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COMMENTARY

Epithelial Peptide Antibiotics

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ABSTRACT. Surfaces of higher eukaryotes such as plants, invertebrates, and vertebrates, including humans, are normally covered with microorganisms but usually are not infected by them. The reason, apart from physical barriers, is the production of gene-encoded antimicrobial peptides by epithelial cells. Many novel antimicrobial peptides have been discovered recently in the epithelia of plants, insects, amphibians, and cattle, and, more recently, also in humans. *In situ* hybridization studies indicate a rather organ-specific expression of the genes for peptide antibiotics, which, due to their antimicrobial spectrum and conditions of expression, may also define the physiologic microflora. Some epithelial antimicrobial peptides are constitutively expressed; others are inducible, either by the presence of microorganisms via as of yet not well characterized elicitor receptors or by endogenous proinflammatory cytokines. Most antimicrobial peptides kill microorganisms by forming pores in the cell membrane, and the sensitivity of some peptide antibiotics towards cholesterol, a major mammalian cell membrane constituent, may indicate why these peptide antibiotics are not toxic for mammalian cells. Thus, it seems to be difficult for microorganisms to acquire resistance, making these peptides very attractive for therapeutic use as antibiotics. The first clinical studies are very promising, and after solving the problems of a large-scale biotechnical synthesis, which is more complicated due to the principally suicidal activity of these peptides, a number of new natural structure-based peptides may be developed. Furthermore, discovery of the inducibility of many antimicrobial peptides may also lead to the development of compounds that elicit epithelial defense reactions by stimulating the synthesis of endogenous peptide antibiotics. BIOCHEM PHARMACOL **57**;2: 121–134, 1999. © 1998 Elsevier Science Inc.

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Pathogenic microorganisms usually interact with higher organisms at epithelial surfaces where they adhere, and, if they survive, either multiply locally or invade deeper tissues. Because pathogen-specific immune responses occur slowly, it is not surprising that epithelial cells are equipped with various antimicrobial substances to act rapidly and control the growth of a broad range of potentially pathogenic microorganisms on body surfaces. Some of these innate host-defense molecules restrain microbes by depriving them of essential nutrients. Others kill them with antimicrobial peptides by causing structural disruption [1].

Surfaces of higher eukaryotes such as plants, invertebrates, and vertebrates, including humans, are normally covered with microorganisms but usually are not infected by them. The reason, apart from physical barriers, is the production of antimicrobial peptides as a chemical shield that controls the growth of microorganisms.

This article summarizes our present knowledge about nature's answer to the permanent presence of microorganisms on surfaces and discusses current views of the role of these molecules in innate epithelial defense, along with their physiological significance. Particular attention is paid to the mechanisms of these molecules that kill microorganisms and thus control microbial growth on epithelia.

PEPTIDE ANTIBIOTICS FROM PLANTS

Higher vertebrates contain, as one important component of the defense system, an adaptive immune response, which is unique in the sense that it displays specificity towards the infectious agent and features a memory function via T-lymphocytes and immunoglobulins. Plants do not contain any comparable adaptive immune system; however, they share with animals components of the so-called innate immune system, in which, either constitutively or upon perception of microbial signals, chemical substances are produced that control microbial growth on their surfaces. A number of antimicrobial peptide families have been discovered recently in plants and are listed in Table 1.

Thionins comprise a family of cysteine-rich antimicrobial peptides containing 45–47 amino acids; they are divided into two subgroups possessing either four or three

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* *Abbreviations:* ALP, antileukoprotease; CAMP, cathelicidin antimicrobial peptide; CF, cystic fibrosis; EBD, enteric β-defensin; hBD, human β-defensin; HD, human defensin; HNP, human neutrophil peptide; IL, interleukin; LAP, lingual antimicrobial peptide; LD_{90}, dose killing 90% of the microorganisms; LPS, bacterial lipopolysaccharide; mBD, murine β-defensin; PMA, phorbol-myristate-acetate; RT–PCR, reverse transcription–polymerase chain reaction; TAP, tracheal antimicrobial peptide; and TNF-α, tumour necrosis factor α.

TABLE 1. Plant antimicrobial peptides*

Thionins (6 cysteines)
Thionins (8 cysteines)
Plant defensins
Hevein-type peptides
Knottin-type peptides

* See Ref. 16 for a review.

disulfide bonds. Thionin sequences are highly divergent. Residues conserved are restricted to the six cysteines at identical relative positions.

For more than 50 years, it has been known that thionins inhibit the growth of bacteria and fungi *in vitro* [2]. In 1972, it was first demonstrated that thionins inhibit the growth of a number of plant pathogenic bacteria, thus suggesting that thionins may fulfill a protective role in plants [3]. In subsequent studies [4–6], the antimicrobial spectrum of thionins has been analyzed in more detail. It was found that the growth of several Gram-positive and Gram-negative bacteria, as well as that of phytopathogenic fungi, was inhibited, with IC_{50} values (concentrations required for 50% growth inhibition) near 1–15 μg/mL [4, 6]. Interestingly, antifungal activity of thionins is inhibited by Ca^{2+} at concentrations higher than 5 mM, but not by Mg^{2+} or monovalent cations [7–9]. In contrast, antifungal activity has been potentiated by the addition of other cysteine-rich peptides [10].

Expression of some thionin genes has been found predominantly in flowers and leaves, whereas that of others has been seen at low levels in seedlings; these levels were strongly up-regulated upon infection with fungal species [11]. Induction of the expression of thionin genes after pathogen exposure may involve methyljasmonate (a hormone-like compound structurally related to prostaglandins, which is synthesized upon release of α-linolenic acid from phospholipid membranes) as an endogenous signal transducer; this compound has been shown to accumulate after wounding [12] or exposure of plant cells to invading microorganisms. Another endogenous hormone-like compound in plants, salicylic acid, which mediates the expression of a number of other antimicrobial compounds in plants, does not induce thionins [13].

As seen by immunocytochemistry, thionins have been found to be most abundant in plant epidermal cells, further supporting the idea of a first line chemical defense system [14].

Apart from thionins, other cysteine-rich antimicrobial peptides, such as defensins [15], also are produced by plants. Plant defensins act similarly against a broad spectrum of microorganisms, but show the strongest activity against fungi and lesser activity against bacteria [16, 17]. Interestingly, antifungal activity is reduced by increasing the ion strength; divalent cations are at least one order of magnitude more potent than monovalent cations [18, 19].

