Defensins and Other Antimicrobial Peptides: A Historical Perspective and an Update

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Abstract: Antimicrobial peptides are effectors of innate immunity in phagocytes, body fluids and epithelia. In mammals, defensins, peptides with a characteristic six-cysteine framework, are particularly abundant and widely distributed in various animal species and tissues. The first part of this review provides a historical overview of the ideas that led to the current state-of-the-art in antimicrobial peptides, and the second part is an update on mammalian defensins and their role in host defense to infections.

Keywords: History, membrane interactions, peptide structure.

1. INTRODUCTION AND HISTORICAL OVERVIEW

In the first part of this review, I was offered the opportunity to provide a personal view of the discovery of defensins and other antimicrobial peptides. In response to this request, I have made an attempt to trace the origin of ideas that influenced my own work and that of my closest colleagues. I do not pretend to be objective or exhaustive, and have therefore refrained from providing historical references.

Humans and animals mount an active defense against microbial infections (1860-1920):—Largely under the influence of Louis Pasteur, Robert Koch and their contemporaries, the notion that microbes caused most infectious diseases as well as spoilage of foodstuffs became widely accepted. Implicit in this view was the concept that if the microbes could be destroyed before they started multiplying, diseases would be prevented or cured, and food (and importantly, beer and wine) would not spoil. Pasteur used heat to kill bacteria but Joseph Lister successfully tested a chemical, carbolic acid, as a surgical disinfectant, based on its ability to inhibit the bad odor of decaying garbage. Carbolic acid was too toxic to use internally but Paul Ehrlich began a systematic search for antimicrobial chemicals that could be used to treat infectious diseases, and found that a tolerably toxic arsenical derivative (Salvarsan) was active against syphilis. The idea of biological resistance to microbes probably developed from centuries-old clinical observation of variable severity of infections during epidemics but the notion of struggle between microbes and their animal and plant hosts also fit well with the widespread influence of Darwinian thought. Regarding the mechanisms of host resistance to microbes, two distinct concepts soon dominated the field. Paul Ehrlich was the most prominent proponent of the idea that antimicrobial substances resided in blood plasma, as "antibodies" and "complement". His main intellectual opponent, and fellow Nobel prize winner, was Elie Metchnikoff who proposed that phagocytic cells located and ingested invading microbes Once inside the phagocytes, ingested microbes could be observed to lyse, and Metchnikoff concluded that ferments (enzymes) within the phagocytes were responsible for the killing and digestion of microbes. Thus from the notion that chemicals could kill bacteria, it was a relatively small step to searching for the chemicals that killed microbes within living organisms. However, the technology was not yet in hand to establish the specific chemical composition of these substances.

Living organisms contain proteins and peptides with antimicrobial activity (1920-1970)—The methods for separating simple and complex chemicals from mixtures by selective precipitation, selective crystallization, filtration, chromatography, and other techniques soon made it possible to characterize newly discovered bioactive substances in progressively greater detail. In the late 1920's Alexander Fleming identified and partially purified lysozyme as the first enzyme that showed antimicrobial properties. He also noted that many forms of lysozyme were widely distributed throughout the plant and animal kingdoms. His later discovery of penicillin, the development of synthetic sulfonamide-based antimicrobials by Gerhard Domagk and his team at I.G. Farben in the 1930s, and the discovery of many more antibiotics derived from fungi and bacteria in the 1940s and 1950s clearly influenced the search for mechanisms of phagocytic killing. Indeed, in the 1950's, James Hirsch found that extracts of human neutrophils contained a microbicidal substance which he dubbed phagocytin, a name resembling those of conventional antibiotics. In the 1960s, before the nature of this substance could be fully characterized, the attention of the scientific community was attracted by the discovery of the phagocytic respiratory burst. In this reaction, phagocytes that ingest microbes convert large amounts of oxygen to such antimicrobial chemicals as hydrogen peroxide and hypochlorous acid (Clorox). Neutrophils and macrophages from patients with chronic granulomatous disease (CGD) lacked the ability to mount a respiratory burst and the patients with the disease suffered recurrent infections with *Staphylococcus aureus, Aspergillus* species and certain other microbes. It soon became clear however that the respiratory burst did not account for much of the microbicidal power of

