1997. Isolation and characterization of pleurocidin, an antimicrobial peptide in the skin secretions of winter flounder. J. Biol. Chem. *272*:12008-12013.
10. **Cole, A.M., M. Wu, Y.-H. Kim and T. Ganz.** High resolution techniques for analyzing antimicrobial properties of scant human fluids. J. Microbiol. Method. *41*:135-143.
11. **Couto, M.A., S.S. Harwig, J.S. Cullor, J.P. Hughes and R.I. Lehrer.** 1992. Identification of eNAP-1, an antimicrobial peptide from equine neutrophils. Infect. Immun. *60*:3065-3071.
12. **Cowland, J.B., A.H. Johnsen and N. Borregaard.** 1995. hCAP-18, a cathelin/pro-bactenecin-like protein of human neutrophil specific granules. FEBS Lett. *368*:173-176.
13. **Daher, K.A., M.E. Selsted and R.I. Lehrer.** 1986. Direct inactivation of viruses by human granulocyte defensins. J. Virol. *60*:1068-1074.
14. **Destoumieux, D., P. Bulet, D. Loew, A. Van Dorsselaer, J. Rodriguez and E. Bachere.** 1997. Penaeidins, a new family of antimicrobial peptides isolated from the shrimp *Penaeus vannamei* (Decapoda). J. Biol. Chem. *272*:28398-28406.
15. **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.
16. **Driscoll, J., Y. Zuo, T. Xu, J.R. Choi, R.F. Troxler and F.G. Oppenheim.** 1995. Functional comparison of native and recombinant human salivary histatin 1. J. Dent. Res. *74*:1837-1844.
17. **Ganz, T.** 1999. Defensins and host defense. Science *286*:420-421.
18. **Ganz, T. and R.I. Lehrer.** 1997. Antimicrobial peptides of leukocytes. Curr. Opin. Hematol. *4*:53-58.
19. **Ganz, T., M.E. Selsted, D. Szklarek, S.S.**

Harwig, K. Daher, D.F. Bainton and R.I. Lehrer. 1985. Defensins. Natural peptide antibiotics of human neutrophils. J. Clin. Invest. *76*:1427-1435.

20. Giacometti, A., O. Cirioni, F. Barchiesi, M. Fortuna and G. Scalise. 1999. In vitro activity of cationic peptides alone and in combination with clinically used antimicrobial agents against *Pseudomonas aeruginosa*. J. Antimicrob. Chemother. *44*:641-645.

21. Goldman, M., G. Anderson, E.D. Stolzenberg, U.P. Kari, M. Zasloff and J.M. Wilson. 1997. Human β-defensin-1 is a salt-sensitive antibiotic in lung that is inactivated in cystic fibrosis. Cell *88*:553-560.

22. Hancock, R.E. 1999. Host defence (cationic) peptides: what is their future clinical potential? Drugs *57*:469-473.

23. Hancock, R.E. and D.S. Chapple. 1999. Peptide antibiotics. Antimicrob. Agents Chemother. *43*:1317-1323.

24. Harder, J., J. Bartels, E. Christophers and J.-M. Schroeder. 1997. A peptide antibiotic from human skin. Nature *387*:861-862.

25. Harder, J., R. Siebert, Y. Zhang, P. Matthiesen, E. Christophers, B. Schlegelberger and J.M. Schroder. 1997. Mapping of the gene encoding human β-defensin-2 (DEFB2) to chromosome region 8p22-p23.1. Genomics *46*:472-475.

26. Harwig, S.S., N.P. Chen, A.S. Park and R.I. Lehrer. 1993. Purification of cysteine-rich bioactive peptides from leukocytes by continuous acid-urea-polyacrylamide gel electrophoresis. Anal. Biochem. *208*:382-386.

27. Huttner, K.M. and C.L. Bevins. 1999. Antimicrobial peptides as mediators of epithelial host defense. Pediatr. Res. *45*:785-794.

28. Jensen, J.L., T. Xu, M.S. Lamkin, P. Brodin, H. Aars, T. Berg and F.G. Oppenheim. 1994. Physiological regulation of the secretion of histatins and statherins in human parotid saliva. J. Dent. Res. *73*:1811-1817.

29. Jones, D.E. and C.L. Bevins. 1992. Paneth cells of the human small intestine express an antimicrobial peptide gene. J. Biol. Chem. *267*: 23216-23225.

