

## The Role of Antimicrobial Peptides in Innate Immunity<sup>1</sup>

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**SYNOPSIS.** Production of antimicrobial peptides and proteins is an important means of host defense in eukaryotes. The larger antimicrobial proteins, containing more than 100 amino acids, are often lytic enzymes, nutrient-binding proteins or contain sites that target specific microbial macromolecules. The smaller antimicrobial peptides act largely by disrupting the structure or function of microbial cell membranes. Hundreds of antimicrobial peptides have been found in the epithelial layers, phagocytic cells and body fluids of multicellular animals, from mollusks to humans. Some antimicrobial peptides are produced constitutively, others are induced in response to infection or inflammation. Studies of the regulation of antimicrobial peptide synthesis in *Drosophila* have been particularly fruitful, and have provided a new paradigm for the analysis of mammalian host defense responses. It now appears that the general patterns of antimicrobial responses of invertebrates have been preserved in vertebrates (“innate immunity”) where they contribute to host defense both independently and in complex interplay with adaptive immunity.

### ANTIMICROBIAL PEPTIDES—BACKGROUND AND DEFINITION

Multicellular organisms continually defend themselves against parasitization by potentially harmful microbes. In the absence of penetrating injury, the most common sites of initial encounter with microbes are the epithelial surfaces (skin, the moist surfaces of the eyes, nose, airways and the lungs, mouth and the digestive tract, and the urinary and reproductive systems). Because mechanisms requiring specific antigen recognition depend on clonal proliferation of immunocytes, and therefore take days to weeks to develop fully, the initial host resistance mechanisms must recognize or target microbe-specific class characteristics and employ mechanisms that are either constitutive or rapidly inducible. Some unique microbial molecular features are recognized by complementary receptors that trigger localized effector mechanisms (“pattern recognition”) while other structural or metabolic characteristics make the microbes selectively susceptible to the action of injurious antimicrobial substances including chemically highly reactive molecules, lytic enzymes, pore-forming molecules, or substances that sequester essential nutrients. Certain antimicrobial substances may be present constitutively; the local synthesis or release of others is provoked by invading microbes; and yet other antimicrobial substances can be brought into the area of invasion by mobile cells. Unlike innate immunity, adaptive immunity (antibodies and antigen-recognizing cytotoxic lymphocytes) is a late evolutionary development developed fully only in higher vertebrates. Specific antigen recognition by lymphocytes probably plays a limited role during the initial encounter but it is especially effective against

persistent microbes or against microbes previously encountered by the host.

The innate antimicrobial properties of epithelial surfaces were noted a century ago by Metchnikoff, who emphasized the cleansing role of mechanical factors such as the continuous movement of the tear film across the frontal surface of the eye. Metchnikoff also observed that microbes that breached the epithelial surfaces were met by mobile cells (phagocytes) that ingested and killed the invaders. Having described the phagocytic killing of microbes, Metchnikoff surmised that microbicidal substances must be present in phagocytes and thought that these were “ferments” (enzymes). In the 1920s, Fleming discovered that the fluid coating the epithelia contained an antimicrobial enzyme which he named lysozyme, and showed that the same substance was also found in abundance in phagocytes. Later studies identified the main target of lysozyme as a sugar linkage in the peptidoglycan cell wall of bacteria.

Over the past 40 years, a number of additional antimicrobial substances produced by epithelia and phagocytes have been characterized, ranging in size from small inorganic molecules such as hydrogen peroxide to large protein complexes such as those generated by the activation of the complement cascade. Antimicrobial peptides are conventionally defined as polypeptide antimicrobial substances, encoded by genes and synthesized by ribosomes, with fewer than 100 amino acid residues. This definition distinguishes them from most (but not all) peptide antibiotics of bacteria and fungi, which are synthesized by specialized metabolic pathways and often incorporate exotic amino acids.

### DISTRIBUTION OF ANTIMICROBIAL PEPTIDES

The highest concentrations of antimicrobial peptides are found in animal tissues exposed to microbes or cell types that are involved in host defense (Table 1). Epithelial surfaces secrete antimicrobial peptides from

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TABLE 1. Structures, distribution and activities of antimicrobial peptides.

