Antimicrobial Peptides in Mucosal Secretions: The Importance of Local Secretions in Mitigating Infection\textsuperscript{*2}

Shruti M. Phadke, Berthony Deslouches,* Sara E. Hileman,* Ronald C. Montelaro,* Harold C. Wiesenfeld,† and Timothy A. Mietzner*\textsuperscript{3}

Department of Pediatrics, Children’s Hospital of Pittsburgh, Pittsburgh, PA 15213; *Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261; and †Department of Obstetrics, Gynecology, and Reproductive Services, Magee Women’s Hospital, Pittsburgh, PA 15261

ABSTRACT The antimicrobial activity of the collective molecules comprising human milk reflects an evolutionarily successful paradigm for preventing and limiting microbial infection. Understanding the molecules that participate in this process and how they work can yield insight into potentially new antimicrobial therapies. Upon proteolytic processing, antimicrobial peptides can be derived from milk proteins, such as lactoferrin, casein, and lysozyme. Similarly, using the HIV-1 gp41 protein template, we have demonstrated that the 28-residue C-terminus, when produced as an independent peptide, exhibits selective toxicity for bacteria over eukaryotic cells. Upon optimizing this sequence for cationic charge and hydrophobic character presented as an α-helical structure, we show improved capability of the parent LLP1 sequence to selectively kill bacteria in the host environment and that this activity is increased by the inclusion of Trp residues on the hydrophobic face. We report that it is possible to (i) design de novo antimicrobial peptides that demonstrate optimal antimicrobial activity with minimal inflammatory activity and (ii) design antimicrobial peptides to function in a defined environment. In the end, we describe a de novo designed antimicrobial peptide, WLBU2, which is selectively toxic to microbial pathogens in complex environments and does not stimulate a significant immunomodulatory response. In spite of these properties, WLBU2 activity against Pseudomonas aeruginosa in human milk is inferior to the host peptide LL37 with regard to antimicrobial potency. These studies demonstrate that antimicrobial peptides can be engineered for greater potency in one medium but may not be optimal for working in a different medium such as human milk. J. Nutr. 135: 1289–1293, 2005.

KEY WORDS: antimicrobial peptide Pseudomonas aeruginosa Staphylococcus aureus antibiotic

Antimicrobial composition of human milk

As a modified secretion, human milk demonstrates remarkable biologic versatility. This nutrient-rich fluid is specifically adapted to the needs of the newborn. Antenatally, the mother transfers nutrients and bioactive components through the placenta. Postnatally, these substances are transferred through colostrum and milk. Like other secretions (e.g., vaginal fluid, airway surface liquid of the pulmonary tissues), human milk has a unique composition. On a gram per liter basis, human milk is composed of a carbohydrate:lipid:protein ratio of 7:4:1 (1), a perfectly formulated bacterial growth medium. It is remarkable that contaminating bacteria do not overgrow this biologic fluid. Rather, the diverse group of molecules present in milk operates in concert to prohibit the growth of contaminating pathogenic microorganisms. A nonexhaustive list of the molecules responsible for this antimicrobial activity is summarized in Table 1. Immunoglobulins that are transferred by human milk provide passive immunity based on previous maternal exposure to microbial pathogens (2). Carbohydrates and glycolipids act as soluble decoys, competing for microbial attachment factors and preventing adherence to cellular receptors. For example, it has been demonstrated recently that the sugar α1,2-fucosylated carbohydrate moieties found in high concentrations in milk inhibit binding of Campylobacter jejuni to host cell tissues and protects against the establishment of infection (3). Lipids...
can play a role in disrupting the organization of bacterial or viral membrane lipids (4). Lactoperoxidase catalyzes the formation of oxygen radicals that inhibit microbial processes. Lysozyme hydrolyzes the peptidoglycan cell wall associated with nearly all bacteria. Lactoferrin plays bacteriostatic and bactericidal roles. Its bacteriostatic role is fulfilled by sequestering growth-essential iron, rendering the secretion “hypoferremic” and therefore unable to support multiplication of bacterial organisms. As reviewed by Vogel and colleagues (5), lactoferrin is also bactericidal because it binds the microbial cell surface where its C-terminus structurally reorganizes and inserts into bacterial membranes. Interestingly, proteolyzed lactoferrin can contribute peptides that are highly stimulatory to the growth of normal flora bifidobacteria, the predominant bacteria in the large intestine of healthy breast-fed infants (6).

Recently, substantial quantities (between 1 and 10 mg/L) of antimicrobial peptide human β-defensin 2 have been demonstrated in human milk (7). In addition, antimicrobial peptides can be generated by the proteolytic processing of milk proteins (8). Based upon these studies, one can conclude that antimicrobial peptides are abundant in human milk both pre- and postdigestion, which clearly benefits the ingesting host.