In a single plant genus, different defensin genes exist, all of which have a distinct organ-specific expression pattern [18], where they are expressed always in the outer cell wall lining the epidermis [19]. Likewise, in potato tubers, the highest transcription is in the epidermis [18]. It has been proposed that plant defensins play an important role in the protection of seed from infection [16]. Indeed, defensins account for 30% of released proteins upon germination of seed [16]. Most plant defensins seem to be inducible [16, 20], showing a systemic response upon fungal infection.

Two other cysteine-rich antimicrobial peptide families have been discovered in plants. One family, originally called lipid transfer proteins due to their ability to transfer phospholipids, revealed varying activities against different microorganisms [16]. The other family, called hevein- and knottin-type antimicrobial peptides, members of which are cysteine-rich and have a characteristic knot-like fold [21], inhibit a whole range of fungi and Gram-positive bacteria [22]. Again, antimicrobial activity is sensitive towards the presence of divalent cations above a concentration of 1 mM [16]. The expression pattern in plants is, at present, still fragmentary, but it seems to be found mainly in seed. A recent review summarizes our knowledge about plant-derived antimicrobial peptides [16].

TABLE 2. Insect-derived antimicrobial peptides

Antimicrobial peptide	Cysteine bridges	Inducibility	Activity against: Gram+ bacteria	Gram− bacteria	Fungi
Cecropins	None	Yes	(+)	+++	(+)
Drosocin	None	Yes	(+)	+++	−
Apidaecins	None	Yes	(+)	+++	−
Diptericin	None	Yes	(+)	+++	−
Metchnikowin	None	Yes	+++	+++	+++
Thanatin	1	Yes	+++	+++	+++
Defensins	3	Yes	+++	(+)	−
Drosomycin	4	Yes	−	−	+++

INSECT PEPTIDE ANTIBIOTICS

Although pioneering studies after the First World War showed, in a number of investigations [23], that injections of bacterial cultures into insects induced the appearance of bacteriolytic substances, it was not until 1980 that any of the antimicrobial peptides, apart from the ubiquitous lysozyme, were characterized. Boman and coworkers were able to analyze the structure of the major inducible bactericidal factor from bacteria-challenged silkworms (*Hyalophora cecropia*), and named it cecropin [24]. In the following decade, a number of other insect-derived antimicrobial peptides were identified (Table 2).

Cecropins represent 4-kDa cationic peptides that are active mainly against Gram-negative bacteria. Small-sized (2–4 kDa) cationic proline-rich peptides like apidaecins [25], abaecin [26], and drosocin [27] are primarily bactericidal against Gram-negative bacteria. All members of these three families are devoid of cysteines. The proline-rich

drosomycin, which represents a 5-kDa peptide, contains four disulfide bridges, acts primarily against fungi [28]. Several distinct polypeptides (e.g. attacins [29], sarcotoxins [30]) ranging in size from 8 to 27 kDa, mostly cationic and frequently rich in glycine, affect Gram-negative bacteria and are either bactericidal or bacteriostatic.

In contrast, the fourth group of antimicrobial peptides in insects, insect defensins, are composed of 38–43 amino acids and have six cysteines engaged in three intramolecular cysteine bridges [31]. These molecules are moderately cationic, primarily affect Gram-positive bacteria, and appear to be the most widespread group of antibacterial peptides in insects. Many insect defensins are constitutively expressed at a low level and are induced by injection of bacteria into larvae [31]. The main sites of gene expression are fat bodies and epidermal cells of tracheas. In the hemolymph of insects, the concentration of antimicrobial peptides may reach 200 μM after injury.

Drosomycin production after infection is controlled by the dorsoventral regulatory gene cassette *spätzle/Toll/cactus*, which is structurally related to the mammal IL-1/I-κB/NF-κB system [32]. Interestingly, when *Drosophila* flies having a recessive immune deficiency mutation, which impairs the inducibility of all genes encoding antibacterial peptides involved in the immune response, were challenged with bacteria, a lower survival rate than in wild-type flies was seen, whereas the antifungal peptide drosomycin remained inducible [33].

In insects, antimicrobial responses seem to show some degree of specificity. In *Drosophila*, a discrimination between various classes of microorganisms occurs because genes encoding antibacterial and antifungal peptides are differentially expressed after injection of distinct microorganisms. More strikingly, infection of *Drosophila* with pathogenic fungi exhibits an adapted response by producing only peptides with antifungal activities [34].

VERTEBRATE PEPTIDE ANTIBIOTICS

One group of vertebrates that has been studied extensively for the presence of antimicrobial peptides is frogs. Skin extracts of frogs are a rich source of pharmacologically active peptides such as kinins, caeruleins, bombesin-like peptides, and opioid peptides [35]. Vertebrate skin has the same embryonic-ectodermal origin as the brain, and many frog skin peptides have been found to have counterparts in the mammalian gastrointestinal tract and brain.

The antimicrobial peptide bombinin was first isolated 20 years ago from the skin secretion of *Bombina variegata*, but it was in the late 1980s that antimicrobial peptides from the skin of different frog species were studied in more detail (for review, see Ref. 36). Systematic analyses of antimicrobial peptides in *Xenopus laevis* led to the discovery of magainin/peptidyl-glycine-serine (PGS) peptides [37, 38], peptidyl-glycine-leucine carboxamide (PGLa), and a multitude of fragments derived from the precursors of caerulein, xenopsin, and laevitide.

In the meantime, a number of related peptides were discovered in different frog species. Each of these linear peptides, which can form amphipathic helices, contains more than 20 amino acids and has a broad spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi, and protozoa [39].

Two peptide families have been described from the skin of the *Bombina* species: the bombinins and type H bombinins. Whereas bombinins are related to the original bombinin, type H bombinins are more hydrophobic molecules, possessing both antibacterial and hemolytic activities [40]. Interestingly, some H bombinins contain a D-alloisoleucine as the second amino acid of their sequence. D-Alloisoleucine is not essential for cytolytic activity of these peptides, but it may increase their biostability [40]. As yet, the presence of a D-amino acid in peptides of animal origin has been demonstrated only for opioid peptides that were isolated from South American frogs [41].

Several related peptides, called dermaseptins, were isolated from the skin of *Phyllomedusa sauvagii*. These are particularly active against pathogenic fungi [42].