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phagocytes. CGD phagocytes retained a remarkable ability to kill many microbes rapidly, and even *S. aureus* if given enough time. Meanwhile the attempts to identify additional antimicrobial substances continued, and became successful when H.I. Zeya and John K. Spitznagel partially characterized arginine-rich cationic peptides in rabbit and guinea pig neutrophil granules. In parallel, biochemical methods that eventually resulted in the identification of antimicrobial peptides began to be applied to the study of antimicrobial activity in insect hemolymph (the equivalent of blood in vertebrates) by Hans Boman and his colleagues, and antimicrobial substances in plants by Francisco Garcia-Olmedo and his colleagues. However, until the early 1990s there was little if any mutual awareness among the research groups that studied phagocytes, insects and plants.

Antimicrobial peptides are distinct gene products and belong to gene families (1970-1990) —In the 1970s and 1980s, developing technology, especially the use of high performance liquid chromatography and automated peptide sequencing made it possible to purify individual peptides and to determine their amino acid sequences. Later developments in molecular biology led to the cloning of the genes that encoded these peptides. The granules of neutrophils proved to be a particularly rich source of antimicrobial proteins and peptides. Robert Lehrer and his group purified and sequenced the various antimicrobial peptides of rabbit and guinea pig neutrophils, and showed that they were cationic and amphipathic, and had a broad spectrum of activity under permissive conditions of low salt and serum concentrations. In 1984, Robert Lehrer, Michael Selsted and Tomas Ganz found related peptides in human neutrophils, and named the family "defensins" based on their presumed role in host defense. These had been missed in earlier comparative studies of human, rabbit and guinea pig granulocytes because the human defensins were much less cationic than their rabbit and guinea pig counterparts. Nevertheless, as confirmed by the cloning of the respective cDNAs, all defensins belonged to the same gene family. They were encoded as 90-94 amino acid precursors that were processed to mature 29-35 amino acid peptides before storage in granules. Robert Lehrer also initiated the search for the mechanism of action of defensins, and identified permeabilization of microbial membranes as an early and necessary step in the sequence that eventually led to the death of the target microbe.

In parallel studies, D. Romeo and his group found antimicrobial activity in the granules of bovine neutrophils, leading eventually to a group of peptides named bactenecins. Peter Elsbach and Jerrold Weiss noted that rabbit neutrophils also contained small non-defensin polypeptides, named p15a and p15b. The cloning of the cDNAs that encoded the p15s, bactenecins, as well as other non-defensin mammalian peptides led to their reclassification, by Margherita Zanetti and her colleagues, as "cathelicidins" based on the similarity of their proforms and genes. Larger neutrophil proteins that had microbicidal activity were also found by several groups but differed from antimicrobial peptides by having specific enzymatic properties (proteases, phospholipases) that contributed to their activity against microbes or by binding specific bacterial molecules or nutrients essential for microbes (bactericidal permeability-increasing protein that bound lipopolysaccharide, or lactoferrin, an iron-binding protein). Although clearly biologically interesting as well as important, these larger antimicrobial proteins were very different from peptides in both their chemistry and the mechanism of their activity.