30. Jones, D.E. and C.L. Bevins. 1993. Defensin-6 mRNA in human Paneth cells: implications for antimicrobial peptides in host defense of the human bowel. FEBS Lett. *315*:187-192.

31. Kuwata, H., T.T. Yip, M. Tomita and T.W. Hutchens. 1998. Direct evidence of the generation in human stomach of an antimicrobial peptide domain (lactoferricin) from ingested lactoferrin. Biochim. Biophys. Acta *1429*:129-141.

32. Lehrer, R.I., A. Barton, K.A. Daher, S.S. Harwig, T. Ganz and M.E. Selsted. 1989. Interaction of human defensins with *Escherichia coli*. Mechanism of bactericidal activity. J. Clin. Invest. *84*:553-561.

33. Lehrer, R.I., C.L. Bevins and T. Ganz. 1999. Defensins and other antimicrobial peptides, p. 89-99. *In* P.L. Ogra, J. Mestecky, M.E. Lamm, W. Strober, J. Bienenstock and J.R. McGhee (Eds.), Mucosal Immunology. Academic Press, San Diego.

34. Lehrer, R.I., M. Rosenman, S.S. Harwig, R. Jackson and P. Eisenhauer. 1991. Ultrasensitive assays for endogenous antimicrobial polypeptides. J. Immunol. Methods *137*:167-173.

35. Lehrer, R.I., G. Xu, A. Abduragimov, N.N.

Dinh, X.D. Qu, D. Martin and B.J. Glasgow. 1998. Lipophilin, a novel heterodimeric protein of human tears. FEBS Lett. *432*:163-167.

36. Martin, E., T. Ganz and R.I. Lehrer. 1995. Defensins and other endogenous peptide antibiotics of vertebrates. J. Leukoc. Biol. *58*:128-136.

37. Mathews, M., H.P. Jia, J.M. Guthmiller, G. Losh, S. Graham, G.K. Johnson, B.F. Tack and P.B. McCray, Jr. 1999. Production of β-defensin antimicrobial peptides by the oral mucosa and salivary glands. Infect. Immun. *67*: 2740-2745.

38. McCray, P.B. and L. Bentley. 1997. Human airway epithelia express a β-defensin. Am. J. Respir. Cell. Mol. Biol. *16*:343-349.

39. Miyasaki, K.T., A.L. Bodeau, T. Ganz, M.E. Selsted and R.I. Lehrer. 1990. In vitro sensitivity of oral, gram-negative, facultative bacteria to the bactericidal activity of human neutrophil defensins. Infect. Immun. *58*:3934-3940.

40. Mor, A., V.H. Nguyen, A. Delfour, D. Migliore-Samour and P. Nicolas. 1991. Isolation, amino acid sequence, and synthesis of dermaseptin, a novel antimicrobial peptide of amphibian skin. Biochemistry *30*:8824-8830.

41. Ogata, K., B.A. Linzer, R.I. Zuberi, T. Ganz, R.I. Lehrer and A. Catanzaro. 1992. Activity of defensins from human neutrophilic granulocytes against *Mycobacterium avium-Mycobacterium intracellulare*. Infect. Immun. *60*:4720-4725.

42. Oppenheim, F.G., T. Xu, F.M. McMillian, S.M. Levitz, R.D. Diamond, G.D. Offner and R.F. Troxler. 1988. Histatins, a novel family of histidine-rich proteins in human parotid secretion. Isolation, characterization, primary structure, and fungistatic effects on *Candida albicans*. J. Biol. Chem. *263*:7472-7477.

43. Panyim, S. and R. Chalkley. 1969. High resolution acrylamide gel electrophoresis of histones. Arch. Biochem. Biophys. *130*:337-346.

44. Pena, S.V. and A.M. Krensky. 1997. Granulysin, a new human cytolytic granule-associated protein with possible involvement in cell-mediated cytotoxicity. Semin. Immunol. *9*:117-125.

45. Schagger, 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.

46. Schnapp, D. and A. Harris. 1998. Antibacterial peptides in bronchoalveolar lavage fluid. Am. J. Respir. Cell. Mol. Biol. *19*:352-356.

