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Review

Epithelial antimicrobial peptides: innate local host response elements

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Abstract. Multicellular organisms have to survive in an environment laden with numerous microorganisms, which represent a potential hazard to life. Different strategies have been developed to ward off infections by preventing microorganisms from entering surfaces and by preventing the attack of microorganisms that have already entered the epithelia. Therefore, it is not surprising that epithelia are equipped with various antimicrobial substances that act rapidly to kill a broad range of microorganisms. This review summarizes our present knowledge about epithelial peptide antibiotics produced in plants, invertebrates, and vertebrates including humans. There is now strong evidence that in addition to constitutively secreted peptide antibiotics, others are induced upon contact with microorganisms or by proinflammatory cytokines. β-Defensins represent one family of vertebrate antimicrobial peptides, members of which are inducible and have recently been identified in humans. The defensin-characteristic local expression pattern may indicate that specialized surfaces express a characteristic surface antimicrobial peptide pattern that might define the characteristic microflora as well as the density of microorganisms present on the surface.

Key words. Antimicrobial peptides; cystic fibrosis; defensin; epithelia; innate immunity; peptide antibiotics.

Introduction

Macroorganisms including plants, invertebrates, and vertebrates have to survive in an environment laden with numerous microorganisms, which represent potential hazards for multicellular organisms. All macroorganisms have developed strategies to prevent infections. They achieve this by preventing the entrance of microorganisms into epithelia, the surface in contact with the environment laden with microorganisms, and through defense mechanisms directed against microorganisms that have already entered the epithelia.

Because activation and deployment of pathogen-specific immune responses occurs slowly relative to the potential kinetics of microbial proliferation and are restricted to higher eukaryotes which contain immune cells capable of recognizing antigens and responding with effector cells, it is not surprising that surfaces are equipped with various antimicrobial substances. These substances, mainly peptides, act rapidly to kill a broad range of potentially pathogenic microbes. These epithelia-derived molecules can restrain microbes by depriving them of essential nutrients, such as iron, or may decimate them by causing structural disruption or metabolic injury. Host-derived antimicrobial substances produced by eukaryote epithelial cells range in complexity from a few relatively simple inorganic molecules such as nitric oxide to a large number of antimicrobial peptides and proteins.

In contrast to simple inorganic molecules as well as classical antibiotics, all of which are made by microorganisms, antimicrobial peptides are gene encoded and

made from an RNA template read by ribosomes. Higher organisms, therefore, might be flexible in adjusting the structure of epithelia-derived peptide antibiotics to changes in the peptide-antibiotic sensitivity of the microflora on their surfaces. Thus, this epithelial chemical barrier system represents an important part of the innate immune system preventing primary infection rather than by preventing reinfection, which is achieved by our immune system with elements allowing recognition of specificity and memory. The absence of a functionally important immune system in lower vertebrates, invertebrates, and plants indicates that survival in an environment laden with microorganisms is possible without an adaptive immune system.

Plants as a source of antimicrobial peptides

Plants share with animals several components of the innate immune system. These include the capacity to produce—after contact with microorganisms—various antimicrobial components such as hydrogen peroxide, proteases, antimicrobial peptides and proteins, in addition to producing the majority of these molecules constitutively in epithelial cells [for a detailed review see ref. 1].

As this review focuses on antimicrobial peptides as defense components, it will briefly highlight the role of such peptides in the defense system of plants. Many peptide antibiotics have been identified. Their discovery was made by the observation of in vitro antimicrobial activity; however, this alone is an insufficient argument to claim their possible involvement in plant host defense. Full proof for a functional role in defense must rely on multiple lines of evidence and ultimately include the demonstration that plant mutants with a reduced content or inducibility of antimicrobial peptides show, in contrast to wild-type plants, enhanced susceptibility toward microbial attacks. As yet, however, no direct proof has been provided.

A number of antimicrobial peptide families have been discovered in plants. One group, termed thionins, represents peptides that mostly range from 45 to 47 amino acids in length. Thionins contain a high number of cysteine residues with eight or six cysteines at the same relative position. These peptides are inhibitory to a number of plant pathogenic bacteria, thus suggesting that thionins may fulfill a protective role in plants. Analyses of the antimicrobial activity spectrum of thionins revealed activity against various Gram-positive and Gram-negative plant pathogenic bacteria as well as about 20 different phytopathogenic fungi with IC_{50} values (concentration required for 50% growth inhibition) ranging from 0.3 to 10 μM [2, 3]. Interestingly, some Gram-negative bacteria such as *Pseudomonas* species are apparently insensitive to thionins. Furthermore, some of these antimicrobial peptides are inhibited by the presence of Ca^{2+} but not Mg^{2+} or monovalent ions [4–6].

In addition to their effects on microorganisms, thionins have also been shown to exert adverse effects on various cultured mammalian and insect cells. They are toxic to insects and mammals when injected but not when applied topically [7]. Thionins seem to be present in most, if not all, plants and are expressed in tissues such as seed endosperm, leaf epidermal cells, and root epithelia. Thionins expressed in these different organs are encoded by different genes displaying organ-specific expression.

Some of these genes are strongly expressed, predominantly in flowers, siliques, and leaves, whilst others are expressed at only a low level, as shown in seedlings, where they are strongly induced upon infection with e.g., *Fusarium* fungi [8]. It is interesting to note that expression of thionin genes is induced by methyljasmonate, an endogenous signal transducer lipid formed from α-linolenic acid, and they have structural similarities to prostaglandins [9].

Accumulation of thionin transcripts occurs in differentiated epithelial cells of plants as seen by in situ hybridization. By immunocytochemistry, thionin peptides are seen to be most abundant in epidermal cells, which is consistent with their role as molecules involved in the first line of defense. Over 95% of thionins are present in vacuolar compartments, whereas only a minor fraction is present in cell walls.