We have been involved in identifying (9), engineering (10), and determining the mechanism of action (11,12) of the antimicrobial lentivirus lytic peptide 1 (LLP1) derived from an unlikely source, the HIV-1 gp41 transmembrane protein. In considering the potent antimicrobial activity of the parent LLP1 sequence, several questions arise pertaining to the larger literature on antimicrobial peptides: (i) why has nature evolved so many different antimicrobial peptide motifs, and is there a common theme; (ii) do all antimicrobial peptides work by the same mechanism of action; and (iii) can peptides be engineered to work in a specific environment and against a specific subset of microbial pathogens? This report reflects the current thinking on these questions.

### Innate immunity and the diversity of host-derived antimicrobial peptides

The innate immune system participates as the first line of defense against infection and comprises a variety of cells and humoral effector molecules poised to prevent the initiation of microbial infection. Characteristics of innate immunity include responses that (i) are broad spectrum (nonspecific), (ii) lack memory or lasting protective immunity, and (iii) target a limited repertoire of recognition molecules. Host-derived antimicrobial peptides are an important component of the innate immune system and play a significant role in the innate immunity to bacterial, fungal, parasitic, and viral infections (13–15).

Surveys of vertebrate tissues have revealed thousands of antimicrobial peptides with widely diverse sequences. Host-derived antimicrobial peptides generally range in length from 20 to 40 residues. Whereas these sequences are diverse, the majority of these peptides can be structurally classified into relatively few conformational paradigms (16). For example, many host-derived antimicrobial peptides assume cationic, amphipathic α-helical structures (e.g., cathelicidin, also known as LL37). Others are organized as β-sheet structures (e.g., protegrins) or are rich in a particular amino acid, such as Trp or His (e.g., indoliscin or histatin). Still other antimicrobial peptides contain thio-ether rings with attached lipid groups (e.g., colistin) or have macrocyclic Cys knots (e.g., defensins). Remarkably, changes in a single amino acid within these sequences can have a profound effect on antimicrobial activity (12). Despite the diversity of sequences and structure, a common feature of antimicrobial peptides is their organization as a cationic amphipathic structure. It is this fundamental structural motif that gives rise to their binding specificity for microbial lipid interfaces and their propensity to disrupt these membranes.

The broad diversity of host-derived antimicrobial sequences and structures raises the question why nature has not evolved a single peptide sequence to be singularly effective in all infectious settings. Several assumptions underlie this point. At the forefront are the preinflammatory vs. the inflammatory environments in which host-derived antimicrobial peptides have evolved to function. By definition, as mediators of innate immunity, many host-derived antimicrobial peptides are positioned to prevent infection and therefore have been optimized for the preinflammatory or nondiseased state. Other antimicrobial peptides have evolved to function in the inflammatory environment that might result, for example, upon degranulation of a neutrophil. Cationic antimicrobial peptides comprise a substantial proportion of a macrophage granule (17). It is well known that antimicrobial peptides function differently, depending on the organism and the environment. For example, human β-defensin 2 has been demonstrated to be a salt-sensitive peptide (18). We have demonstrated that the LLP1 peptide is effective against prototype strains of *Pseudomonas aeruginosa* and *Staphylococcus aureus* suspended in phosphate buffer at pH 7.0. When saline (150 mmol/L NaCl) is added, only *P. aeruginosa* is killed (9). In addition, antimicrobial peptides are secreted by macrophages and epithelial cells in response to infection and are found in “biologically active” concentrations to prevent infection on compromised mucosal surfaces. This spectrum of peptide function is relevant to the host innate immune response and suggests that (i) at low concentrations, they have the ability to stimulate immunomodulatory function (15,19); and (ii) at higher concentrations, they have the capability for antimicrobial activity and selective toxicity to the membranes of bacteria and other pathogens (14).

Studies from our laboratory have addressed the issue of the diversity of antimicrobial peptides that function in diverse host environments through the design of de novo peptides. Analyses of the LLP1 of gp41 from diverse HIV-1 isolates have shown that Arg and Val are the most common residues among these sequences (Timothy A. Mietzner, unpublished results). In addition, substantial evidence that the aromatic side chain

---

**TABLE 1**

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunoglobulin, maternal slgA, IgG, IgM, IgD</td>
<td>Passive immunization against potential pathogenic bacteria</td>
</tr>
<tr>
<td>Soluble carbohydrates or glycolipids</td>
<td>Surrogate receptor analogs that compete with bacterial adhesions for binding</td>
</tr>
<tr>
<td>Antimicrobial lipids</td>
<td>Disruption of bacterial membranes</td>
</tr>
<tr>
<td>Lactoperoxidase</td>
<td>Oxygen-dependent killing</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>Cleavage of the bacterial cell wall contributing to osmotic instability</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>Iron-binding protein with antimicrobial peptide activity (lactoferricin)</td>
</tr>
<tr>
<td>Human β-defensin 1</td>
<td>Antimicrobial peptides</td>
</tr>
</tbody>
</table>
of Trp is membrane interactive (20) led to consideration of its inclusion on the hydrophobic face of this motif. What was not clear from previous studies was the minimum peptide length required for optimal antimicrobial activity. To address this issue, the series of peptides described in Figure 1 was designed based on the concept of a repeating lytic base unit of 12 residues.