A large number of antimicrobial peptides have been isolated from the *Rana* species. These peptides were found to have a single intramolecular disulfide bridge located at the C-terminal end in common and are grouped into several families on the basis of length and distinct activity. Most of these peptides are active against Gram-positive and Gram-negative bacteria as well as fungi, and were found to kill microorganisms in the range of 0.5 to 4 μM.

cDNA cloning of some of these antimicrobial peptides revealed that precursors of esculetin, brevinins, ranalexin, and dermaseptin contain signal sequences similar to those found in the precursors of several opioid peptides from the skin of the *Phyllomedusa* species [43].

HIGHER VERTEBRATE EPITHELIAL ANTIMICROBIAL PEPTIDES

Although primitive lymphoidal tissues are present in invertebrates, cell-mediated, long-lasting immunity does not seem to play a major role. Invertebrates very efficiently combat microorganisms that enter their bodies by synthesis and delivery of a fixed repertoire of broad-spectrum antimicrobial peptides produced mainly by epithelia and related cells.

Vertebrates and, to a far lesser degree, invertebrates have in their blood professional phagocytes, mainly polymorphonuclear leukocytes and monocytes, that allow a directed defense strategy to eliminate bacteria that have entered the body. These phagocytes have the capability of killing ingested microorganisms through both oxygen-dependent and oxygen-independent mechanisms.

Leukocytes are a rich source of endogenous antimicrobial peptides termed α-defensins (for recent reviews, see Refs. 44–46). The term "defensin" was devised about a decade ago to describe a family of antimicrobial peptides found in the primary granules of rabbit and human neutrophils. Six

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HUMAN
HD5                 ARATCYCRTGRCATRESLSGVCEISGRLYRLCCR
HD6               TRAFTCHCRR-SCYSTEYSYGTCTVMGINHRFCCL

MOUSE
Cryp-1            LRDLVCYCRSRGCKGRERMNGTCRKGHLLYTLCCR
Cryp-2            LRDLVCYCRTRGCKRRERMNGTCRKGHLMYTLCCR
Cryp-3            LRDLVCYCRKRGCKRRERMNGTCRKGHLMYTLCCR
Cryp-4              GLLCYCRKGHCKRGERVRGTC--G-IRFLYCCPRR
Cryp-5            LSKKLICYCRIRGCKRRERVFGTCRNLFLTFVFCCS
Cryp-6            LRDLVCYCRARGCKGRERMNGTCRKGHLLYMLCCR

RABBIT
NP6                  GICACRRRFCLNFEQFSGYCRVNGARYVRCCSRR

α-DEFENSIN
consensus          ---CXC----C---------C---------CC---
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FIG. 1. Primary amino acid sequence of mammalian enteric α-defensins. The single letter code of amino acids was used. Conserved cysteines are presented in bold.

human α-defensins are presently known. Whereas neutrophils produce four of them (defensins HNP*-1, 2, 3, and 4), epithelial granulocytes that are found at the base of the small intestinal crypts produce two (HD5 and HD6).

Figure 1 shows the primary amino acid sequences of a number of mammalian enteric α-defensins, which are cysteine-rich and have, in addition, a single conserved glycine, glutamic acid, and arginine. α-Defensins differ in their antimicrobial spectrum and relative potency; the latter seems to parallel their net positive charge. The antimicrobial spectrum of α-defensins encompasses Gram-positive and Gram-negative bacteria, yeast phase and filamentous fungi, many enveloped viruses, and mycobacterial species. Curiously, HDs are far less potent than other mammalian α-defensins against many human pathogens, which may suggest that relative resistance to autogenous defensins may be a species-specific virulence factor.

α-Defensins are produced in remarkably large amounts (5–10 mg/kg body weight/day) by human bone marrow during polymorphonuclear leukocyte maturation. They are synthesized as 10-kDa pre-pro-peptides that are stored in cytoplasmic primary (or azurophilic) granules after stepwise processing. Regulation of α-defensin expression in phagocytic leukocytes varies considerably. Guinea pig and rat neutrophil defensins are produced early in myeloid differentiation, and mature neutrophils have no detectable defensin mRNA [47]. In rabbits, alveolar macrophages express only two of the six α-defensin genes, whereas peritoneal macrophages express none and neutrophils express all six.

Enteric defensins of mice and humans seem to be expressed exclusively in the Paneth cells of the small intestine [44, 48]. Paneth cells are epithelial granulocytes located at the base of the crypts of Lieberkühn—hence, the name cryptdins. Curiously, despite the largest known repertoire of defensin-coding sequences, mice express defensin genes only in Paneth cells [49] (mouse neutrophils lack defensins at levels found in other species [50]). Instead, up to 17 murine defensin mRNAs with distinct coding sequences are produced in a single intestinal crypt [51]. In humans, Paneth cells contain high levels of mRNA encoding the putative pre-pro-defensin peptides HNP-5 and HNP-6.

Biochemical and immunological analyses of the luminal content in the small intestines suggest that cryptdin peptides are localized in eosinophilic secretory granules of the Paneth cells [52] and are secreted into the lumen, in a pattern that is known for lysozyme. Intestinal defensins can be distinguished from phagocyte defensins by the ability to be actively secreted and not primarily targeted for intracellular delivery to phagolysosomes. Recent investigations indicate that cryptdins 2 and 3, but not the others also have a role in salt and water secretion from intestinal epithelia by selective permeabilization of the apical cell membrane of intestinal epithelial cells [53].

β-DEFENSINS

Unlike rodents and humans, neutrophils of cattle and birds do not seem to produce α-defensins. Instead, myeloid defensins belong to a structurally related family called β-defensins. In bovine neutrophils, thirteen β-defensin genes have been identified [54]. These ~4-kDa peptides contain 38–42 residues and are highly cationic. Half of these β-defensins had blocked NH_2-termini that resulted from cyclization of an amino-terminal glutamine residue.

The disulfide connectivity of β-defensins has been investigated in bovine β-defensin-12 and differs from that of α-defensins.

Three β-defensins (Gal1α, Gal1, and Gal2) were isolated from domestic chicken (*Gallus gallus*) leukocytes [55]. They were found to contain 36–39 amino acid residues including numerous arginines and lysines. Very similar β-defensins have been purified recently from turkey neutrophils [55].