Most plant pathogens grow on the plant surface at least during some stages of infection. During these stages they are unlikely to be affected by thionins. In many plant-pathogen interactions, however, plant epithelial cells and their vacuoles (where thionins are stored) are disrupted, as a result of which antimicrobial compounds can reach the microorganisms and kill them [1].

A second family of plant antimicrobial peptides have been termed plant defensins. Although these compounds resemble thionins in both size (45–54 amino acids) and number of disulfide-linked cysteines, they are structurally unrelated [10]. Similar to thionins, comparison of plant defensins from many different species demonstrates very few conserved residues, conservation being restricted to the cysteines and only a few other residues. Hence, the majority of non-structural residues are variable, sustaining divergence in biological activities.

In general, plant defensins possess strong antifungal activity (table 1). Different groups of defensins cause morphological distortion and reduced hyphal elongation with marked differences in activity [1, 11]. It is not known at present exactly how plant defensins inhibit fungal growth. Interestingly, some plant defensins do

not cause substantial permeabilization of *Neurospora* hyphae and have no effects on electric currents measured in artificial membrane systems [12].

Plant defensins are less active against bacteria and, in contrast to thionins, have not been found to cause detrimental effects on cultured human or plant cells [5]. Similar to thionins, it has been noted that a single plant species can contain different plant defensin genes, all of which have a distinct, organ-specific expression pattern [13]. Immunostaining and in situ hybridization techniques have revealed that defensins preferentially accumulate in peripheral cell layers, i.e., in seeds, in the outer cell wall lining the epidermis of cotyledons, hypocotyls and endosperm, or in the epidermis of potato tubers and leaf primordia [14]. Again, this preferential location in epithelial layers is consistent with a role in the protection of the organ against microbial challenge [1]. Plant defensins are induced after fungal infection of a vegetative tissue [15] in a fashion similar to that demonstrated by thionins.

Surprisingly, such an infection sometimes triggers the appearance of plant defensins only in cells surrounding the infection zone. In other plants, contact with pathogenic fungi triggers a systemic effect, i.e., it can be detected not only in infected leaves, but also in unaffected leaves. Interestingly, induction of some plant defensins can be also achieved by the external application of methyljasomate [15] (which is a volatile substance), which might explain the systemic effect after infection of plants with fungi, where this compound is liberated.

Apart from these two major classes of antimicrobial compounds, plants contain a few additional plant antimicrobial peptide families including lipid transfer proteins, hevein-like antimicrobial peptides, knottin-like antimicrobial peptides, four cysteine-type peptides, as well as enzyme-inhibiting peptides [for a detailed review see ref. 1].

Invertebrate antimicrobial peptides

In contrast to plants, invertebrates (in particular insects) contain (primitive) blood cells with the capacity to phagocytose invading microorganisms. Although initially believed to be the most important defense strategy in these organisms, there were hints that this mechanism could not alone explain the particular resistance of insects to bacterial infection.

This hypothesis was supported by the fact that bacteriolytic substances were induced in insects when bacteria were inoculated. Surprisingly, these pioneering studies were followed by a long period of stagnation and it was not until 1980 that any of these induced antibacterial peptides were characterized by H. Boman and associates, who identified and characterized peptide antibiotics from the silkworm *Hyalophora cecropia*. Hence, the novel peptide antibiotic was termed cecropin [16]. Thereafter, numerous small-sized, mostly cationic antimicrobial peptides were identified that included members of structurally different families such as cecropins, cationic proline-rich peptides termed apidaecins, cationic glycine-rich peptides termed attacins, sarcotoxins II and diptericins, all of which are devoid of cysteines [17–19].

In contrast to these families, insect defensins are the most widespread group of inducible insect peptide antibiotics [20]. They have six cysteines engaged in three intramolecular disulfide bridges mainly produced by

Table 1. Antimicrobial peptides from plants and invertebrates.

Antimicrobial peptide	Species	Active against		
		Gram+ bacteria	Gram− bacteria	Fungi
Thionins	plants	++	++	++
Plant defensins	plants	(+)	(+)	+++
Knottin-type peptides	plants	++	(+)	++
Hevein-type peptides	plants	+	+	+
Cecropins	insects	(+)	+++	(+)
Drosocin	insects	(+)	+++	−
Metchnikowin	insects	+++	+++	+++
Insect defensins	insects	+++	(+)	−
Drosomycin	insects	−	−	+++
Clavanins	tunicates	++	++	++
Styelins	tunicates	+++	+++	(+)
Tachyplesin	shrimps	++	++	?
Penaeidins	shrimps	++	(+)	++
Lycotoxins	wolf spider	?	++	++

epithelial cells of the fat body and the trachea as well as by some blood cells. Insect defensins are predominantly active against Gram-positive cells with only a few Gram-negative bacteria being affected by these compounds [20]. Interestingly, it has not been possible so far to detect any activity against fungi or enveloped viruses. Synthesis of insect defensins is elicited by a variety of stimuli including Gram-positive and Gram-negative bacteria. These peptides are released as systemic responses by fat body cells (the cells in insects having a similar function to liver cells in vertebrates) or local responses by epithelial cells. Although only some molecular data are available on the mechanism by which bacteria are recognized by insects, it is believed that non-clonal receptors detect common constituents of microorganisms, such as lipopolysaccharides (LPSs), mannan, glycans, and double-stranded RNA [20]. As argued by Janeway [21], such pattern-recognition receptors are the prime recognition structures that have been selected over evolutionary time to provide broad-spectrum recognition of harmful microorganisms.