The 24-residue WLBU2 derivative (3 Trp residues on the hydrophobic face) displayed potent activity against both index organisms in the presence or the absence of 150 mmol/L NaCl. The surprising finding from this study was that increasing the length of the WLBU series of peptides beyond WLBU2 did not appreciably increase their molar antimicrobial potency. This was a significant finding, because it is generally regarded that a “threshold” peptide concentration is required for activity, presumably because they need to oligomerize to function. Carrying this thought further, by covalently presenting multiple copies of the same sequence, it seems predictable that multimers of the sequence would be more effective on a molar basis. It is shown, in Table 2, that WLBU2 is equally effective against either index organisms in the absence (phosphate buffer) or the presence (phosphate buffered saline) of 150 mmol/L NaCl.

The immunomodulatory activity of host-derived antimicrobial peptides can include a proinflammatory response (15). This is an obvious advantage when trying to clear an infection; it could also be a disadvantage when discouraging the genesis of infections in a specific environment. Additional constraints on these peptides that may contribute to their biologic diversity must also be appreciated. They must be transcribed in a nontoxic form, secreted into the host-cell environment, and processed to be active. The lessons learned from studying the biologic nature of host-derived antimicrobial peptides can be exploited for the de novo design of new antimicrobial agents that can obviate the biologic constraints of processing, delivery, and immunopotentiating.

**Why has nature evolved so many different antimicrobial peptide motifs?**

Our demonstration that a de novo derived antimicrobial peptide is more potent than the host antimicrobial peptide suggests that peptides can be derived under specific conditions and that their diversity has evolved to “prevent” specific infections in a specific environment. Additional constraints on these peptides that may contribute to their biologic diversity must also be appreciated. They must be transcribed in a nontoxic form, secreted into the host-cell environment, and processed to be active. The lessons learned from studying the biologic nature of host-derived antimicrobial peptides can be exploited for the de novo design of new antimicrobial agents that can obviate the biologic constraints of processing, delivery, and immunopotentiating.

**Do all antimicrobial peptides work by the same mechanisms of action?**

Historically, it has been well established that antimicrobial peptides interact with bacteria in different ways. It is clear that different antimicrobial peptides use different mechanisms of action. We have demonstrated this by using LLP1 and polymyxin B, which are both cationic antimicrobial peptides demonstrating similar antimicrobial potencies. By electron microscopy, LLP1 acted at the outer and the cytoplasmic membranes, forming ridges and valleys on the outer membrane and leading

---

**TABLE 2**

Activity expressed as minimum bactericidal concentrations (MBC) of peptide derivatives described in Figure 1 for P. aeruginosa or S. aureus in the presence of phosphate buffer (PB) of phosphate buffered saline (PBS)

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Length</th>
<th>MBC P. aeruginosa</th>
<th>MBC S. aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PB</td>
<td>PBS</td>
</tr>
<tr>
<td>WLBU1</td>
<td>12</td>
<td>0.6</td>
<td>&gt;10</td>
</tr>
<tr>
<td>WLBU2</td>
<td>24</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>WLBU3</td>
<td>36</td>
<td>1.2</td>
<td>0.2</td>
</tr>
<tr>
<td>WLBU4</td>
<td>48</td>
<td>1.2</td>
<td>0.6</td>
</tr>
<tr>
<td>LL37</td>
<td>37</td>
<td>1.2</td>
<td>1.2</td>
</tr>
</tbody>
</table>

**FIGURE 2** Human bronchial epithelial (HBE) cell stimulation of IL-8 production. HBE cells grown in polarized culture were stimulated on the apical side with no peptide, LL37, or WLSA5.
to fusion and dissolution of the outer and cytoplasmic membranes. This activity is similar to that of gramicidin, an antimicrobial peptide known to interact with both the outer and cytoplasmic bacterial membranes (7). In contrast, polymyxin B produced characteristic changes in the outer membrane by thin-section electron microscopy (data not shown), suggesting severe alteration of outer membrane curvature via intercalation into its outer leaflet. Treatment of bacteria well below the minimum bactericidal concentration of other cationic antimicrobial peptides by electron microscopic analysis showed that other antimicrobial peptides such as lactoferricin and indolicidin were similar to polymyxin B, whereas magainin-2 resembled the activity of LLP1. Bacterial membrane disruption at an achievable minimum bactericidal concentration is relevant to the design of antimicrobial agents. The findings from this study suggest that LLP1 acts in synergy with other conventional antimicrobial agents through physical disruption of the outer and cytoplasmic membranes.