Structure	Representative peptides	Species and tissue	Antimicrobial activity (reported)
4-disulfide $\alpha$ -helix + $\beta$ -sheet	plant defensins drosomycin	plants arthropod hemolymph	fungi
3-disulfide $\beta$ -sheet-rich	$\alpha$ -defensins $\beta$ -defensins	vertebrate neutrophils epithelia	bacteria, fungi, enveloped viruses
3-disulfide $\alpha$ -helix + $\beta$ -sheet	insect defensins	arthropod hemolymph	Gram + bacteria
3-disulfide 2 $\alpha$ -helices + $\beta$ -sheet	$\gamma$ -thionins (crambin)	plants	bacteria, fungi, mammalian cells
2-disulfide $\beta$ -sheet	protegrins tachyplesins, polyphemusins	pig neutrophils horseshoe crab hemocytes	bacteria, fungi, enveloped viruses
1-disulfide cyclic	bactenecin-1, cyclic dodecapeptide ranalexin, brevinin	ruminant leukocytes amphibian skin	bacteria
$\alpha$ -helix	cecropins magainin, PGLa LL-37	insect hemolymph amphibian skin mammalian leukocytes	bacteria
linear with repeating motifs	bactenecins 5 and 7, PR-39, indolicidin diptericin, apidaecin	mammalian leukocytes insect hemolymph	bacteria

both barrier epithelia and glandular structures (Zasloff, 1987; Diamond *et al.*, 1991; Ouellette and Selsted, 1996; Jones and Bevins, 1992). Phagocytic cells contain several types of storage organelles (granules) for microbicidal substances and digestive enzymes (Levy, 1996; Ganz and Lehrer, 1997). In the process of phagocytosis, granules fuse to phagocytic vacuoles that contain ingested microbes, thereby exposing the microbes to very high concentrations of microbicidal and digestive substances. Other granules are secreted into the extracellular fluid where their contents kill microbes or inhibit their multiplication. Both types of granules contain abundant antimicrobial peptides (Ganz *et al.*, 1985; Selsted *et al.*, 1984; Cowland *et al.*, 1995).

In invertebrates, the fluid portion of blood (hemolymph) as well as the granules of phagocytic cells (hemocytes) contain antimicrobial peptides (Boman *et al.*, 1991; Iwanaga *et al.*, 1994). Secretion of antimicrobial peptides from the fat body (equivalent to the liver in vertebrates) into hemolymph appears to be the dominant mechanism in injured or infected insects, while hemocytes may be the more important source in horseshoe crabs. Like in vertebrates, insect epithelia, most prominently the gut, secrete tissue-specific antimicrobial peptides (Richman and Kafatos, 1996; Richman *et al.*, 1997), a response which is likely to be important in insect resistance to intestinal parasites.

#### STRUCTURES AND MECHANISM OF ACTION

Almost all antimicrobial peptides are cationic and amphipathic. The simplest antimicrobial peptide structures whose mechanism of action has been investigated are either  $\alpha$ -helices or  $\beta$ -hairpins. Both types of peptides can form transmembrane channels. The length of a simple  $\alpha$ -helix is approximately 1.5 Å per amino acid residue whereas that of a  $\beta$ -hairpin is roughly 3.5 Å per two residues. Since the hydrocarbon core of the phospholipid membrane is roughly 30 Å across it takes

about twenty amino acids to span the membrane by either an  $\alpha$ -helical or  $\beta$ -hairpin peptide. Indeed, the simplest antimicrobial peptides of these two classes are the frog skin peptide magainin (23 amino acids) (Bechinger *et al.*, 1993; Luttko *et al.*, 1996) and the pig leukocyte peptide protegrin (16–18 amino acids) (Aumelas *et al.*, 1996; Fahrner *et al.*, 1996). Smaller natural antimicrobial peptides exist (*e.g.*, the 12 amino acid cyclic dodecapeptide (Romeo *et al.*, 1988)) but their structure in membranes and their mechanism of action have not been extensively investigated. More recently, it has been demonstrated that even smaller artificial peptides (6 or 8 amino acids) can generate pores in membranes by assembling into nanotubes (Fernandez-Lopez *et al.*, 2001). It is possible that naturally occurring small peptides that employ similar mechanisms will be discovered.

There are three major hypotheses about how the disruption of membrane integrity kills the target microbes. The loss of microbial viability may be due to the cumulative effects of energy drain due to the equilibration of intracellular and extracellular ion concentrations through the disrupted membrane. Alternatively, antimicrobial peptides may enter the target cell through the disrupted membrane, bind to as yet unknown intracellular molecules and interfere with their metabolic function. Finally, some peptides may generate pores that admit water but do not allow osmotically active substances to pass. The entry of water generates osmotic pressure that eventually stretches and breaks the microbial membrane (Lehrer *et al.*, unpublished). Either way, repair processes may limit or reverse these lesions when peptide concentrations are low or limited in time. Prolonged exposure to higher concentrations of antimicrobial peptides overwhelms the repair capacity of the microbe and the damage becomes irreversible.