Drosomycin is a 44-residue peptide containing eight cysteine residues. It consists of a central α-helix, which is linked to an antiparallel β-sheet via two disulfide bridges (as seen in insect defensins); however, it has an extended N-terminal sequence [22]. Its structure is reminiscent of that of plant defensins. Similar to these antimicrobial peptides, drosomycin is predominantly active against filamentous fungi by inhibiting spore germination or delaying growth of hyphae [22]. Synthesis of drosomycin by fat body cells is induced in *Drosophila*, e.g., by application of fungi, via the dorsoventral regulatory gene cassette Spätzle/Toll/Cactus, which is structurally related to the mammal interleukin (IL)-1/I-κB/NF-κB. Interestingly, it has recently been shown [23] that insect epithelia also produce drosomycin using, however, a Toll-independent pathway.

The serendipitous discovery of a recessive mutant, imd (for immunodeficiency) [23], indicates the existence of a second regulatory pathway: in homozygous imd mutant flies, expression of antibacterial peptides is dramatically affected, whereas the drosomycin gene remains inducible. Analyses of *Drosophila* mutants revealed that in mutants deficient for any of the Toll regulatory gene cassette members, the induction of the drosomycin gene is dramatically affected, whereas expression of antibacterial peptides varies from gene to gene: some are reduced, others are not affected.

In this context, it seems evident that in insects, antimicrobial responses show some degree of specificity: in *Drosophila*, there is discrimination between various classes of microorganisms because genes encoding antibacterial or antifungal peptides are differentially expressed after injection of distinct microorganisms [24]. More strikingly, infection of *Drosophila* with fungi elicits an adapted response, with the production mainly of peptides with antifungal properties [25].

Apart from insects, other invertebrates such as the marine tunicates (sea squirts), which belong to the phylum Chordata, produce antimicrobial peptides, first discovered in their hemocytes. *Styela clava* forms clavanins [26], which are histidine-rich, C-terminally aminated, α-helical peptides that contain 23 amino acids, including (in some) a methylated tyrosine residue. Clavanins are active against Gram-positive and Gram-negative bacteria as well as *Candida albicans*. The most interesting aspect of these antimicrobial peptides, however, is their higher activity at pH 5.5 than at pH 7.4.

Other more recently isolated antimicrobial peptides from *S. cava* have been termed styelins [27]. These antimicrobial peptides have molecular masses near 3700 Da, are active against a panel of human Gram-negative and Gram-positive bacterial pathogens and are usually active with minimal inhibitory concentrations < 1 μM. The presence of such molecules in tunicates is evidence that they are ancient mediators of host defense within the vertebrate lineage. Living in an aquatic environment rich in microorganisms means that crustaceans have developed effective mechanisms for killing bacteria. They produce antimicrobial peptides such as tachyplesin or big defensin which are synthesized by bivalve molluscs [28]. More recently, penaeidins were isolated from shrimps (*Penaeus vannamei*); they are a group of cysteine- and proline-rich 50-to-62-amino-acid peptides, which have no homology to hitherto described antimicrobial peptides [29]. A summary of invertebrate antimicrobial peptides is given in table 1.

Epithelial peptide antibiotics from vertebrates

Among vertebrates, frogs represent the first species investigated for the presence of epithelial peptide antibiotics. M. Zasloff [30] wondered why freshly operated African clawed frogs (*Xenopus laevis*), which are often used for the isolation of oocytes, do not show any problems with infections when kept in the laboratory pond and speculated that the frogs' skin produced antibiotics. He identified two closely related, linear peptides which he termed magainins. These molecules are identical to the peptidyl-glycine-serine (PGS) peptides that were discovered independently [31]. Magainins/PGS are non-hemolytic, amphiphilic peptides containing about 23 residues, which inhibit growth of numerous species of bacteria and fungi and induce osmotic lysis of protozoa at low concentration [32]. A number of other, partially structurally related peptides were discovered in different frog species at about this time [for a review see ref. 33].

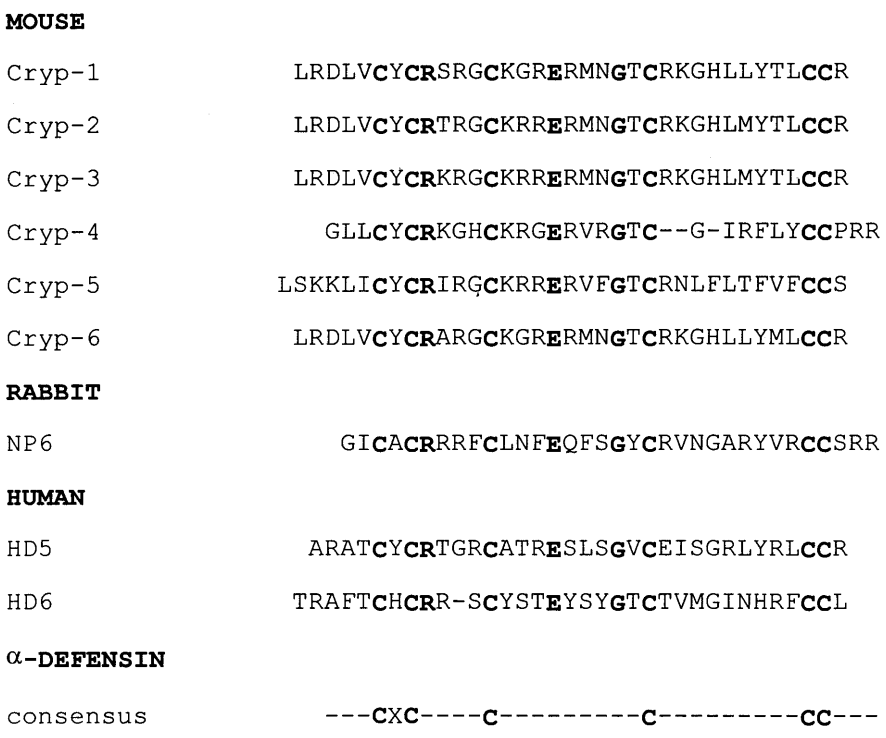

Figure 1. Alignment of the amino acid sequences of six mice, one rabbit and two human members of the α-defensin family produced by epithelial cells. Amino acid sequences were translated from nucleotide sequences. The single-letter code is used. Amino acids conserved in all epithelial α-defensins are presented in bold.

