Editorial: "Antimicrobial" or "host defense" peptides

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Short cationic amphipathic peptides were first demonstrated in the 1970s to be present in amphibians, insects, and human phagocytes. When examined by use of in vitro assays of antimicrobial activity, they could be demonstrated to kill bacteria and other microorganisms and were thus accorded the general names "cationic antimicrobial peptides" or "antibiotic peptides" and were lauded as "Nature's antibiotics." As is clear from recent research summarized in this book and in leading journal review articles (e.g., Boman, 1995; Andreu and Rivas, 1998; Gudmundsson and Agerberth, 1999; Hancock and Diamond, 2000), they have many other activities that are relevant to the anti-infective host defense process known as innate immunity. We would like to propose here that, with some prominent exceptions, most of these peptides have no *relevant* antibiotic activities at physiological concentrations and conditions and, because they have multiple impacts on innate immunity, they should be classed as "host (innate) defense peptides" or "peptides of the innate immune system."

The prevailing conditions in vivo do not favor the antimicrobial activity of cationic peptides. Often these activities are assessed ex vivo by either a 10-mM phosphate buffer or, for example, a tenfold diluted bacterial growth medium. Of necessity, such conditions are artificial and certainly do not reflect most mammalian tissue environments. Some papers in the literature have considered the higher levels of salt in vivo; however, sodium and chloride ions have a relatively modest effect on antimicrobial peptide activity. Indeed, divalent cations have a much stronger effect and, at the millimolar concentration found in vivo (e.g., blood has approximately 2-mM Ca^{2+} and 1-mM Mg^{2+}), can completely ablate the activity of many or most natural peptides. This happens only with 200-mM monovalent cations (Friedrich et al., 1999). Other highly antagonistic agents include polyanionic saccharides

(e.g., mucins) and cell surfaces, and serum factors, including lipoproteins and proteases. Such considerations have been made for the cationic aminoglycosides, for which it has been shown that in vivo conditions can be partly reflected by supplementation of in vitro minimum inhibitory concentration (MIC) assays (Reller et al., 1974); however, they rarely are for cationic peptides. For example, human LL-37 is classed by many as antimicrobial in that it has MICs of around 1–8 μg/ml in diluted broth containing up to 100-mM NaCl (Turner et al., 1998). However, in normal Mueller Hinton medium, which has moderate divalent cation levels, MICs of ≥32 μg/ml are observed (Turner et al., 1998) as confirmed in the Hancock laboratory, far higher than the 2–5 μg/ml found at mucosal surfaces. It will be important as this field moves forward to rate as "antimicrobial" only those peptides that are functionally antimicrobial at physiologically meaningful concentrations and under physiological conditions.

We do not intend to imply that direct antimicrobial activity never occurs with such peptides, but rather that many of these peptides do not act as antimicrobials in most locations where they are found in the host. For example, the work of Lehrer and colleagues has indicated that cationic α-defensins constitute 5% or more of total neutrophil proteins (Spitznagel, 1990), and this means that the concentration would be around 10–100 mg/ml in the compartments where they are found (azurophilic granules and, during phagocytosis, phagolysosomes). Also, estimates of defensin concentrations in intestinal crypts are around 25 mg/ml (Charles Bevins, personal communication). Similarly, peptides can be found at concentrations of >100 μg/ml at sites of chronic inflammation (Hancock and Diamond, 2000). Other peptides, such as polyphemusins from horseshoe crabs (Zhang et al., 2000) and protegrins from pigs, and so on (Steinberg et al., 1997), are far more active than most of the peptides discussed here. In addition, synergy between individual peptides is possible, although such studies have not been performed under physiologically meaningful conditions. Nevertheless, direct killing of microbes would be a part of the host defenses constituting innate immunity, and we submit that a more accurate description for this class of molecules is "host defense peptides." It is likely that some peptides have antimicrobial functions at one body site (e.g., in a phagosome) and other host defence roles at other sites (e.g., at epithelial surfaces when released by degranulation). Also, these peptides may play different roles at heavily colonized sites compared with those that are normally sterile (e.g., intestinal compared with lung epithelia).