1990-2000: Convergence and search for general principles—In the late 1980s, Andre Ouellette and his colleagues studied developmentally-regulated mRNAs in the gastrointestinal tract, and discovered cryptdins, as abundant mRNAs in the murine small intestine. Soon it became clear that cryptdins were defensins expressed in Paneth cells, specialized host defense cells of intestinal crypts. Other epithelial antimicrobial peptides followed. The skin of various frogs turned out to be a goldmine for antimicrobial peptides, and the discovery of α-helical magainins by Michael Zasloff and his colleagues inspired a search for mammalian epithelial peptides. A new type of defensin, a β-defensin named "tracheal antimicrobial peptide", was discovered in the bovine trachea by Gill Diamond in Zasloff's group, and then Charles Bevins identified two human defensins, HD-5 and 6, in intestinal Paneth cells. Antimicrobial peptides were no longer seen as special products of phagocytes but as ubiquitous host defense substances expressed at the interface between microbes and their hosts. At the CIBA symposium in London in January 1994, researchers who studied antimicrobial peptides in invertebrates, vertebrates, plants and bacteria (bacteriocins) came together for the first time, and it is at this point that we all became acutely aware that antimicrobial peptides constituted a general means by which biological organisms controlled microbial colonization and parasitisation. The interactions between insect biologists and mammalian biologists became more frequent and more intensive as the power of Drosophila genetics began to be applied to the problem of how antimicrobial peptides were induced by infections. The groups of Jules Hoffmann and Hans Boman were particularly influential in providing a bridge between the worlds of medicine, mammalian innate immunity and insect innate immunity. These efforts culminated in the description of the central role of Drosophila Toll and mammalian toll-like receptors in triggering innate immune responses to infections. Meanwhile, the groups of Huey Huang, Katsumi Matsuzaki, Yechiel Shai and Karl Lohner focused on another common theme, the mechanism by which antimicrobial peptides (regardless of their origin) interacted with biological membranes and manifested relative selectivity for microbial over animal cell membranes. The interactions of antimicrobial peptides with the more complex structures of bacteria was explored by the groups of Robert Lehrer and Robert Hancock. The bacterial genetics approach taken by the groups of Eduardo Groisman, Samuel Miller and William Shafer revealed that bacteria are not merely passive targets of antimicrobial peptides but respond defensively by modifying vulnerable cell wall structures or by activating systems that export or destroy antimicrobial peptides. Studies by Hans Georg Sahl and others of lantibiotic peptides derived from bacteria (e.g. nisin) provided important insights into how high affinity interactions with specific cell wall molecules of target bacteria can yield highly potent antimicrobial peptides. In the 1990s, industrial researchers began to focus on the potential use of antimicrobial peptides as therapeutics, and companies such as Magainin Pharmaceuticals and

Intrabiotics brought the first products into clinical trials. These efforts have not yet matured. However, daptomycin, a fungal antimicrobial (lipo)peptide that acts on the membranes of bacteria [1] was recently approved for human use.

2000- A return to integrative biology—Recent studies by the groups of William Parks, James Wilson, Richard Gallo, Julia Dorin, Charles Bevins and Nita Salzman have attempted to define the biological contribution of antimicrobial peptides to the integrated function of whole animals. In vertebrates, technology has so far limited these studies to mice because of the facile way in which new genes can be introduced into these animals and existing genes can be selectively disabled. This may be unfortunate for those of us who take an anthropocentric perspective because antimicrobial peptides evolve rapidly and the mouse is quite distant from humans in the distribution and chemistry of its antimicrobial peptides. Nevertheless, on the whole, these studies demonstrate that individual defensins and cathelicidins contribute significantly to resistance to gastrointestinal, cutaneous, pulmonary and urinary tract infections. However, since in most situations multiple antimicrobial peptides and proteins contribute to the activity at a given infected site, this examination of the role of individual peptides probably grossly underestimates their collective contribution. Similar functional studies in Drosophila by Bruno Lemaitre and the group of Jules Hoffmann have suggested an important role for antimicrobial peptides in the resistance of insects to infections. These studies will no doubt continue and expand in the future, as other animals become available for genetic manipulation.

2. DEFINITIONS

Antimicrobial peptides are polypeptides of fewer than 100 amino acids, found in host defense settings, and exhibiting antimicrobial activity at physiologic ambient conditions and peptide concentrations. Two large families of antimicrobial peptides, defensins [2] and cathelicidins [3], are abundant and widely distributed in mammalian epithelia and phagocytes. Many more families of antimicrobial peptides are found in invertebrates[4], including most prominently various insects, horseshoe crabs and mussels.

3. ANTIMICROBIAL PEPTIDES: ACTIVITIES AND THEIR MECHANISMS

Spectrum—Magainins from frog skin, tachyplesins and polyphemusins from horseshoe crab hemocytes, and protegrins from pig leukocytes, have been extensively studied and served as models because of their structural simplicity, small size (16-22 amino acids) and potential pharmaceutical applications. These peptides exhibit a broad spectrum of antimicrobial activity that includes gram-negative and gram-positive bacteria [5,6] and fungi [7], at similar concentrations (in the range of 1-10 μg/ml) and under similar testing conditions to those used for other antimicrobial pharmaceuticals. Protegrins and tachyplesins are also active against some enveloped viruses [8]. The antimicrobial activity of these peptides is remarkably specific, with little cytotoxicity to mammalian cells even at concentrations ten-fold or more higher than those required for antimicrobial activity [9].