Epithelial antimicrobial peptides from higher vertebrates

In contrast to invertebrates and lower vertebrates, higher vertebrates have a well-developed immune system with lymphoid tissues allowing cell-mediated long-lasting immunity. Microorganisms that have entered the body are controlled by blood-borne professional phagocytes, mainly polymorphonuclear leukocytes and monocytes. These phagocytes are capable of killing ingested microorganisms through both oxygen-dependent and oxygen-independent mechanisms. Leukocytes are a rich source of antimicrobial peptides, including defensins [for recent reviews see refs 34–36]. The term 'defensin' was devised about a decade ago to describe a family of antimicrobial peptides found in granules of rabbit and human neutrophils.

Two structurally related families have since been identified: members of one family, termed 'α-defensins' consist of about 35 arginine-rich amino acid residues generating highly cationic peptides. Whereas human neutrophils produce four α-defensins (HNP-1, -2, -3, and -4) and rabbit macrophages and neutrophils synthesize five (Rab-MCP-1, Rab-MCP-2, Rab-NP3a, Rab-NP4 and Rab-NP5), epithelial cells at the base of human small intestinal crypts produce two (HD5 and HD6). Figure 1 shows the primary amino acid sequences of a number of mammalian epithelial α-defensins, which are cysteine rich and have, in addition, a single conserved glycine, glutamic acid, and arginine. These epithelial α-defensins differ in their antimicrobial spectrum and relative potency, the latter seeming to parallel their net positive charge.

Epithelial α-defensins of mice ('cryptdins') and humans are predominantly expressed in Paneth cells of the small intestine [37]. Paneth cells are epithelial granulocytes located at the base of the crypts of Lieberkühn, hence the name cryptdins for mouse enteric α-defensins. Curiously, despite having the largest known repertoire of defensin-coding sequences, mice express defensin genes only in Paneth cells [38] (mouse neutrophils lack defensins at levels found in other species [39]). Up to 17 murine defensin mRNAs with distinct coding sequences are produced in a single intestinal crypt [40].

Interestingly, analyses of cryptdin gene expression in adult mouse small bowel revealed that the cryptdin-4 isoform is differentially expressed along the proximal to distal intestinal axis: it was found to be absent from the proximal small bowel, increasing to maximal levels in the ileum. In contrast, cryptdin-1 and -5 mRNA levels were found to be equivalent in duodenum, jejunum, and

ileum. Similarly, individual crypts of duodenum contained mRNA for cryptdin-1 but not for cryptdin-4. These findings indicate the positional specificity of defensin gene expression and thus the heterogeneity of Paneth cells [41]. Studies on cryptdin gene expression in developing mouse small intestine revealed cryptdin-6 mRNA as the most abundant cryptdin species in 1-day-old mice before crypt formation and maturation of the epithelium [42]. This indicates that cryptdin-6 secretion may contribute to the innate immunity of the neonatal intestine before the presence of distinguishable Paneth cells. It is interesting to note that enteric α-defensins are secreted into the lumen, in a fashion that is known for lysozyme [43].

Enteric defensins can be distinguished from phagocytic defensins, being actively secreted and not primarily targeted for intracellular delivery to phagolysosomes. Recent investigations [44] revealed an additional biological function for enteric defensins apart from antimicrobial activity. When administered apically, mouse cryptdin-2 and -3, but not the others, can reversibly stimulate intestinal epithelial cells to secrete chloride ions, suggesting that Paneth cells may also be multifunctional. Cryptdins might also be important in testis. A recent report indicates that Sertoli cells (testis-derived epithelial granulocytes) release antimicrobial peptides which belong to the cryptdin-1, -2, -3 and -6 group, with differential localization in Sertoli cells at stages corresponding to the maturation of spermatids and in cells of the interstitial space [45].

Using an anchored polymerase chain reaction (PCR) strategy on human intestine RNA samples, only two α-defensin mRNA species, termed HD-5 and HD-6, a number far below that seen in mice, were found. By in situ hybridization, HD-5 and HD-6 mRNA was also localized to human Paneth cells [46, 47]. Using polyclonal antibodies raised against natural HD-5, Paneth cells of the normal human adult small intestine were selectively stained [48]. Immunogold electron microscopy further localized HD-5 to the Paneth cell secretory granules [48]. A recent review summarizes the role of α-defensins in the crypt microenvironment [49].

Naturally occurring HD-5 and HD-6 have been recently isolated from neobladder urine. The identification of multiple N-terminally processed forms of HD-5 in the urine suggests that Paneth cells store prodefensin-5 that is processed to the mature defensin during or after degranulation, in contrast to HD-6, which is found only as the 69-to-100-residue form [50].