The nonantimicrobial activities of these peptides include stimulation of chemotaxis of phagocytic cells, vasodilation (through encouragement

of histamine release from mast cells), neutralization of bacterial-signaling molecules such as lipopolysaccharide (LPS) and lipoteichoic acid (LTA), cell differentiation, and so forth (Boman, 1995; Andreu and Rivas, 1998; Gudmundsson and Agerberth, 1999; Hancock and Diamond, 2000). Given these activities, we must explain why the mammalian host contains measurable activities of many peptides at a range of body sites, which at the same time harbor large numbers of bacteria that constitute the normal resident microbiota. Added to this is the fact that these resident populations produce most of the same surface molecules that signal Toll-like (pattern-recognition) receptors (TLRs). We hypothesize that the background expression of innate immunity peptides in the normal host provides a homeostatic balance to signaling by the natural flora, preventing undesirable induction of innate immunity. When this situation is locally perturbed by the introduction of new microbes onto a mucosal/epidermal surface, by increases in certain populations, or by released microbial components above threshold levels, TLRs are activated, leading to local upregulation of innate immunity. At the same time, signaling through TLRs leads to an increased expression of host (innate) defense peptides. These peptides themselves induce novel gene responses that block the upregulation of gene responses signaled by bacterial surface molecules, permitting reestablishment of homeostasis. If this model is correct, then peptides have a central role in the process of innate immunity and may also assist in the decision to induce both chronic inflammation and adaptive immunity.

The reviews presented in this book discuss a variety of the aspects previously discussed. Many interesting perspectives are presented and, especially, we invite reviewers to read, consider, and make up their own minds about how these peptides might function.

ACKNOWLEDGMENT

The terms host defense peptides and peptides of the innate immune system were suggested to us by Alex Tossi and Tim Falla, respectively.

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CHAPTER 1

Overview: Antimicrobial peptides, as seen from a rearview mirror

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Here I sit, having just celebrated my sixty-fifth birthday, wondering why I agreed to write this overview and also why I never learned to type. I will be brief. If these reflections seem uninteresting, remember that nobody is forcing you to read them. The other chapters in this volume will provide an up-to-date and "serious" introduction to the antimicrobial peptides of mammals.

The gene-encoded antimicrobial peptides of mammals are very old, because such peptides also exist in archaea, eubacteria, protists, plants, and invertebrates. Nevertheless their study is relatively new. Consequently it may be helpful to recall the following dialogue. After William Gladstone (1809–98), Chancellor of the Exchequer, witnessed a demonstration of the generation of electricity by Michael Faraday (1791–1867), Gladstone said "It is very interesting, Mr. Faraday, but what practical worth is it?" Faraday replied "One day, sir, you may tax it." To date, mammalian antimicrobial peptides have been tax exempt.

I complete this overview by recounting how the field began and how I got into it and by mentioning some other early investigators. The search for endogenous antimicrobial molecules arose in the middle third of the nineteenth century. Eli Metchnikoff (1845–1916), an insightful Russian émigré who spent his later years at the Pasteur Institute, first recognized the vital role of phagocytes in host defense and also inquired into their microbicidal mechanisms. In those pre-Sigma Catalogue days only trypsin and pepsin preparations were readily available to him. Finding that these did not kill bacteria, Metchnikoff surmised that other leukocyte enzymes might do so. His speculation was proven correct when, over 30 years later, Alexander Fleming described lysozyme. According to the accounts of Lady Fleming, lysozyme's discovery was largely ignored by the medical community of the day because

it was effective only against nonpathogens. When Fleming later described penicillin, this discovery also received little attention, and the industrial development of penicillin had to wait for the exigencies of World War II.

Recognizing the implications of the nascent science of bacteriology, a Scottish surgeon named Joseph Lister (1827–1912) revolutionized surgical practice by using aerosolized phenol (carbolic acid) to prevent infection and by using phenol-soaked lint to dress wounds. No less than the introduction of ether anesthesia in 1846, a generation before, disinfection and antisepsis revolutionized surgical practice. Although Lister knew of Metchnikoff's work, neither knew that phagocytes used disinfectants that were less cytotoxic than phenol. They produced these substances "on demand" through the agencies of two tightly regulated enzyme complexes: nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase and inducible nitric oxide synthase.

Mammalian neutrophils contain myeloperoxidase, an enzyme that converts hydrogen peroxide, a product of NADPH oxidase, into more potent microbicidal oxidants that include hypochlorite and chloramines. During World War I, Henry Drysdale Dakin (1880–1952), an English-born biochemist who once worked at the Lister Institute, joined Alexis Carrel in introducing dilute sodium hypochlorite irrigations to treat wound infections. "Carrel–Dakins solution" was highly effective, and, unlike Lister's phenol, it retained activity in blood. Sodium hypochlorite is also the active ingredient in Clorox, a common household bleach and disinfectant that was "invented" in 1916.