The structurally more complex mammalian defensins (29-50 amino acids) are also active against bacteria and fungi, especially when tested under low ionic strength conditions [10-12] and with low concentrations of divalent cations, plasma proteins or other interfering substances. Under these optimal conditions, antimicrobial activity is observed at concentrations as low as 1- 10 μg/ml (low μM). Increasing concentrations of salts and plasma proteins competitively inhibit the antimicrobial activity of defensins, in a manner that is dependent on both the specific defensin and its microbial target. In general, metabolically active bacteria are much more sensitive to defensins than bacteria made inactive by nutrient deprivation or metabolic inhibitors. At higher concentrations, some defensins are cytotoxic to mammalian cells [13-15]. Certain enveloped viruses are also inactivated by defensins [16,17], including prominently the human immunodeficiency virus [18-25] but the specific molecular targets important for this effect have not yet been defined.

Mechanisms of antimicrobial activity—Antimicrobial peptides are almost always cationic and amphipathic. This allows them to interact with biological membranes so that the cationic domains are near the negatively-charged phospholipid headgroups while the hydrophobic portions of the peptide are submerged within the hydrophobic interior of the membrane. The simplest antimicrobial peptides, typified by magainins, form an alpha-helix, with cationic and hydrophobic side chains radially arranged on opposite surfaces of the helix. Another simple structure is the beta-sheet hairpin (e.g. protegrins, tachyplesins, polyphemusins) containing positively charged clusters separated by hydrophobic regions. The interactions between simple antimicrobial peptides and model membranes have been extensively explored [26-28]. In these systems, there is strong evidence of a peptide concentration-dependent phase transition. During the first phase, the peptides, attracted to the membrane by electrostatic forces, form a carpet within but near the surface of the membrane, with the long axes of the peptides parallel to the membrane. As more and more peptide molecules accumulate, the membrane becomes distorted and strained, favoring a transition to an energetically more favorable state where the peptides are oriented with their long axes across the membrane, creating toroidal wormholes in the membrane or otherwise disrupting membrane integrity. These interactions are favored by the presence of anionic phospholipids in the membrane and inhibited by neutral phospholipids or cholesterol. Anionic phospholipids are characteristic of bacteria but neutral phospholipids and cholesterol are found in animal cell membranes, explaining the preferential effect of antimicrobial peptides on bacterial targets.

Structurally more complex antimicrobial peptides are thought to act by similar mechanisms. Model bacteria (E. coli ML-35) [29] and mammalian cell line K562 [15] treated by defensins become permeable to small molecules (small sugars and trypan respectively). In bacteria, permeabilization coincides with inhibition of RNA, DNA and protein synthesis and decreased bacterial viability as assessed by the colony forming assay. In the model cell line, the

permeabilized cells can be rescued for up to 1 hour by removing the defensin, and there is evidence that additional intracellular sites of action contribute to cell death [15].

In experiments with artificial phospholipid membranes, defensins NP-1 (rabbit) and HNP-1 (human) formed voltage-dependent channels, requiring negative potential on the membrane side opposite to where defensins where applied [30]. This is consistent with the idea that the insertion of defensin molecules into the membrane is dependent on electrical forces acting on the positively charged defensin molecule. The effect of these forces is evident even when no transmembrane potential is applied externally. Unlike another cationic peptide, melittin, that indiscriminately permeabilized vesicles composed of neutral or anionic phospholipids, defensins were much more active against vesicles that included negatively charged phospholipids [31]. In general, the activity of defensins against vesicles was diminished in the presence of increased salt concentrations, supporting the importance of electrostatic forces between the anionic phospholipids headgroups and the cationic defensins. Interestingly, the permeabilizing activity of the most highly cationic defensins was not inhibited by moderate salt concentrations indicating that electrostatic screening by salt ions may not have been complete. In other experiments, large unilamellar vesicles composed of the negatively charged phospholipid palmitoyloleoylphosphatidylglycerol were permeabilized by human defensin HNP-2 but the addition of neutral phospholipids to the lipid mix inhibited both defensin binding and permeabilization [32]. Using a very different methodology, the importance of anionic phospholipids for the membrane interactions with defensins was clearly shown by calorimetric measurements of the effects of defensins on phase transitions in membranes [33]. These studies suggest that defensin molecules enter into the membrane under the influence of both externally applied and local electric fields.