Analyses of the antimicrobial activity of recombinant HD-5 (rHD-5) revealed concentration-dependent microbicidal activity against *Escherichia coli*, *Listeria monocytogenes*, *Salmonella typhimorium* (including its phoP mutant), as well as *C. albicans*, with a minimal inhibitory concentration (MIC) in the nanomolar range [51]. Interestingly antimicrobial activity of rHD-5 withstood treatment with trypsin. Like HNP-1, HNP-2 and HNP-3, however, antibacterial activity was found to be inhibited in the presence of salt [51]. It is tempting therefore to speculate that enteric epithelia-derived α-defensins are important host defense elements protecting the intestinal epithelial surface against microbial invasion and parasitization. Very recent investigations indicate that HD-5 represents an important peptide antibiotic in the human female reproductive tract. Using immunohistochemistry and confocal laser microscopy, immunoreactive HD-5 was found to be localized in the upper half of the stratified squamous epithelium of the vagina and ectocervix, with the intensity of cellular staining increasing toward the lumen. In positive endocervix, endometrium, and fallopian tube specimens, immunoreactive HD-5 was located in apically oriented granules and on the apical surface of a proportion of columnar epithelial cells [52]. Western blot analyses revealed HD-5 to be present in cervicovaginal lavages, with highest concentrations found during the secretory phase of the menstrual cycle [52].

Vertebrate epithelial β-defensins

Apart from α-defensins, mammals produce a second family of defensins, which due to their structural similarity to α-defensins are termed the β-defensin family. β-Defensins, which have also been found in birds, occur as $\sim$4-kDa peptides that contain 38–42 amino acids and are highly cationic [53].

Unlike rodents and humans, neutrophils of cattle and birds contain β-defensins instead of α-defensins. In bovine neutrophils, 13 β-defensin genes have been identified [54], whereas from bird (domestic chicken) neutrophils, 3 β-defensins were isolated [55]. Thus, in these animals, α-defensins are apparently substituted by β-defensins in their phagocytes.

Phagocyte-derived antimicrobial peptides, however, do not explain why mammalian skin epithelia, gastrointestinal and oral mucosa are normally free of infection, despite hosting a constant epithelium-specific bacterial and fungal flora. It was investigations asking whether bovine tracheal mucosa produces antimicrobial peptides that led to the discovery of the first mammalian epithelia-derived β-defensin, which was termed tracheal antimicrobial peptide, TAP [56].

TAP is similar to but clearly distinct from bovine phagocyte cell-derived β-defensins in size, charge, and location of conserved amino acids (fig. 2). Unlike α-defensins, molecular cloning revealed the TAP precursor to contain 64 amino acids with a mature peptide that is bracketed by a short putative propeptide region [56]. Bovine TAP

was found to have antibacterial activity in vitro against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *E. coli* at MICs of 12–50 μg/ml and is also active against *C. albicans* at 6–12 μg/ml. Unlike the α-defensins, TAP is an inducible peptide antibiotic [57]. Heat-killed *P. aeruginosa* bacteria and *P. aeruginosa* LPS induced TAP mRNA expression in cultured bovine tracheal epithelial cells. Analyses of the promoter of the TAP gene [57] revealed NF-κB motifs that were found at a similar site upstream from the inducible promoter of an antimicrobial peptide gene in *Drosophila* [58], which would have been expected in light of the finding that LPS induces TAP gene expression [59].

Addressing the question why does abrasion of the tongue surface heal rapidly and why do cattle tongues usually remain free of infection, a second bovine epithelial β-defensin named lingual antimicrobial peptide, LAP, was isolated from bovine tongue [60]. LAP, which shows a very high similarity to TAP (fig. 2), is highly active against *E. coli* and *C. tropicalis*, but less active against *P. aeruginosa*, *S. aureus* and *C. albicans*. LAP mRNA is highly expressed in tongue and lung tissue including bronchi and trachea, colon and rectum, but not in the urogenital tract [60].

Similar to TAP, LAP is also an inducible β-defensin; again, bacteria as well as bacterial LPS induced LAP