MAMMALIAN EPITHELIAL β-DEFENSINS

Mammalian epithelia are normally free of infection. Instead, a constant, epithelium-specific bacterial and fungal

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MOUSE
mBD-1          GILTSLGRRTDQYKCLQHGGFCLRSSCPSNTKLQGTCKPDKPNCCKS

COW
TAP              GVGNPV---SCVRNKGICVPIRCPGSMKQIGTCVGRAVKCCRKK
LAP              GVRNSQ---SCRRNKGICVPIRCPGSMRQIGTCLGAQVKCCRKK
EBD            GFTQGISNPLSCRLNRGICVPIRCGPNLRQIGTCFTPSVKCCRWR

SHEEP
SBD-1          GFTQGVRNLRSCHRNKGVCVPSRCPRHMRQIGTCRGPPVKCCRKK
SBD-2          GFTHGVTDSLSCRWKKGICVLTRCPGTMRQIGTCFGPPVKCCRLK

HUMAN
hBD-1            GLGHRSDHYNCVSSGGQCLYSACPIFTKIQGTCYRGKAKCCK
hBD-2            GIGDPV---TCLKSGAICHPVFCPRRYKQIGTCGLPGTKCCKKP

ß-DEFENSIN
consensus        ----------C------C----C---------C------CC---
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FIG. 2. Primary amino acid sequence of mammalian epithelial β-defensins. The single letter code for amino acids was used.

flora is present, which only under certain circumstances is out of control.

The question of what keeps bovine tracheal mucosa free of infection led to the discovery of the first mammalian epithelia-derived defensin named TAP [56]. TAP is similar but clearly distinct from cattle phagocytic cell-derived β-defensins in charge, size, and the location of cysteines (Fig. 2) [56]. Molecular cloning revealed the putative TAP precursor to contain 64 amino acids; the mature peptide resides at the extreme carboxyl terminus and is bracketed by a short putative pro-peptide region. Bovine TAP had antibacterial activity *in vitro* against *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* at a minimal inhibitory concentration (12–50 μg/mL), and is also active against *Candida albicans* at 6–12 μg/mL [56].

Northern blot analyses revealed TAP mRNA to be expressed in tracheal mucosa and to a far lesser degree in the lung [56], but failed to detect it in fetal trachea or in tissue other than airway, indicating a rather tissue-specific expression of TAP [57]. *In situ* hybridization of airway sections localized TAP mRNA to columnar epithelial cells of the trachea and bronchi [57].

Analysis of the TAP gene revealed 2 exons [57], similar to the human α-defensin HD5 gene [58]. Similarities between TAP and α-defensins in both structure and activity may suggest a common ancestral gene. Evolutionary relationship, however, remains uncertain because the conserved cysteine motif is different for TAP and the α-defensins, and the highly conserved putative signal sequences are dissimilar for these two classes of pre-pro-peptides [59].

Unlike the α-defensins, TAP is an inducible antimicrobial peptide. Heat-killed *P. aeruginosa* bacteria and *P. aeruginosa* LPS induced in cultured bovine tracheal epithelial cells TAP mRNA expression [60], indicating further that bovine tracheal epithelial cells express CD14, which was also proven in the same study [60]. When analyzing the promoter region of the TAP gene, NF-κB motifs were found at a similar site upstream from the inducible promoter of an antimicrobial peptide gene in *Drosophila* [61], which would have been expected in light of the finding that LPS modulates TAP expression [62].

Upon addressing the questions as to what keeps the tongue of cattle free of infection and why do abrasions to the tongue surface heal rapidly, another β-defensin called LAP was isolated from bovine tongue [63]. LAP shows high homology to bovine TAP (Fig. 2) and is highly active against *E. coli* and *Candida tropicalis*, but less active against *P. aeruginosa*, *S. aureus* and *C. albicans* [63]. Polymerase chain reaction analyses revealed LAP mRNA to be expressed in tongue and lung tissue including bronchi and trachea, colon and rectum, but not in the urogenital tract [63].

cDNA cloning of LAP from a lingual library revealed slight differences in a few clones, likely representing alternative alleles of the same gene [64]. Similar to TAP but in contrast to bovine alveolar macrophage-derived β-defensins [65], LAP is also an inducible β-defensin. Both killed bacteria, and bacterial LPS induced, in a dose-dependent fashion, LAP mRNA expression in cultured tracheal epithelial cells [62]. Interestingly, TNF-α also stimulates LAP mRNA expression in these cells, raising the question as to whether the LAP gene, like the TAP gene, contains NF-κB binding sites, offering attractive hypotheses of possible *cis*-acting sequences, which may be important in inducible gene expression. A recent *in situ* hybridization study revealed that LAP mRNA is widely expressed in numerous epithelia, but was found at higher levels in tissues that are constantly exposed to or colonized by microorganisms [66].

Infection with *Mycobacterium paratuberculosis* led to LAP mRNA expression in ileal mucosa, whereas infection with *Pasteurella haemolytica* induced LAP mRNA in bronchial epithelium. Furthermore, the strongest induction of LAP was seen in the epidermis upon infection of the skin [66].

A recent study revealed that distal small intestine and colon of the cow also express an epithelial β-defensin designated enteric β-defensin (EBD). EBD mRNA was localized to epithelial cells of the colon and small intestine crypts, which was highly up-regulated upon infection with

Cryptosporidium parvum [67]. The inducible expression of antibiotic peptides encoding genes like TAP and LAP following challenge of epithelial cells with bacteria, LPS, or TNF-α supports the hypothesis that antimicrobial peptides contribute to a dynamic host defense system at mammalian organ surfaces.

HUMAN β-DEFENSIN 1

The finding that epithelia of cattle secrete β-defensins stimulated a search for human β-defensins. However, it was not until 1995 that the first human β-defensin, called hBD-1 was discovered [68]. hBD-1 has been isolated as a trace peptide from human hemofiltrate obtained from patients with end stage renal disease [68]. hBD-1 represents a 3.9-kDa basic peptide consisting of 36 amino acid residues (Fig. 2) [68].

Molecular cloning of hBD-1 cDNA supported the amino acid sequence found for the hBD-1 peptide [68]. Antimicrobial activity has not been ascribed to the hBD-1 preparation originally isolated from hemofiltrate. Based on the partial cDNA sequence, the full-length hBD-1 cDNA has been cloned more recently [69], indicating a putative signal sequence that has some homology to that deduced from bovine TAP. Chemical synthesis of hBD-1 by the use of solid phase methodology with regioselective formation of the three disulfide bridges gave material that was active against Gram-negative bacteria at concentrations ranging from 60 to 500 μg/mL [69].