Leukocytes also have much to teach about antimicrobial peptides. The antimicrobial properties of crude leukocyte extracts were noted in the 1940s and 1950s. Although memorable names, such as leukins or phagocytin, were created to describe the phenomenon, precise molecular characterization of the active principle was not yet feasible. The modern era of antimicrobial peptide research began in the mid–1960s when Hussein Zeya and John Spitznagel described highly cationic polypeptides ("lysosomal cationic proteins") in leukocytes from rabbits and guinea pigs. Considering that their most powerful preparative tools were cellulose and free boundary electrophoresis, they had remarkable success in characterizing these peptides. Unfortunately, their progress stopped when most workers in the field became enthralled with an inherited condition called chronic granulomatous disease (CGD).

Indeed, there were many reasons to be interested in CGD. Although the condition was rare, it was serious; most of the affected children sustained frequent infections, and many died by their late teens. The blood neutrophils and monocytes of CGD patients could ingest various bacteria and fungi normally, but showed defective killing of many of them because of deficient production of hydrogen peroxide and related oxidants by their NADPH oxidase.

Over the next two decades, many laboratories worked to define NADPH oxidase, to ascertain the details of its regulation and structure, and to identify the molecular defects responsible for CGD. During this time, NADPH oxidase was a Holy Grail, and only heretics or skeptics began other quests.

I was also involved in these mainstream issues, but as I tested the neutrophils and monocytes of individuals with CGD or hereditary myeloperoxidase deficiency, I found that they killed many bacteria and fungi with normal or near-normal efficacy. Hence I began to look for other antimicrobial components in leukocytes. By 1974, I had learned how to obtain large numbers of "activated" rabbit alveolar macrophages in considerable purity by using a technique developed by Eva S. Leake and Quentin N. Myrvik. I extracted these macrophages with acid, and subjected the clarified extracts to nondenaturing polyacrylamide gel electrophoresis (PAGE) in pencil-sized tube gels. After the gels were hemisected longitudinally, one half was stained and the other half was sliced at 1-mm intervals with an array of single-edged razor blades. The 60 or so little gel pieces were transferred to test tubes, pulverized in a small volume of distilled water, and the eluted contents were tested against various bacteria and fungi. This simple and direct preparative procedure identified two highly cationic antibacterial and antifungal components. With this preliminary data in hand, I applied for National Institutes of Health funding and six years and three proposals later secured it. Although it is amusing to read the reviewer's comments now, it was less amusing then. Fortunately, I had grants to study postphagocytic ion fluxes in neutrophils and the activation of NADPH oxidase, so the work could continue "on the side."

In the early 1980s, work on insect antimicrobial peptides from Hans Boman's lab in Sweden began to appear. At the same time, the UCLA group (including myself, Judith Delafield, Michael Selsted, Tomas Ganz, and the late Sylvia Harwig) began to isolate and characterize the peptides now called α-defensins. Gradually others began to join the search. I recall that, when I found Bob Hancock's 1989 publication on rabbit NP-1, I sent him a letter (I did not then know him) welcoming him to the "defensin club." A recent Medline keyword search on defensins retrieved well over 1,000 hits. Had I continued to write welcoming letters, I would surely have become an expert typist by now.

By the end of that decade, the first β-defensins had been described in the tracheal epithelial cells and leukocytes of cattle, and Michael Zasloff had captured the imagination of the public with his description of magainins. The first cathelicidin peptides had been recognized, largely through the efforts of Dominico Romeo, Margarita Zanetti, and Renato Gennaro. The first three human β-defensin (HBD) peptides, HBD1, HBD2, and HBD3, were isolated and

described by Harder and Schroeder between 1996 and 2001. More recently, powerful genomics-based search strategies identified 28 "new" β-defensin genes (DEFB) in humans and 43 "new" DEFB genes in mice. Although these numbers are small compared with odorant receptor genes (approximately 900 in humans and 1,500 in the mouse) and some other mammalian multigene families, they are nevertheless impressive. HBD1 is prominently expressed in the human vagina and multiple β-defensin genes are expressed in the human and murine epididymis, suggesting that these peptides play significant roles in reproductive processes.

In any rapidly developing field, surprises can be expected. I end by mentioning two that come from our recent studies. We recently established that several θ-(and α-) defensins are lectins. This property enables them to bind surface glycoproteins and glycolipids involved in cell entry by HIV-1 and herpes simplex viruses. I suspect that the ability to bind sugars could contribute to many other properties, including pathogen recognition and receptor-mediated signaling. At the least, in the words of Linda Loman, "Attention must be paid!"

We have formed somewhat heretical views about the mechanism of action of two exceptionally potent antimicrobial peptides: protegrins and sheep myeloid antimicrobial peptide (SMAP-29). We have evidence that these peptides kill susceptible microbes by inducing a process akin to fresh water drowning – namely, a massive influx of water that overwhelms the microbe's osmoregulatory apparatus. I named this the HOTTER (an acronym for hydro-osmotic transtesseral extrusion and rupture) mechanism. As soon as I get my typing up to speed, I intend to put the supporting data into a manuscript.