It is much less certain what happens once the defensin molecules are in the membrane. The observed leakage of dye markers from liposomes implies that pores (we use this term to refer to any ion or water permeable structure within the membrane) form either stably or transiently. For some defensins, the release of internal markers from each vesicle occurred in all or none fashion [32] indicating that the pores formed were stable. By measuring the ability of pores to allow the passage of marker molecules of various sizes, the pore diameter was estimated at 25 Å. The authors proposed a model of a defensin pore --a hexamer of dimers-- that generates an opening of the observed size. However, stable pore formation is not the only mechanism of defensin interaction with membranes. The more cationic rabbit defensins induced a partial release of markers from individual vesicles indicating that the pores formed were not stable. It is possible that electrostatic repulsion between the highly cationic rabbit defensin molecules destabilizes the pores.

How the pores lead to bacterial death is not fully known. Recent studies with protegrins [34] showed that the pores selectively allow the entry of water into bacteria, eventually causing such swelling that the cytoplasmic contents of the bacteria herniate through the relatively rigid cell wall. This causes severe mechanical damage to bacteria. Once pores form, peptides should readily gain access to the interior of the bacteria. There they probably encounter other as yet uncharacterized intracellular targets which may be specific for different antimicrobial peptides [35].

4. VERTEBRATE DEFENSINS

The structure of defensins—Defensins [2,36] are a family of vertebrate antimicrobial peptides with a characteristic β-sheet rich fold and a framework of six disulfide-linked cysteines [2,36]. The two major defensin subfamilies, α- and β-defensins, differ slightly in cysteine spacing and connectivity. Several structures representative of these two families have been solved by 2D-NMR and by X-ray crystallography [37-44]. Both α-and β-defensins consist of a triple stranded β-sheet with a distinctive "defensin" fold (Fig. **1**). Whereas in α-defensins the six cysteines are linked in the 1-6, 2-4, 3-5 pattern [45], in β-defensins the pattern is 1-5, 2-4, 3-6 [46]. Because cysteines 5 and 6 are adjacent in both types of defensins, this difference in connectivity does not substantially alter the structure [42]. More recently, another structurally very distinct subfamily of θ-defensins [47] has been identified in the rhesus macaque monkey leukocytes. The mature θ-defensin peptides arise by an as yet uncharacterized process that generates a cyclic peptide by splicing and cyclization from two 9-amino acid segments of α-defensin-like precursor peptides. Based on their adjacent chromosomal location and similar peptide precursor and gene structure it is highly likely that all vertebrate defensins arose from a common gene precursor [48]. Antimicrobial peptides from invertebrates and plants containing six or eight cysteines in disulfide linkage have also been called defensins (e.g. insect and plant defensins). Their evolutionary relationship to vertebrate defensins is uncertain.

Distribution of defensins—During studies of the antimicrobial activity of rabbit and guinea pig leukocyte lysates in the 1960's, the peptides originally attracted attention because of their abundance and broad spectrum of antimicrobial activity [49]. Subsequent technical developments facilitated their isolation and detailed chemical characterization [10,50,51]. Their discovery in human leukocytes [2,36] suggested that the peptides were widely distributed in nature. After their isolation from leukocytes, defensins were found in other host defense settings where they were produced by epithelial cells [52,53]. Typical defensin peptides have been found in all mammals that have been carefully examined, as well as in chickens and turkeys [54-57]. Defensin-like peptides (growth arresting peptide [58] and crotamines) have been also isolated from snake venom where they may exemplify the evolutionary acquisition of broad target specificity against eukaryotic targets.