The finding that hBD-1 peptide has been isolated from blood filtrates led to the hypothesis that kidney cells represent a major cellular source. Using RT–PCR techniques, kidney and salivary gland showed mRNA expression when 25 cycles were used [70]. At 30 cycles, prostate, placenta, and trachea became strongly positive, whereas thymus, testis, and small intestine were weakly positive. Peripheral blood leukocytes, spleen, and skeletal muscle were always devoid of hBD-1 message [70]. Interestingly, using northern blot analysis, only the kidney and pancreas showed hBD-1 mRNA expression [70].

hBD-1 has been hypothesized to be an important component that normally keeps the airways free of infection; thus, a defect in its production or action might result in chronic lung infection such as CF. Indeed, in CF, a defect of antimicrobial activity of airway surface fluid has been described [71]. Interestingly, CF bronchial xenografts treated with a recombinant adenovirus expressing CF transmembrane conductance regulator (that represents a chloride channel, which is defective in CF and leads to elevated NaCl concentrations) restored bactericidal activity [69].

It could be shown that hBD-1 bactericidal activity is salt sensitive, giving an IC_{50} of 75 mM NaCl [69]. The most compelling evidence for the role of hBD-1 in CF lung pathogenesis was provided by antisense experiments, where specific ablation of hBD-1 function in four non-CF xenografts abolished bactericidal activity in airway surface fluid [69]. These findings led to the hypothesis that bactericidal activity in human airways to some Gram-negative organisms is primarily constituted through the action of hBD-1 [69]. Indeed, *in situ* hybridization experiments revealed that mRNA encoding hBD-1 is expressed in airway epithelia [69], which was further supported by northern blot analyses [72].

The finding of hBD-1 message in different organ epithelia led to the hypothesis that it may also be expressed in skin. More strikingly, recent *in situ* hybridization experiments revealed hBD-1 transcripts in suprabasal keratinocytes of normal, healthy skin as well as in sweat ducts within the dermis [73]. As yet, however, it is not known whether hBD-1 peptide is secreted, either in lung or skin. Our own attempts to identify bioactive hBD-1 peptide in normal skin, supernatants of cultured skin cells, lesional psoriatic scale extracts as well as supernatants of a cultured lung epithelial cell line have failed thus far (unpublished results).

Sequencing of the hBD-1 gene revealed two exons and one intron spanning 6962 bp. As expected for defensins, the two exons are relatively small; the first exon encodes the signal sequence and propiece peptide, the second exon encodes the mature hBD-1 peptide [74]. Fluorescence *in situ* hybridization revealed the hBD-1 gene to map to chromosomal region 8p23.1–p23.2, which is in close proximity (within 100–150 kb) to the gene for the human neutrophil α-defensin HNP-1 [74], indicating that the α- and β-defensin families appear to have evolved from a premammalian defensin gene.

The mouse homolog of human β-defensin 1 (mBD-1) was cloned very recently. The mBD-1 gene also maps to mouse chromosome 8 at or near the location of the mouse α-defensin genes [75]. RT–PCR data revealed mBD-1 to be expressed mainly in the urogenital tract [76], whereas northern blot analyses revealed mBD-1 mRNA to be expressed exclusively in the kidney [75]. A comparison of its expression pattern supports the idea that it represents the mouse homologue of hBD-1.

hBD-1 has been thought to represent the human homologue to cattle TAP and LAP. No data however, exist to demonstrate that hBD-1 represents an inducible β-defensin. Attempts have failed to see any induction of mRNA expression upon stimulation of cultured lung epithelial cells with bacterial LPS, IL-6, and PMA [70] and heat-inactivated bacteria or TNF-α (unpublished results). Furthermore, the previously reported genomic hBD-1 sequence does not contain transcription factor regulatory elements for NF-κB and AP-1 [74], making it likely that hBD-1 is not the human homologue to bovine TAP/LAP.

HUMAN β-DEFENSIN-2

Independent of these studies, we were interested to know what keeps skin normally healthy and why skin infections, especially those with Gram-negative bacteria, are rather rare. Similar to the approach with frog skin [37], bovine

trachea [56] or tongue [63], we investigated human skin material for the presence of antimicrobial peptides. Lesional scales obtained from patients with psoriasis were chosen because psoriatics have fewer skin infections than expected [77]. To enrich antimicrobial peptides prior to purification, an affinity column, where bacteria were covalently linked with the matrix [78], was used. With this technique, we were able to isolate a novel human antimicrobial peptide having an amino acid sequence similar to that of TAP, LAP, and hBD-1, indicating it to be the second human β-defensin. Thus, it was called hBD-2 [78].

hBD-2 consists of 41 amino acids, being a 4-kDa basic polypeptide (Fig. 2). When tested for antimicrobial activity, it was seen to be highly effective against Gram-negative bacteria like *E. coli* and *P. aeruginosa* (LD_{90}: 10 μg/mL), whereas Gram-positive *S. aureus* was only inhibited in growth at concentrations >100 μg/mL [78]. In addition to bacteria, *C. albicans* was also killed effectively (LD_{90}: 25 μg/mL).

Full-length cDNA cloning of hBD-2 from human keratinocytes revealed a 64-residue precursor having homology to hBD-1 and bovine TAP and LAP. Indeed, comparison of hBD-2 cDNA with other mammalian β-defensins reveals a similarity closest to bovine TAP (62% similarity), but comparably less similarity to hBD-1 (45% similarity), indicating that hBD-2 but not hBD-1 represents the human equivalent of TAP.

This was supported further by experiments that showed hBD-2 to be an inducible gene. Both HaCat-keratinocytes and cultured skin-derived keratinocytes can be stimulated with heat-inactivated *P. aeruginosa* bacteria [78], as has been shown for TAP [60]. Interestingly, heat-inactivated Gram-positive bacteria such as *S. aureus* as well as the eukaryotic yeast *C. albicans* were able to stimulate hBD-2 gene transcription [78], which points towards the existence of mechanisms other than those mediated by CD 14 for hBD-2 gene induction in keratinocytes.