The assembly of membrane pores by magainin (Luttko *et al.*, 1996; Matsuzaki, 1998; Shai, 1999) and

tachyplesins ( $\beta$ -hairpin peptides from horseshoe crab hemocytes) (Matsuzaki *et al.*, 1991) is favored by membranes that are rich in anionic phospholipids, a characteristic property of bacterial membranes. Conversely, the cell membranes of animals are rich in neutral phospholipids and cholesterol, substances that inhibit the incorporation of these peptides into membranes and the formation of pores. This mechanism explains why the concentrations necessary to kill eukaryotic cells are much higher than those required for killing most bacteria. Current evidence favors similar mechanisms of action for other peptides commonly found in the animal and plant kingdoms (Lohner *et al.*, 1997).

Defensins (Ganz and Lehrer, 1995) are particularly abundant and widely distributed antimicrobial peptides characterized by a cationic  $\beta$ -sheet rich amphipathic structure stabilized by a conserved three-disulfide motif. They range in size from 29 to 47 amino acids, and are abundant in many vertebrate granulocytes, Paneth cells (specialized granule-rich intestinal host defense cells), and on epithelial surfaces. Like the simpler magainins and protegrins, defensins also form pores in target membranes. There is evidence that the permeabilization of target cells is nonlethal unless followed by defensin entry into the cell and additional intracellular damage (Lichtenstein, 1991).

#### REGULATION OF SYNTHESIS AND RELEASE

In invertebrates and plants, organisms that lack adaptive immunity, antimicrobial peptides constitute a major component of host defense (Fritig *et al.*, 1998; Meister *et al.*, 1997). Many of the plant and invertebrate peptides (e.g., insect defensins and plant defensins) structurally and functionally resemble their vertebrate counterparts but a comprehensive evolutionary lineage has not yet been established. Both plants and invertebrates induce the synthesis of antimicrobial peptides in response to infection. The signaling pathways that mediate this response are similar to the acute phase response in animals and employ similar transcriptional regulators, most prominently the rel/NF- $\kappa$ B family. In vertebrates, antimicrobial peptide synthesis is either constitutive or inducible by microbial macromolecules and/or cytokines. The epithelial  $\beta$ -defensin of the bovine trachea, the tracheal antimicrobial peptide (TAP), is synthesized in the airway epithelia when these are exposed to inhaled bacteria or lipopolysaccharide (Diamond *et al.*, 1996). This response is initiated by lipopolysaccharide receptors that ultimately signal to transcriptional regulators including the NF- $\kappa$ B complex, acting on NF- $\kappa$ B binding motifs in the promoter of the TAP gene. In addition to transcriptional regulation of synthesis, stimulus-dependent degranulation provides an additional level of responsiveness and specificity. Thus the granulocytes of many vertebrates contain antimicrobial defensin peptides in their phagocytic granules and another class of antimicrobial peptides, cathelicidins, in granules destined for extracellular secretion (Rice *et al.*, 1987; Sor-

ensen *et al.*, 1997). Intestinal Paneth cells, positioned at the bottom of narrow crypts in the small intestine, release their defensin-rich granules (Ouellette and Selsted, 1996) upon stimulation by cholinergic or bacterial stimuli, both of which are associated with food ingestion (Qu *et al.*, 1996).

All known antimicrobial peptides are synthesized as larger precursors, containing one or multiple copies of the active peptide segment which are released by proteolytic processing. In the simplest cases the cotranslational removal of an N-terminal signal peptide frees the active moiety but more commonly one or more anionic propieces are also removed during processing (Valore and Ganz, 1992; Terry *et al.*, 1988; Zasloff, 1987). Perhaps the most intriguing and as yet unexplained processing pattern is seen with cathelicidins, a group of peptides with a conserved 100 amino acid domain that is frequently proteolytically cleaved from the highly variable C-terminal antimicrobial domain (Zanetti *et al.*, 1995). In phagocytes, the cathelicidins are commonly stored as inactive precursors in secretory granules. In many cases, the processing enzyme is neutrophil elastase contained in a separate set of storage granules. During phagocytosis, this binary system combines to generate active antimicrobial peptides. The function of the highly conserved cathelin domain is not yet known.