Defensins are found predominantly in cells and tissues involved in host defense against microbial infections. The highest concentrations of defensins (> 10 mg/ml) are found in granules, the storage organelles of granulocytic leukocytes (granulocytes) [2,59]. When these leukocytes ingest microbes into phagocytic vacuoles the granules fuse to these vacuoles and deliver their contents onto the target microbe. Since there is little free space in phagocytic vacuoles the microbe is exposed to minimally diluted granule material.

Fig. (1). The β-sheet-rich structures of α-defensins (exemplified by human neutrophil peptide-3 dimer), β-defensins (human β-defensin-2) and θ-defensins (rhesus θ-defensin-1).

Similarly, Paneth cells, specialized host defense cells of the small intestine, contain secretory granules that they release into narrow intestinal pits, called crypts. The concentration of defensins in the crypts may also reach >10 mg/ml [60]. Various epithelia produce defensins, in some cases constitutively [61], in others in response to infection [62]. The average concentration of defensins in these epithelia is in the 10-100 μg/ml range [62,63] but because the peptides are not evenly distributed the local concentrations could be much higher.

Patterns of tissue distribution are quite variable even when closely related species are compared. Among rodents, mice lack leukocyte defensins [64], rats have them [65] and both species have numerous Paneth cell defensins and epithelial β-defensins. In some cases, defensin expression appears to be induced by a combination of a specific cell type and tissue environment. Inflammatory macrophages are leukocytes that arise by differentiation from circulating blood monocytes, under the influence of local tissue signals. In the rabbit, alveolar (lung) macrophages have abundant α-defensins in amounts comparable to rabbit neutrophils but defensins are absent from their peritoneal macrophages [66]. Although defensin expression in monocytes, macrophages and lymphocytes of some mammals can be detected by highly sensitive techniques [67-69] high levels of defensins in macrophages have only been documented in rabbits. We suspect that these peculiarities of the pattern of expression of defensins in certain animal species could be related to the evolutionary pressure from species-specific pathogens.

Microbial resistance to defensins—Specific mechanisms that confer increased bacterial resistance to defensins have been identified by insertional mutagenesis. Disruption of the two-component transcriptional regulator *phoP-phoQ* increases the sensitivity of *Salmonella* to defensins and other cationic peptides [70-73]. *PhoP-PhoQ* directly regulates multiple genes involved in resistance to cationic peptides and also exerts some of its activity by modulating a second two-component regulator, *PmrA-PmrB*. The function of the downstream genes includes covalent modification of lipopolysaccharides that decreases their affinity for cationic peptides [74] and expression of membrane proteases that degrade cationic peptides [75]. In *Neisseria gonorrhoeae,* a bacterium naturally quite resistant to defensins, the energy-dependent efflux system *mtr* increases the resistance to protegrins, potent mini defensin-like peptides of pig neutrophils [76]. In *Staphylococci*, the disruption of either of two genes, dlt or MprF, increases the sensitivity of the bacteria to defensins [77,78]. The gene dlt is required for covalent modification of cell wall teichoic acid by alanine, and MprF is necessary for covalent modification of membrane phosphatidylglycerol with L-lysine. These modifications probably act by decreasing the negative charge of the cell wall and bacterial membrane respectively and diminishing their attraction for the cationic defensins. Homologues of these resistance genes have been identified in many bacterial species indicating that these mechanisms may be widespread.

Other activities of defensins—Various defensins have been reported to have chemotactic activity for monocytes, T-lymphocytes and dendritic cells [79-82]. In the case of human β-defensins 1 and 2, which attract memory T-cells and immature dendritic cells, the chemoattractant activity may be due to defensin binding to the chemokine receptor CCR6 [81]. Although the physiologic significance of this interaction has not yet been demonstrated, the high concentrations of HBD-2 in inflamed skin make it likely that this defensin could compete effectively with the natural chemokine ligand (variously named CCL20, LARC, MIP-3α) despite the higher affinity of the latter for the CCR6 receptor. Recent structural analysis of CCL20 pointed out remarkable similarities to HBD-2 in the putative receptor-binding region of CCL20. The role of this region in the chemotactic activity of HBD-2 needs to be confirmed by mutating the amino acid residues suspected in its interaction with CCR6. Human neutrophil defensins HNP1-3 have been reported to be chemotactic for monocytes [79], naïve T-cells and immature dendritic cells [82] but a specific receptor has not yet been identified.