In addition to stimulation by bacteria, we have observed that proinflammatory cytokines such as TNF-α also stimulate hBD-2 transcription in keratinocytes (unpublished results), which again is similar to TAP [60] and different from hBD-1. It may be possible that stimulation of keratinocytes to hBD-2 production by proinflammatory mediators rather than bacteria is the reason for the detection of huge amounts of hBD-2 peptide in lesional psoriasis scales [78], because psoriasis does not represent an infectious disease.

Interestingly, our attempts to isolate hBD-2 from heel stratum corneum or normal epidermis (obtained from juvenile foreskin) by the use of bacteria-affinity-chromatography and antimicrobial testing have failed thus far (unpublished results). Nevertheless, by the use of RT–PCR techniques, we detected constitutive hBD-2 mRNA expression in freshly isolated foreskin, lung, and trachea, but not in kidney, salivary gland, small intestine, and liver [78]. This pattern is nearly identical to that described for organ distribution of TAP mRNA [56, 57].

The presence of hBD-2 transcripts in lung and trachea, as well as the capacity of hBD-2 to effectively kill *P. aeruginosa*, may indicate that hBD-2 is an important chemical shield component of the airway epithelia that usually protects us from Gram-negative infection in the lung. Studies to elucidate whether this theory is true are still underway. Disruption of either antimicrobial peptide action or production, as is postulated in cystic fibrosis [79], may be one reason for recurrent lung infection. It is intriguing to speculate that hBD-2 rather than hBD-1 may play a critical role in cystic fibrosis.

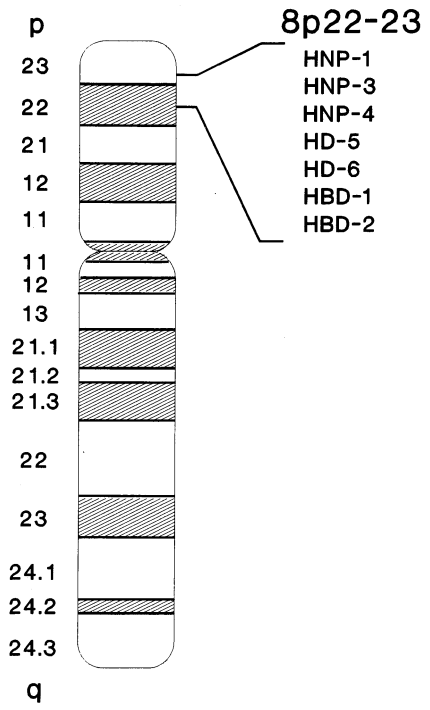

FIG. 3. Human defensin genes map to chromosomal region 8p23.1–p23.2.

By the use of fluorescence *in situ* hybridization as well as PCR with a set of yeast artificial chromosome clones (YACs), we were able to assign the hBD-2 gene to the human chromosome region 8p22–p23.1 [80], which supports the idea that the human chromosome region p22–p23.3 is of major importance for the organization of peptide antibiotics, such as HNP1–4, HD-5, and -6 as well the human β-defensins (74, Fig. 3), participating in innate chemical defense of human epithelia. It is intriguing to speculate that some disorders based on defective innate immunity (unexplained recurrent infections of different organs including skin, lung, gut, and urogenital tract) may be caused by abnormalities of one or more genes encoding defensins or other antimicrobial peptides [80].

OTHER HUMAN EPITHELIAL PEPTIDE ANTIBIOTICS

Apart from defensins and the ubiquitous lysozyme, human epithelial cells also have the capability to synthesize other antimicrobial peptides. ALP, also known as secretory leu-

koprotease inhibitor, is an important protease inhibitor found at mucosa surfaces [81, 82] as well as skin [83]. It is an 11.7-kDa cationic protein composed of two highly homologous domains. In the human lung, ALP is produced in the submucosal glands and in the lining of epithelial cells of the bronchi [84]. In human skin it is produced by keratinocytes [83].

Recombinant ALP displays marked *in vitro* antibacterial activity against *E. coli* and *S. aureus* [82], which, however, is on a molar basis lower than that of lysozyme and HNP-1 [85]. Interestingly, the first but not the second domain of ALP contains an active antibacterial site [86]. ALP also expresses fungicidal activity towards metabolically active *Aspergillus fumigates* conidia and *C. albicans* yeast cells [86].

Another peptide antibiotic belonging to the cathelin family, which is known to be synthesized by granulocytes in bone marrow, and in testis [87], was found by western blot analysis in wound fluid as an immunoreactive band representing both the mature 4-kDa peptide and the cathelin-containing 20-kDa precursor [88]. More recently it was shown that the human genome contains only one CAMP gene, which is up-regulated in inflammatory skin disorders [89]. By *in situ* hybridization and immunohistochemistry, transcripts for CAMP and immunoreactive CAMP were located in keratinocytes of inflammatory regions, but not normal skin [89].

STRUCTURE OF PEPTIDE ANTIBIOTICS

Antimicrobial peptides of plants and animals are typically cationic (i.e. they contain excess numbers of lysine and arginine residues) and amphipathic molecules. Some are α-helical, especially when placed in structure-promoting solvents such as trifluoroethanol or mixed with anionic phospholipid membranes. Others contain β-sheet elements that are stabilized by intramolecular cystine disulfide bridges, sometimes associated with α-helical domains. Furthermore, some antimicrobial peptides are unusually rich in tryptophan, proline, or histidine residues.

α-HELICAL ANTIMICROBIAL PEPTIDES

α-Helical antimicrobial peptides such as cecropins are widely distributed in invertebrates (for review, see Ref. 90). Although they have been most widely studied in Lepidoptera (butterflies and moths) and Diptera (flies), cecropin-like α-helical peptides also exist in other insect orders, in the blood of marine protochordates [91], as well as in porcine intestine [92]. Magainins also belong to the family of α-helical antimicrobial peptides. Their structures and interaction with model membranes and microorganisms have been analyzed extensively [93, 94].

In mammals, leukocytes contain cathelicidins, which represent polypeptides with a conserved N-terminal precursor (termed cathelin) followed by an antimicrobial peptide [95]. Cathelin-associated α-helical peptide antibiotics are found in blood cells of humans, cattle, and rodents. Solution structures of a number of α-helical antimicrobial peptides including magainin [96], buforin [97], protegrin [98, 99], and caerin [100] have been reported.