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RAT
ra BD-1          TSLGRRTDQYRCLQNGGFCLRSSCPSHTKLQGTCKPDKPNCCRS
ra BD-2             SINNPITCLTKGGVCWGP-CTGGFRQIGTCGLPRVRCCKKK
MOUSE
mBD-1              GRRTDQYKCLQHGGFCLRSSCPSNTKLQGTCKPDKPNCCKS
mDefb2             GYEAELDHCHTNGGYCVRAICPPSARRPGSCFPEKNPCCKYMK
COW
TAP               GVGNPV---SCVRNKGICVPIRCPGSMKQIGTCVGRAVKCCRKK
LAP               GVRNSQ---SCRRNKGICVPIRCPGSMRQIGTCLGAQVKCCRKK
EBD               GISNPL---SCRLNRGICVPIRCGPNLRQIGTCFTPSVKCCRWR
PIG
PBD-1         PVPGLLKNIGNSVSCLRNKGVCMPGKCAPKMKQIGTCGMPQVKCCKRK
SHEEP
SBD-1             GVRNLR---SCHRNKGVCVPSRCPRHMRQIGTCRGPPVKCCRKK
SBD-2             GVTDSL---SCRWKKGICVLTRCPGTMRQIGTCFGPPVKCCRLK
GOAT
GBD-2             GIINHR---SCYRNKGVCAPARCPRNMRQIGTCHGPPVKCCPKK
MACAQUE
maBD-1            GLGHRSDHYNCVRSGGQCLYSACPIYTRIQGTCYHGKAKCCK
HUMAN
HBD-1             GLGHRSDHYNCVSSGGQCLYSACPIFTKIQGTCYRGKAKCCK
HBD-2             GIGDPV---TCLKSGAICHPVFCPRRYKQIGTCGLPGTKCCKKP
ß-DEFENSIN
consensus         ----------C------C----C---------C------CC---
```

Figure 2. Alignment of the amino acid sequences of 15 mammalian epithelial β-defensins. Amino acid sequences were translated from nucleotide sequences. The single-letter code is used. Amino acids conserved in all epithelial β-defensins are presented in bold. Note the presence of a three-residue NH_2-terminal gap in TAP, LAP, SBD-1, SBD-2, GBD-2, and HBD-2.

mRNA in cultured tracheal epithelial cells. TNF-α also induced LAP mRNA expression in these cells, indicating that the LAP gene possibly also contains NF-κB binding sites [61].

In situ hybridization studies revealed that LAP mRNA is widely expressed in numerous epithelia but was found at higher levels in tissues that are constantly exposed or colonized by microorganisms. Colonization with *Pasteurella haemolytica*-exposed bronchial epithelium and *Mycobacterium paratuberculosis*-exposed ileal mucosa elicited high mRNA levels; however, the strongest induction of LAP was seen in the epidermis upon skin infection [62].

Another gene for an epithelial β-defensin, designated enteric β-defensin (EBD) has been recently identified in distal small intestine and colon of the cow [64]. mRNA for EBD was found in high amounts in epithelial cells of the colon and small intestine crypts, in low amounts in the trachea, but not in the lung. Infection with *Cryptosporidium parvum* highly upregulated EBD mRNA in colon and small intestine [63].

Two epithelial β-defensins have been recently cloned from sheep (*Ovis aries*). mRNA for these β-defensins termed sheep beta-defensin 1 and 2 (SBD-1 and SBD-2) was found to be expressed in tongue, trachea, ileum, and colon [64]. Thus it is difficult to say which equivalent of the bovine epithelial β-defensins it may represent.

In mice, two epithelial β-defensins (mBD-1, murine β-defensin-1 and murine β-defensin-2, mDefb2) have been recently identified. mBD-1 was cloned by three independent groups [65–67] and its mRNA was found to be expressed in the urogenital tract and kidney when analyzed by Northern blotting. Sensitive RT-PCR techniques on lung, heart, spleen, and uterus tissues revealed mBD-1 gene expression [65].

In contrast to mBD-1, mDefb2 does not appear to be expressed in the airways of untreated mice but it is upregulated in response to LPS [68]. Databank screening reveals that rat also expresses two epithelial β-defensins (raBD-1, raBD-2), rhesus macaques one β-defensin (maBD-1) and the goat (*Capra hircus*) one β-defensin (GBD-2) (fig. 2).

Human β-defensins

Although a number of β-defensins have been discovered in cattle, it was not clear until 1995 whether humans also have this family of antimicrobial peptides. The first human β-defensin, HBD-1 (fig. 2), was originally isolated as a trace peptide from human hemofiltrate obtained from patients with end-stage renal diseases [69], but was recently also found in urine [70]. Multiple natural forms of HBD-1 ranging in length from 36 to 47 amino acids differing from each other by amino-terminal truncation were found in voided urine at levels of 10–100 μg/l [70].

Recombinant and natural HBD-1 forms were found to kill *E. coli* at micromolar concentrations [70]. Antimicrobial activity was not changed by low pH, but was inhibited at high salt concentrations. Interestingly, in contrast to the longer forms, the 36-amino-acid form of HBD-1 was also microbicidal in high-conductance urine [70]. Chemically synthesized HBD-1 was found to be active against Gram-negative bacteria at concentrations ranging from 60 to 500 μg/ml [71]. Using RT-PCR, HBD-1 mRNA expression was seen in kidney and salivary gland, when 25 cycles were used [72]. With 30 cycles, trachea, placenta, and prostate became strongly positive, whereas thymus, small intestine and testis were only weakly positive [72]. When Northern blot techniques were used, only kidney and pancreas were found to show HBD-1 transcripts [72].

Recent investigations using in situ hybridization techniques revealed localization of HBD-1 mRNA in epithelial layers of the loops of Henle, distal tubules and from the collecting ducts of epithelial layers of the vagina, endocervix, ectocervix, uterus, and fallopian tubes in the female reproductive tract [70]. Using monoclonal HBD-1 antibodies, immunostaining was seen in the kidney, within the loops of Henle. Interestingly, no intracellular storage sites were identified in renal or female reproductive tissues [70].

HBD-1 has been hypothesized to play an important role in chronic lung infection such as cystic fibrosis (CF). This hypothesis was based upon the observation that CF airway epithelia-derived surface fluid (ASF) is defective in its antimicrobial activity, which could be restored by dilution of ASF with water [73]. When CF bronchial xenografts were treated with a recombinant adenovirus expressing the CF transmembrane conductance regulator (a chloride channel which is defective in CF and which is believed to lead to elevated NaCl concentrations), bactericidal activity was tested [71]. Antisense experiments in non-CF xenografts, which revealed that ablation of HBD-1 function abolished bactericidal activity in ASF, led to the hypothesis that bactericidal activity in human airways to some Gram-negative bacteria is primarily constituted through the action of HBD-1 [71].

HBD-1 RT-PCR transcripts have also been recently identified in suprabasal keratinocytes of normal skin and in sweat ducts within the dermis [74]. As yet, however, it is not known whether HBD-1 peptide is secreted in lung or skin. Our own attempts to identify bioactive HBD-1 peptide in normal skin, supernatants of cultured skin and lung epithelial cells and lesional