The general assumption is that the mechanism of antimicrobial peptide action is the insurmountable disruption of microbial membranes, leading to ion and metabolite leakage, depolarization, disruption of membrane-coupled respiration, and ultimately cell death. It is likely that these effects contribute to the mechanisms of antimicrobial peptides. However, a mounting body of evidence supports additional or complementary mechanisms, wherein membrane permeabilization alone appears insufficient to cause cell death. Data supporting this come from studies documenting dissociation between membrane perturbation and cell death (23). Cell killing may proceed due to disruption of intracellular processes with relatively little membrane disruption.

From these studies, it is clear that antimicrobial peptides function in a variety of ways, with the most demonstrable being membrane perturbation. Understanding the mechanism of action is important in that it may be possible to devise therapeutic rationales that exploit the membrane-perturbing properties of antimicrobial peptides in combination with antimicrobials that act on the bacterial cytoplasm.

**Engineering peptides to work in specific host environments**

For decades, host-derived antimicrobial peptides have been recognized for their activity but only recently have received interest from the medical community as host defense mechanisms or potential therapeutics. Animal models and unique human populations (e.g., cystic fibrosis) reveal that these host-derived antimicrobial peptides constitute an important first line of defense in the mammalian immune system (24). For example, a recent report by Nizet et al. (25) used a murine background deficient in the ability to produce the antimicrobial peptide designated as CRAMP and to provide protection against necrotic skin infections caused by Group A Streptococcus. This study underscores the importance of antimicrobial peptides for the prevention of infectious diseases that begin on mucosal surfaces (26). However, observations that NaCl, calcium, and magnesium, or serum components inhibit the antimicrobial activity of many antimicrobial peptides raises questions as to whether these peptides can function in extreme environments of inflammation or physiologic dysregulation (e.g., cystic fibrosis).

Toward understanding whether it is possible to create an antimicrobial peptide that is active in environmental extremes, we compared our idealized peptide, WLBU2, described in Figure 1, for the activity of these peptides in the face of inhibitory substances. To more appropriately evaluate antibacterial selectivity, we demonstrated that this idealized peptide is active at concentrations of micromoles per liter against both *S. aureus* and *P. aeruginosa* in the presence of NaCl, MgCl₂, and CaCl₂ (data not shown). Under identical conditions, the host-derived LL37 is inhibited at concentrations that approximate those found in most human mucosal secretions (data not shown). We tested the ability of the de novo derived peptide WLBU2 to selectively kill bacteria in whole blood (Fig. 3) and were able to demonstrate that this peptide is remarkably selective for bacteria over host red blood cells in this setting. Surprisingly, when WLBU2 was compared with LL37 in the presence of human milk that was put through a 0.45-µm filter, LL37 demonstrated superior activity (Fig. 4). One explanation for this is that the concerted efforts of the individual antimicrobial molecules described in Table 1 have uniquely evolved to complement the activity of the antimicrobial peptides comprising the gauntlet of antimicrobial activities found in secretions such as human milk.

![FIGURE 3](image-url) Human whole blood was inoculated with *P. aeruginosa* PAO1 (~10⁶ cells/mL) and treated with peptide at various concentrations for 30–45 min at 37°C. The bacterial count (shown as open triangles) was determined by standard bacterial dilution assay and blood cell lysis by spectrophotometric analysis of hemoglobin release. The right axis shows the erythrolytic effect of red blood cell lysis buffer in comparison to that of water, which was used to generate the standard curve. The peptide WLBU2 demonstrates remarkable selective toxicity against the bacterial cells, with no detectable lytic effects on the blood cells.

![FIGURE 4](image-url) Similar to Figure 3, experiments of the activity of WLBU2 vs. LL37 in human milk were performed using *P. aeruginosa* as the index organism. Surprisingly, the activity of the host-derived LL37 was superior to the activity of the de novo derived WLBU2, presumably as a consequence of molecules that comprise human milk.
What is clear from these studies is that it is possible to generate antimicrobial peptides that incite a reduced host response, demonstrate antimicrobial activity, and may be favorable to bacterial membrane permeabilization but in the end may not coordinate with host antimicrobial molecules that have evolved to protect against bacterial infections. Increased awareness of the synergy between host molecules and antimicrobial peptides should be the next step in devising de novo antimicrobial peptides.

LITERATURE CITED