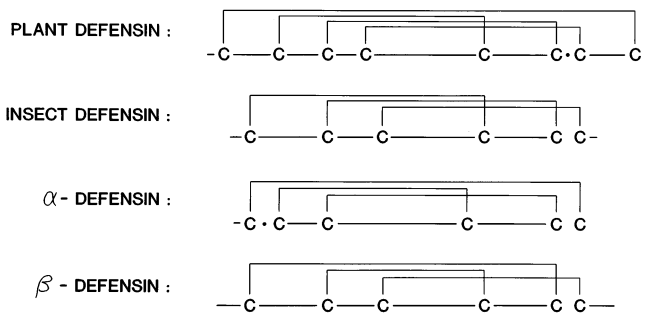

FIG. 4. Characteristic covalent structures of plant defensins, insect defensins, and mammalian α- and β-defensins. Cysteine (C) connectivities are shown schematically as solid lines.

β-SHEET ANTIMICROBIAL PEPTIDES

Unlike amphiphilic α-helical peptides, the defensins represent a rather unusual class of antimicrobials because they are stabilized by three disulfide bonds and have a β hairpin as their principal structural feature. This motif is the defining and unifying feature of all defensins, which are otherwise diverse in terms of evolutionary origin. Several defensin structures have now been solved by x-ray and NMR methods, which allow us to begin to understand how defensins interact with model membranes.

All defensins are cationic. With the exception of human β-defensins, they have arginine as the predominant cationic residues and typical cysteine connectivities. As shown in Fig. 4, these are characteristic for plant defensins, insect defensins, α-defensins, and β-defensins.

Plant defensins possess eight disulfide-linked cysteines and are typified by a triple-stranded antiparallel β-sheet structure with only one α-helix [101]. In the insect defensins, cysteines 5 and 6 have similar positions in the α- and β-defensins except that they are adjacent to one another in the sequence. The solution structures of three α-defensins, rabbit NP-2, rabbit NP-5 [102, 103] and human HNP-1 [1], one insect defensin [104], and three plant defensins [16, 105] have been determined by NMR methods.

The crystal structure for the α-defensin HNP-3 has been analyzed at high resolution [106]. The first β-defensin investigated for its solution structure was bovine neutrophil β-defensin-12 [107]. It was found to be identical to that of the α-defensins. The most obvious common structural feature is a hydrogen-bonded pair of antiparallel β strands connected by a short turn to form a β hairpin comprising the last 15 (or so) residues of the sequence. In the α-defensins, the open end of the hairpin is closed by the C_3–C_5 disulfide bridge [1].

Comparisons of structural measurements revealed rabbit neutrophil defensins to exist in solution as monomers, whereas human α-defensins exist as dimers, possibly stabi-

lized by hydrogen bonds and hydrophobic contacts [1]. A large number of slowly exchanging amide protons are observed in HNP-1, suggesting that HNP-1 forms aggregates of some sort in solution [108].

Amphiphilicity is assumed to be a prerequisite for membrane disruption and pore formation. In the case of HNP-1, charged residues face away from a distinctive hydrophobic surface, when analyzed for solution structure [109], indicating that the monomer has an amphiphilic character. The amphiphilicity of dimers, however, may be of greater importance for the human, dimer-forming defensin. A study of Hill *et al.* [106] described the HNP-3 dimer as being shaped like a basket having a hydrophobic bottom (exposed surfaces of the β hairpins) and a polar top (containing N- and C-termini). As yet, data for the human β-defensins are not available. Migration behavior of hBD-2 upon SDS–PAGE in the absence of urea, however, indicates the presence of oligomeric forms in the absence of urea (unpublished results).

MODE OF ACTION OF ANTIMICROBIAL PEPTIDES IN THE KILLING OF MICROORGANISMS

The mechanisms of action of cationic peptide antibiotics, including the well-studied peptides melittin, magainin, gramicidin, cecropin, and defensins, have been studied in much detail [46, 110]. Several approaches have been tried for its investigations. The effects on known functions like membrane permeability and/or protein and DNA synthesis in a target organism, and the influence of changes of the structure of antimicrobial peptides by synthesis of analogs with amino acid substitutions, including making D-enantiomers or retro-peptides, have been studied. Furthermore, biophysical measurements that can detect channel formation and/or membrane potential changes as well as influence on liposomes or mitochondria (as a model system for bacteria) were used. One (or more) of these approaches has been tried for several of the antimicrobial peptides described above. The main difficulty has been to relate the different findings to the terminal killing of the microorganisms.

Linear α-helical peptides, such as cecropins and magainins, lyse bacteria, sometimes very quickly, making it difficult to assess whether other steps precede cell lysis. Membranes were recognized early on as targets of many antimicrobial peptides. This was proven by experiments with artificial membranes where voltage-dependent channel formation was seen with defensins, magainins, cecropins, and insect defensins [111–114]. Liposome lysis could be demonstrated with cecropins [115, 116], magainin [117], α-defensin [118], and an insect defensin [114].

Whether the formation of channels and the lysis of liposomes mimic early steps of lysis of live bacteria is not yet clear. It is interesting to note that liposomes are lysed by intact defensin as well as linearized (without cysteine bridges) defensin; however, the latter was incapable of killing bacteria [118]. When liposomes contain cholesterol, the lytic activity of a cecropin is reduced dramatically [115]. This might explain why antimicrobial peptides act only on bacteria and not on higher eukaryotic cells, which contain cholesterol in their cytoplasm membrane. In another study, the effects of α-defensins on the outer and inner membrane of *E. coli* were investigated, and it was found that defensins affected the permeability of both membranes [119].

When a number of α-helical antimicrobial peptides were made only from D-amino acids, they exhibited the same antimicrobial activity as the natural L-enantiomers did [120], indicating that these molecules cannot have stereospecific targets (i.e. protein receptors). In contrast, D forms of the proline-rich bee-derived apidaecins were completely inactive in the killing of bacteria, indicating that this peptide antibiotic has a protein target [121]. A few antimicrobial peptides kill bacteria without any signs of lysis. PR-39, a proline- and arginine-rich peptide antibiotic, stopped DNA synthesis, and the number of viable bacteria dropped more than three orders of magnitude [122]. Westerhoff *et al.* [123] have studied the effects of magainin on mitochondria and concluded that uncoupling of respiration and the dissipation of the membrane potential may explain the lethal effects of magainin on bacteria.