psoriatic scale extracts have so far failed (unpublished results). Very recent investigations revealed HBD-1 mRNA expression in gingival epithelial cells, but not gingival fibroblasts. Levels of RT-PCR transcripts in inflamed versus healthy tissue were similar, indicating constitutive expression of the HBD-1 gene, which is apparently not affected by inflammatory stimuli [75].

Cloning of the HBD-1 gene revealed two exons and one large intron spanning 6962 bp. As expected, the two exons are small: the first encodes the signal sequence and propiece peptide, the second encodes the mature HBD-1 peptide [76].

All attempts have so far failed to induce mRNA expression upon stimulation of cultured lung epithelial cells with IL-6, phorbolesters, bacterial LPSs [72], heat-killed bacteria or tumor necrosis factor (TNF)-α (unpublished results).

The previously reported genomic HBD-1 sequence does not contain transcription factor regulatory elements for NF-κB and AP-1 [76], suggesting that HBD-1 is not the human homologue to bovine TAP/LAP, which differ from HBD-1 in both their intron structure, which is small, promoter region and inducibility.

Interestingly, the HBD-1 sequence contains NF-IL-6, despite the lack of inducibility by IL-6, indicating a different role for IL-6 in HBD-1 gene expression [77].

To identify peptides that normally keep skin free of infection, we analyzed lesional psoriatic scales for the presence of antimicrobial peptides, investigating psoriatic scales because patients with psoriasis have less skin infections than expected [78]. We hypothesized that antimicrobial peptides would be produced in high amounts in psoriatic lesions. This was confirmed by initial studies in which we saw extremely high killing activity in such scale extracts.

Using special techniques for trapping antimicrobial peptides, based upon bacteria-affinity chromatography, we were able to purify an antimicrobial peptide from lesional psoriatic scale extracts that has homology to β-defensins and was thus termed human β-defensin 2 (HBD-2) (fig. 2) [79]. HBD-2 is a 41-residue basic peptide with a molecular mass of 4328.3 Da. It is remarkably stable toward digestion with tryptic and chymotryptic enzymes. Its microbicidal activity is restricted mainly toward Gram-negative bacteria such as *E. coli*, and in particular *P. aeruginosa*, as well as yeasts such as *C. albicans* [79], with a MIC in the low micromolar range. We have seen only bacteriostatic activity against *S. aureus* at concentrations higher than 100 μg/ml [79].

Recently, it was reported that chemically synthesized HBD-2 kills *E. coli* in the micromolar range [80], similar to that obtained with natural material.

Recombinant HBD-2 produced in insect cells seems to be less potent and, interestingly, was also reported to be active against *S. aureus* [81].

The most striking feature of HBD-2 is its inducibility. Normal skin as well as normal lung showed some expression of HBD-2 mRNA as demonstrated by sensitive RT-PCR detection techniques [79]. The epidermis surrounding inflamed regions showed a marked increase in HBD-2 mRNA, a distribution that was confirmed at the peptide level by immunostaining with anti-HBD-2 antibodies [82]. In situ hybridization studies revealed weak and diffuse gene expression in various epithelia including the respiratory tract, skin, and colon [81].

When cultured keratinocytes are incubated with either TNF-α or heat-killed bacteria, HBD-2 mRNA expression is highly upregulated [79]. This behavior is not restricted to skin keratinocytes; we and others recently found that cultured bronchial, nasal and tracheal epithelial cells also respond to contact with *P. aeruginosa* bacteria or proinflammatory cytokines such as IL-1β or TNF-α with induction of HBD-2 [83, our unpublished results]. Thus, HBD-2 represents the first human defensin that is inducible in the presence of proinflammatory stimuli.

When different microorganisms were used to stimulate HBD-2 gene expression, it became clear that Gram-negative bacteria, in particular *P. aeruginosa*, act as powerful stimuli. *S. aureus* induced far less HBD-2 gene expression [79] and in some strains, no HBD-2 induction was seen (our unpublished results). *C. albicans* was also able to induce HBD-2 mRNA, but again less than *P. aeruginosa*. Although high concentrations (10 μg/ml) of *P. aeruginosa* LPS stimulate HBD-2 mRNA expression in tracheal epithelial cells, the molecular mechanism of epithelial cell activation by LPS is not yet clear.

It was very recently found that in humans, genes for Toll-like receptors (TLRs), mimicking the situation described in insects, are differentially expressed in human macrophages and tissues [84]. TLR-2 was found to represent a signalling LPS receptor in macrophages [85]. The response depended on the presence of LPS-binding protein (LPB) and was enhanced by CD14. Thus, it is intriguing to speculate that epithelial cells bear similar LPS receptors that would be fully activated only in the presence of LBP thus explaining why only non-physiologically high concentrations of LPS induce HBD-2 in vitro.

Nevertheless, one gets the impression that HBD-2 represents a rather Gram-negative-bacteria-selective antimicrobial peptide that is selectively produced upon contact with Gram-negative bacteria.

Cloning and sequencing of HBD-2 genomic DNA revealed that the HBD-2 gene consists of two exons and one intron [82], supporting the gene organization seen in mammal α- and β-defensins as well as that of plant defensins. The size of the intron is small, similar to that of bovine TAP and LAP. The HBD-2 gene was localized to chromosome 8p23 [82, 86], exactly where all other known human defensin genes cluster [87].

Analysis of the 5′-flanking region revealed the presence of a number of conserved transcription factor consensus sequences in the HBD-2 gene. Consensus sequences for NF-κB binding, which have also been seen in the TAP gene [57] and are known to be implicated in a number of proinflammatory host responses such as bacterial LPS stimulation and TNF-α activation, are found in the HBD-2 gene [87; our unpublished results]. NF-κB-like binding sites had already been identified in the inducible antimicrobial peptide genes of insects [58] indicating a common evolutionary link for mediation of this local innate host defense system.