The principal risk in "naming names" comes from leaving some out. Although I expect no complaints from Metchnikoff or Lister, if I did not mention you in the view from my rearview mirror, then perhaps you were and are in front of me. Please excuse the lack of references. I will learn how to use my citation manager after mastering typing. By the time a second edition comes around, I should have it perfected.

CHAPTER 2

Cationic antimicrobial peptides in regulation of commensal and pathogenic microbial populations

Deirdre A. Devine

2.1. INTRODUCTION

Microbial cells that comprise the diverse resident communities colonizing mucosal sites outnumber cells of the human body by 10:1 (Savage, 1977). It seems remarkable that these potentially overwhelming populations coexist with a host, with harmful effect only if the host becomes immunocompromised or organisms reach sites to which they do not normally have access, for example, through trauma. Effective maintenance and control of resident populations is very important to a colonized host, as these populations contribute to host protection through blocking of colonization by pathogens (e.g., Mead and Barrow, 1990; Roos, Håkansson, and Holm, 2000), development of cell structure and function (Hooper, Falk, and Gordon, 2000; Freitas et al., 2002), and development of the immune system (Cebra, 1999). In addition, nonpathogenic bacteria can downregulate or attenuate inflammatory responses (Neish et al., 2000). Disruption of the host–microbe balance and loss of regulation of these populations may have seriously detrimental effects in development of infections (e.g., in immunocompromised patients) or chronic inflammatory disorders (Neish et al., 2000; Wehkamp et al., 2002). The mammalian host is able, under normal circumstances, to allow the survival and long-term tolerance of these essential resident microbial communities without eliciting a damaging chronic inflammatory response.

The mechanisms involved in this host–microbe homeostasis are not well understood, but cationic antimicrobial peptides possess many characteristics that indicate roles in regulating resident populations as well as defending against specific pathogens. Diverse antimicrobial peptides are components of the innate defenses of a wide range of higher and lower host species, and there is evidence that they have evolved under positive pressures exerted by

colonizing microorganisms. At each site of production, antimicrobial peptides form part of a cocktail of antimicrobial substances that in vivo work synergistically to combat infection (Gudmundsson and Agerberth, 1999; Hancock and Diamond, 2000). Recent research has provided evidence that antimicrobial peptides have multiple activities in host immunity and have a key role in modulating early immune responses (Yang, Kwak, and Oppenheim, 2002, see also chapter 3). This multiplicity of function, combined with the fact that every host species produces such a range of site-specific antimicrobial peptides, has led to the proposal that cationic antimicrobial peptides are of key importance in host responses to highly diverse resident populations (Boman, 1996; Garabedian et al., 1997; Simmaco et al., 1998). The complexity of these host–microbe relationships is illustrated by consideration of the composition and diversity of resident populations, which vary according to site and host species.

2.2. DIVERSITY AND SITE SPECIFICITY OF RESIDENT MICROBIAL POPULATIONS

The complex mechanisms involved in regulating responses to colonizing mammalian hosts must interact with a vast diversity of microorganisms, mostly bacteria, although some protozoa, fungi, and viruses are members of the resident microbiota (Tannock, 1999). In humans and other mammals, some sites are usually sterile (e.g., lung, bladder), whereas others (e.g., oral cavity and colon) are heavily colonized by largely anaerobic bacterial populations (Fig. 2.1). It is estimated that up to 600 species, only 50% of which can be grown in monoculture by conventional methods, are normal inhabitants of the human mouth (Wilson, Weightman, and Wade, 1997; Paster et al., 2001), and the human gut harbors more than 400 bacterial species (Berg, 1996). In spite of gaining access to the gastrointestinal tract through frequent swallowing, few oral organisms colonize the gut. Recent phylogenetic studies have shown that resident populations in the human gut and oral cavity are equally highly diverse, but substantially different in composition (Martin, 2002). Studies of oral and nasopharyngeal resident populations have also shown that sites that are anatomically close or adjacent can nonetheless harbor very distinct microbiota (e.g., Rasmussen et al., 2000; Hohwy, Reinholdt, and Kilian, 2001; Könönen et al., 2002).

In addition to the microbial species diversity evident in resident populations, single species exhibit substantial genetic diversity (e.g., Jolley et al., 2000; Hohwy et al., 2001). The genetic composition of a species at one site can fluctuate significantly, with resident populations comprising persistent