Three possible mechanisms for permeabilization of bacterial membranes by defensins have been discussed: first, a detergent-like action by monomeric dimers; second, a dimer of dimers with a solvent channel between them; or, third, an annulus of defensin dimers that forms a large pore. Using unilamellar vesicles formed from the negatively charged lipid palmitoleoyloleoylphosphatidylglycerol, native HNP-2 formed pores with a diameter of ~25 Å, suggesting a hexamer of dimers [124]. Native defensin causes all-or-no leakage in which some of the vesicles released all of their contents (fluorescence dye), while others released none [125]. A putative mechanism of pore formation is indicated in Fig. 5.

ANTIMICROBIAL PEPTIDES AS THERAPEUTIC AGENTS

The majority of antimicrobial peptides are of epithelial cell origin. Thus, it has been assumed that they may be limited to treatment of topical infections. Indeed, the first clinical trials have been directed towards topical infections [126].

A magainin variant peptide termed MSI-78 was taken into phase-III clinical trials with 926 patients treated for efficacy against polymicrobic diabetic ulcers [127]. As recently announced (http://www.pslgroup.com/dg/2168e.htm), these trials demonstrated effects similar to those of orally administered ofloxacin, but with fewer side-effects. Cecropin-melittin hybrid peptides have been shown to have topical activity against *P. aeruginosa* eye infections of rabbits [128].

There is a reasonable amount of published evidence that systemic infections are treated efficiently with peptide antibiotics such as β-sheet-protegrin, which is active

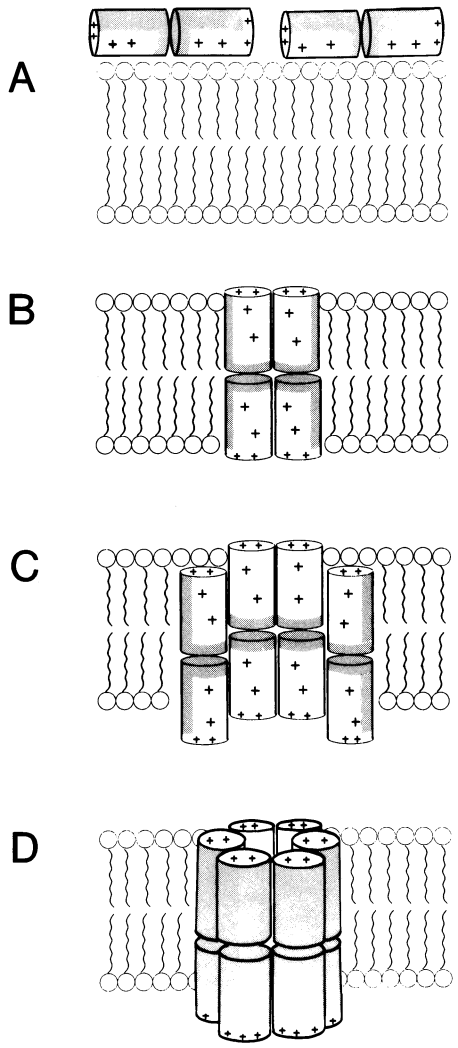

FIG. 5. Model of pore formation by defensin dimers. Amphiphilic defensin dimers (hydrophobic areas are shaded, cationic areas are indicated by ++) first bind to the anionic groups located outside of the bacterial membrane (A), insert into the membrane to form aggregates (B and C), and finally produce an assembly of six (or more) dimers to form a pore (D), having been estimated to be ~25 Å in diameter [124]. This model is based on data published for HNP-2 [124], adapted from reference [44].

against methicillin-resistant *S. aureus*, vancomycin-resistant *Enterococcus faecalis* and *P. aeruginosa* infections [129] and extended-helix indolicidin against *Aspergillus* fungal infections [130]. These findings indicate that antimicrobial peptides may be able to be utilized as injectable antibiotics against microorganisms that are resistant to conventional antibiotics.

PRODUCTION OF ANTIMICROBIAL PEPTIDES

Solid phase chemical synthesis is useful for many linear, α-helical peptide antibiotics that will be used for experimental purposes. This method may give big problems for peptides that contain a high number of cysteines, such as defensins. Indeed, chemically synthesized α- and β-defensins often are far less active than those obtained from natural sources (unpublished results), possibly because of wrong connections of cysteines resulting in wrong folding.

An alternative strategy is recombinant synthesis. Expressing antimicrobial peptides in bacteria or yeasts, however, may create a suicide situation that must be avoided, i.e. by using a vector with a construct for a fusion protein, as has been done with human tumor necrosis factor and cecropin A [131] (see Ref. 132 for a review).

The baculovirus system has also been tested to express a fusion protein with cecropin A in an insect cell line, which, however, gave low yields [133, 134]. Other recombinant procedures that have been used include the production of antimicrobial peptides in a fungal expression system [135] and in tobacco using tobacco-mosaic-virus vectors (Geneware™ technology of Biosource Technologies), as well as in the milk of transgenic mice [136]. Due to the commercial potential of these procedures, few details are available.

In other cases, transgenic approaches also were used to improve the resistance of various species. For example, transgenic symbiotic bacteria have been engineered to produce cecropin A to kill *Trypanosoma cruzi* in the hindgut of the reduviid bug, the vector that carries the Chagas' disease agent [137].

OUTLOOK

Studying the constituents and the mechanism of this ancient epithelial chemical defense system has improved our understanding of how higher organisms can survive in a hostile environment laden with pathogenic microorganisms. New perspectives in the conception of new therapeutic treatments of infections have been opened. Many of the antibiotics available today are made as by-products of fungi. Unfortunately, resistant strains have evolved, as they will almost inevitably do given sufficient time of exposure. A prominent solution to this resistance problem may reside in new antibiotics as different as possible from those that have lost their effectiveness. Because peptide antibiotics have completely different mechanisms of action than traditional antibiotics, these natural antimicrobial peptides, which extend the chemical defense shield that plants and animals have been using for millions of years, may just fulfill that requirement and may be more durable remedies. With the recent knowledge that some epithelial peptide antibiotics are inducible, it is intriguing to speculate that therapeutics also could be developed that specifically induce epithelial production of peptide antibiotics via elicitor receptors and, thus, would help to protect the organism from infection via elevation of its epithelial chemical shield.

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