Some defensins (called "corticostatins") [83-85] oppose the action of adrenocorticotropic hormone (ACTH) by binding to ACTH receptor [86] without activating it. Although such activity would inhibit the production of the immunosuppressive hormone cortisol, and could thus be useful in responding to infections, the physiologic role of this *in vitro* interaction has not yet been demonstrated.

Yet another reported activity of some defensins is their ability to activate nifedipine-sensitive calcium channels in mammalian cells [87,88]. This effect required only

nanomolar concentrations of defensins. The structural basis of this effect is not understood. Certain mouse Paneth cell defensins (cryptdins) activate chloride secretion most likely by forming channels in the apical membrane of epithelial cells [89,90]. This activity is limited to a subset of cryptdins, and its structural basis is not yet known.

Most recently, several peptides genetically and structurally related to defensins have been found in the male reproductive tract, and in particular in the epididymis [91,92]. While some peptides expressed in the male reproductive tract are typical defensins also found in other organs [93], most are larger peptides from genes that undergo complex alternative splicing. These peptides could have an important role in the host defense of germ cells as well as in the regulation of sperm maturation.

Defensin biosynthetic pathways—At least eight genes encoding α- and β-defensins are located in a cluster on chromosome 8p23 [48,94-97] and recent studies document additional defensin clusters with multiple transcribed defensin genes [98]. Mapping of the 8p23 cluster has been problematic, probably due to its polymorphic nature, with individuals and their chromosomes differing in the number of copies of individual defensin genes [99,100]. Alpha-defensins are generally encoded as a tripartite prepropeptide sequence, wherein a 90-100 amino acid precursor contains an N-terminal ~19 amino signal sequence, ~45 amino acid anionic propiece and a C-terminal ~30 amino acid mature cationic defensin [101] (Fig. **3**). In many cases, the charge of the propiece and the mature defensin approximately balance [102], and this arrangement may be important for folding and/or to prevent intracellular interactions with membranes [103,104]. For neutrophil α-defensins, synthesis takes place in the bone marrow, in neutrophil precursor cells, promyelocytes [105-107]. Mature neutrophils circulating in blood or found in inflamed tissues contain large amounts of defensins but are no longer synthesizing the peptides or their mRNAs. During defensin synthesis in myeloid cell lines, the signal sequence is rapidly removed but the subsequent proteolytic processing to mature defensins takes many hours, and the final proteolytic cleavage may take place in maturing granules [108]. The process is very efficient so that only small amounts of partially processed intermediates are detectable in mature neutrophils [109]. In the case of murine Paneth cell defensins (cryptdins) the metalloproteinase matrilysin (MMP-7) is required for processing since mice with homozygous disruption of the matrilysin gene do not process Paneth cell defensin past the removal of the signal sequence. The structure of β-defensin precursors is simpler, consisting of a signal sequence, a short or no propiece and the mature defensin peptide at the C-terminus. The lack of anionic propiece in β-defensin precursors contrasts with the relatively large anionic propiece in α-defensin precursors, a difference that has not been satisfactorily explained.

Amino acid sequence and composition of defensins—The amino acid sequences of mature defensins are highly variable except for the conservation of the cystine framework in each defensin subfamily. Clusters of positively charged amino acids are characteristic of most α- and β-defensins but their specific distribution within the defensin molecule is variable. In leukocytes and in Paneth cells of the small intestine, defensins are stored in granules, subcellular storage organelles rich in negatively charged glycosaminoglycans. With the exception of chicken gallinacins, these α- and β-defensins contain arginine as the predominant cationic amino acid. In contrast, β-defensins that are secreted from epithelial cells contain similar amounts of arginine and lysine. The preferential use of arginine in defensins stored in granules may reflect the constrains imposed by packing defensin molecules into the glycosaminoglycan matrix of granules [110,111].