47. Schnapp, D., G.D. Kemp and V.J. Smith. 1996. Purification and characterization of a proline-rich antibacterial peptide, with sequence similarity to bactenecin-7, from the haemocytes of the shore crab, *Carcinus maenas*. Eur. J. Biochem. *240*:532-539.

48. Schnapp, D., C.J. Reid and A. Harris. 1998. Localization of expression of human β-defensin-1 in the pancreas and kidney. J. Pathol. *186*: 99-103.

49. Schroder, J.M. and J. Harder. 1999. Human β-defensin-2. Int. J. Biochem. Cell Biol. *31*:645-651.

50. Selsted, M.E. 1997. HPLC methods for purification of antimicrobial peptides. Methods Mol. Biol. *78*:17-33.

51. Selsted, M.E., D. Szklarek and R.I. Lehrer. 1984. Purification and antibacterial activity of

antimicrobial peptides of rabbit granulocytes. Infect. Immun. *45*:150-154.

52. Shiloh, M.U., J. Ruan and C. Nathan. 1997. Evaluation of bacterial survival and phagocyte function with a fluorescence-based microplate assay. Infect. Immun. *65*:3193-3198.

53. Sorensen, O., K. Arnljots, J.B. Cowland, D.F. Bainton and N. Borregaard. 1997. The human antibacterial cathelicidin, hCAP-18, is synthesized in myelocytes and metamyelocytes and localized to specific granules in neutrophils. Blood *90*:2796-2803.

54. Steinberg, D.A. and R.I. Lehrer. 1997. Designer assays for antimicrobial peptides, p. 169-187. *In* W.M. Shafer (Ed.), Antimicrobial Peptide Protocols. Humana Press, Totowa, NJ.

55. Stevens, M.G. and S.C. Olsen. 1993. Comparative analysis of using MTT and XTT in colorimetric assays for quantitating bovine neutrophil bactericidal activity. J. Immunol. Methods *157*:225-231.

56. Tang, Y.Q., J. Yuan, C.J. Miller and M.E. Selsted. 1999. Isolation, characterization, cDNA cloning, and antimicrobial properties of two distinct subfamilies of α-defensins from rhesus macaque leukocytes. Infect. Immun. *67*:6139-6144.

57. Travis, S.M., N.N. Anderson, W.R. Forsyth, C. Espiritu, B.D. Conway, E.P. Greenberg, P.B. McCray, Jr., R.I. Lehrer et al. 2000. Bactericidal activity of mammalian cathelicidin-derived peptides. Infect. Immun. *68*:2748-2755.

58. Travis, S.M., B.A. Conway, J. Zabner, J.J. Smith, N.N. Anderson, P.K. Singh, E.P. Greenberg and M.J. Welsh. 1999. Activity of abundant antimicrobials of the human airway. Am. J. Respir. Cell. Mol. Biol. *20*:872-879.

59. Uttenweiler-Joseph, S., M. Moniatte, M. Lagueux, A. Van Dorsselaer, J.A. Hoffmann and P. Bulet. 1998. Differential display of peptides induced during the immune response of *Drosophila*: a matrix-assisted laser desorption ionization time-of-flight mass spectrometry study. Proc. Natl. Acad. Sci. USA *95*:11342-11347.

60. Valore, E.V., C.H. Park, A.J. Quayle, K.R. Wiles, P.B. McCray and T. Ganz. 1998. Human β-defensin-1: an antimicrobial peptide of urogenital tissues. J. Clin. Invest. *101*:1633-1642.

61. Westerhoff, H.V., M. Zasloff, J.L. Rosner, R.W. Hendler, A. De Waal, G.A. Vaz, P.M. Jongsma, A. Riethorst and D. Juretic. 1995. Functional synergism of the magainins PGLa and magainin-2 in *Escherichia coli*, tumor cells and liposomes. Eur. J. Biochem. *228*:257-264.

62. Zhao, C.Q., I. Wang and R.I. Lehrer. 1996. Widespread expression of β-defensin HBD-1 in human secretory glands and epithelial cells. FEBS Lett. *396*:319-322.

Address correspondence to:

Dr. Alexander M. Cole
Department of Medicine
Division of Pulmonary and Critical Care
UCLA School of Medicine, CHS 37-055
10833 Le Conte Ave.
Los Angeles, CA 90095-1690, USA
e-mail: acole@ucla.edu