#### SPECTRUM OF ACTIVITY

Many antimicrobial peptides display activity against gram-positive and gram-negative bacteria, yeasts and fungi, and even certain enveloped viruses and protozoa. Other peptides are more restricted in their spectrum. Even minor variations in peptide structure can influence activity, and a systematic understanding of the relationship between peptide structure and activity is an important area for future investigations. Evidence is accumulating that many peptides act synergistically with larger polypeptides whose antimicrobial activity is enzymatic (e.g., lysozyme) or is dependent on specific recognition of bacterial macromolecules (e.g., the bactericidal permeability-inducing protein, BPI) (Levy *et al.*, 1994). Synergistic interactions between two antimicrobial peptides in the frog skin, magainin 2 and PGLa, have also been reported (Westerhoff *et al.*, 1995). In addition to their action on microbes, some antimicrobial peptides can function as regulatory molecules in the host. For example, *in vitro* studies suggest that defensins can attract phagocytes and lymphocytes to sites of infection, inhibit the release of cortisol from adrenal cells, induce the proliferation of fibroblasts and modify ionic fluxes in epithelial cells (Ganz and Lehrer, 1995).

#### BIOLOGICAL ROLE AND CONSEQUENCES OF DEFECTS IN THE FUNCTION OF ANTIMICROBIAL PEPTIDES

In insects, injury or infection elicits the production of antimicrobial peptides in the fat body (the insect equivalent of the vertebrate liver) and within a few hours renders the insect hemolymph (the insect equiv-

alent of blood) antimicrobial. At least two distinct pathways participate in the induction response. In *Drosophila*, the antifungal response is induced by the Toll signaling pathway that is very similar to the dorso-ventral morphogenic pathway as well as to the acute phase response in mammals, which involves the cytokine interleukin-1 (IL-1). The antibacterial response involves a less extensively characterized *imd* (immune deficiency gene) system. Genetic disruption of these two pathways in *Drosophila* blocks the induction of two distinct sets of antimicrobial peptides and causes increased susceptibility to fungal or bacterial infections, respectively (Lemaitre *et al.*, 1996; Meister *et al.*, 1997).

Evidence for the significant role of antimicrobial peptides in the host defense of mammals is also accumulating. In mouse knockout models, the disruption of the matrilysin gene prevented the normal proteolytic activation of intestinal defensins (cryptidins) and increased the susceptibility of these mice to intestinal infections (Wilson *et al.*, 1999). In pigs, the application of an exogenous inhibitor of the proteolytic activation of cathelicidins increased bacterial proliferation in skin wounds (Cole *et al.*, 2001). In the most direct experiment to date, mice with disrupted genes for the cathelin-related antimicrobial peptide (CRAMP) showed increased susceptibility to skin infections with group A streptococci (Nizet *et al.*, 2001). Thus, interference with synthesis and posttranslational processing of antimicrobial peptides weakens host resistance to infections.

In a rare human disease, specific granule deficiency, the content of defensins (and probably several other antimicrobial peptides and proteins as well) in neutrophil granulocytes is severely decreased. The patients develop recurrent and severe bacterial infections. However, the interpretation and attribution of this defect is made complex by the multiple proteins affected (Ganz *et al.*, 1988).

#### INTERACTION OF ANTIMICROBIAL PEPTIDES WITH THE ADAPTIVE IMMUNE SYSTEM

In addition to the innate immune system present in all animals, vertebrates evolved an adaptive immune system based on specific recognition of antigens. The immunocytes involved in adaptive immunity possess highly diverse antigen recognition receptors generated by somatic gene rearrangements and somatic mutations in the genes that encode their antigen recognition sites. When its receptor binds an antigen, the immunocyte undergoes clonal expansion to generate an effector population (either antibody-producing or capable of directly killing the invader or the cell harboring it). It has long been known that many microbial antigens are more effective in eliciting this response than other environmental antigens. A well-known practical application of this observation is the use of microbe-derived adjuvants to increase antibody responses to artificial immunogens. Broadly speaking, the ability of the adaptive immune system to respond more vigor-

ously to pathogenic microbes can be accounted for by positing that the activation of the innate immune responses generates signals that make the activation of adaptive responses more likely or more intense (Hoffmann *et al.*, 1999). There is increasing evidence that antimicrobial peptides released in response to microbial invasion can activate adaptive immunity (Lillard, Jr. *et al.*, 1999), at least in part by attracting antigen-presenting dendritic cells to the site of invasion (Yang *et al.*, 2001).

#### Summary and conclusions

Antimicrobial peptides participate in host defense of invertebrates and vertebrates by contributing to the killing of invading microbes. In higher vertebrates, antimicrobial peptides may also activate adaptive immunity.

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