Structure-function considerations—A unitary hypothesis of how defensins permeabilize membranes is complicated by the marked differences in net charge, amino acid sequence and quaternary structure (monomers vs. dimers) among the defensins. It is possible that these differences evolved so that various defensins can target different types of bacteria with differing structures of cell walls and membranes. Further complexity is introduced by the flexibility of the basic amino acid side chains that permit a variety of potential spatial interactions with phospholipids headgroups or water. Although the interactions of defensins with membranes have been modeled [32] there are only rudimentary experimental data on the structure of the defensin complexes within the membrane. Further work in this area is clearly needed since the considerable progress in understanding the interactions of amphipathic α-helical peptides with membranes does not readily translate to defensins, which are larger, more complicated and more variable structures.

Evolution of defensins—One of the initially puzzling features of defensins was the great variety of sequences found in similar functional settings in different animals. Evolutionary analysis of these differences indicates that the rates of nucleotide substitutions that change the amino acid encoded exceed the rates of synonymous substitutions [112,113]. This would indicate that the differences in defensin peptides between species are selected for, presumably by the pressures of potentially pathogenic microbial flora in each animal species. In α-defensins, this selection involves almost all residues of the mature defensin peptide except those that are most critical for the maintenance of the molecular shape and stability, i.e. the disulfide-bonded cysteines, and residues involved in salt bridging [114].

Functions of defensins *in vivo*—When initially proposed [2], the name "defensins" represented a lucky guess since it was largely based on *in vitro* antimicrobial activity and the peptides' location in neutrophils, the prototypic host defense cells. Since then, experiments with transgenic mice have largely supported the idea that the dominant function of defensins is antimicrobial. Mice with homozygous disruption of the matrilysin gene failed to activate intestinal prodefensins to defensins, and were more susceptible to infection with Salmonella typhimurium, requiring an eight-fold lower oral dose for 50% mortality [115]. After oral administration of *E. coli* test bacteria, the counts of viable bacteria were similar in the proximal intestine of wild-type and matrilysin-knockout mice but the wild-type mice had lower bacterial counts in the mid- and distal small intestine, where Paneth cells are present at higher density. In vitro, segments of intestine from wild-type mice contained and secreted more antimicrobial activity than those of matrilysin knockout mice [60]. Morever, in wild-type mice the

antimicrobial activity could be largely neutralized by anti-defensin antibody, indicating that defensins were responsible for much of the activity. Taken together, these experiments provided important circumstantial evidence for the protective role of defensins in the early stages of infection.

More recently, a gain-of-function model was reported, in transgenic mice expressing the human Paneth cell defensin gene HD-5 [116]. HD-5 compared to murine Paneth cell defensins has greater antibacterial potency against the murine pathogen Salmonella typhimurium. HD-5 mice were fully protected against death from Salmonella typhimurium infection at oral doses that killed all of the wild-type mice. Protection from infection was seen early, already at 6 hours, and correlated with lower *Salmonella typhimurium* counts in the intestinal lumen, and prevention of the spread of infection to other organs. The effect of transgenic defensin was local, since intraperitoneal inoculation that bypassed the intestine caused equal mortality in the transgenic and wild-type strains. The intestinal lumen-specific effects of transgenic defensin early in the course of infection provide the strongest evidence to date that defensins act as locally secreted antibiotics.

Mice deficient in murine β-defensin-1 show only very mild defects [117,118] in host defense of the urinary and respiratory tracts, most likely due to the redundancy amongst mouse defensin genes. Unlike the many defensin genes present in the mouse genome, there is only one, or at most very few murine cathelicidins (the number depends on how the family is defined). The murine cathelicidin (cathelin-related antimicrobial peptide, CRAMP) is similar to its human ortholog, LL-37, and both are expressed in predominantly in neutrophils. Mice with homozygous disruption of the CRAMP gene showed diminished resistance to skin infection with group A Streptococcus [119]. Taken together, data from loss of function models support a host defense role for cathelicidins and defensins.

5. CONCLUSIONS

Defensins and other antimicrobial peptides are widely distributed and abundant effectors of innate immunity. The analyses of the great variety of sequences seen in various animal species would indicate that they have evolved in response to specific pressures from microbial flora in each species. This raises the possibility that combinatorial approaches could be used to develop defensins active against specific targets. Other antimicrobial peptides could also be useful as templates for combinatorial development.

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