The finding of HBD-2 transcripts in lung and trachea and its strong inducibility by contact with *P. aeruginosa* as well as its high activity against these bacteria may indicate that HBD-2 represents an important innate defense component of the airways. Disruption of either antimicrobial peptide action or production might result in recurrent lung infection.

Smith et al. [88] have seen that the ASF of healthy people was capable of killing *P. aeruginosa*, whereas CF ASF failed to do so, possibly due to elevated NaCl concentrations in CF ASF, which would inactivate endogenous peptide antibiotics released by CF epithelia. The antimicrobial factor secreted by human airway epithelia was a small peptide, but it was not characterized. In a very recent investigation, we were able to show that HBD-2 is the major antimicrobial peptide released upon stimulation of cultured respiratory tract epithelial cells with *P. aeruginosa* (our unpublished results) and that sputum of CF patients contains HBD-2. Similarly, it was also recently shown that under the air-liquid phase, cultured lung epithelial cells secrete the HBD-2 protein when stimulated with IL-1β [83]. That study also showed that cultured lung epithelial cells obtained from CF patients were able to produce HBD-2 when stimulated with IL-1β.

Therefore CF patients in principle have the capacity to produce HBD-2. It remains to be elucidated whether the HBD-2 production rate of CF patient epithelia under various stimulation conditions is identical to that seen in healthy epithelia. This is of particular importance because the antimicrobial activity of HBD-2 depends upon the NaCl concentration as well as the concentration of HBD-2. Thus HBD-2 still can kill *P. aeruginosa* at supraphysiologic salt concentration, when its concentration is sufficiently high [83].

Apart from β-defensins and the ubiquitous lysozyme, human epithelial cells also have the capacity to produce other antimicrobial peptides. Antileukoprotease (ALP), also known as secretory leukocyte protease inhibitor (SLPI), an 11.7-kDa cationic inhibitor of proteases, is produced by submucosal glands, lining epithelial cells of bronchi [89] and keratinocytes of skin [90], and displays marked in vitro antibacterial activity against *E. coli* and *S. aureus* [91, 92]. On a molar basis, however, it is less effective than lysozyme or HNP-1.

In the past few years, many propeptides (cathelicidins) with a conserved cathelin domain have been identified in the neutrophils of vertebrates including cows, pigs, sheep, rabbits, mice, and humans. The cathelin domain (containing nearly 100 amino acids) is not microbicidal. Its carboxyl terminus contains a potential (and, in some cases documented) cleavage site that is followed by the antimicrobial-peptide-encoding domain. Proteolysis of the 20-kDa precursor of the human cathelicidin, named hCAP-18, forms an antimicrobial peptide named LL-37, which represents an α-helical 37-amino-acid arginine-rich antimicrobial peptide [for a review see ref. 93]. Apart from the secretory granules of neutrophils, it is also present in testis and in the keratinocytes located close to inflammatory foci, but not in normal skin [94]. More recently it was also found in the epithelia of human lung [95].

In a standard-type broth microdilution assay, synthetic LL-37 shows considerable activity (MIC < 10 μg/ml) against *P. aeruginosa*, *S. typhimurium*, *E. coli*, *L. monocytogenes*, *S. epidermidis*, *S. aureus* and vancomycin-resistant enterococci, even in media that contain 100 mM NaCl. *Burkholderia cepacia* alone was found to be resistant to LL-37 [96].

Killing of microorganisms by antimicrobial peptides

Although detailed analyses of the mechanisms that lead to killing of microorganisms by contact with antimicrobial peptides are still incomplete, there is now circumstantial evidence that pore formation in the microorganisms cell walls is of major importance [for a review see ref. 97]. For some peptides, lipid-peptide interaction studies do not support the formation of transmembrane pores. It has been suggested that the peptides bind to the surface of bacterial membranes, cover it in a 'carpet-like' manner, and dissolve it like a detergent [98]. For pore formation, antimicrobial peptides would have to fulfil structural criteria that allow (i) binding to bacterial membranes, (ii) aggregation within the membrane, and (iii) channel formation.

Among the peptide antibiotics, a number of structurally different types have been found. The majority are cationic and amphipathic. Some, such as magainins and cecropins, are α-helical peptides devoid of cysteines [99–101]. Others, like defensins, are rather unusual in having as their principal structural features a β sheet hairpin which is stabilized by three disulfide bonds as well as an α helix [102].

Structural analyses (by nuclear magnetic resonance) revealed identical solution structures of rabbit neutrophil α-defensins and bovine leukocyte β-defensins. Com-

parison of structural measurements showed that human α-defensins exist as dimers, which in solution look like a basket with a hydrophobic base (exposed surfaces of the β hairpins) and a polar top [103, 104]. Data on mammalian epithelial β-defensins are as yet not available. Pore formation results in membrane potential differences due to increased permeability for water and/or ions. Furthermore, there are some studies that reveal that protein synthesis and/or DNA synthesis is influenced in a target microorganism [105–107]. It will be difficult, however, to determine which mechanism is critical for terminal killing of the microorganisms!

It is interesting to speculate about the principles that allow killing of microorganisms but not host cells. One hypothesis would be that membrane components that are present only in higher eukaryotic cells interfere with the cytotoxic properties of antimicrobial peptides. Indeed cholesterol, one such compound, dramatically reduces lytic activity of a cecropin when added to liposomes [108].

In summary, there are still a number of open questions concerning the molecular mechanisms of the microbicidal activity of antimicrobial peptides. In particular, it is not yet clear why some microorganisms are efficiently killed, whereas others are killed not nearly as much or are only affected at extremely high concentrations.

Apart from microbicidal properties, antimicrobial peptides seem to have additional functions. Enteric defensins (cryptdins), which are known to form anion-conductive pores in phospholipid bilayers, have been shown to stimulate chloride secretion from polarized monolayers of human intestinal epithelial cells. This response was found to be dose dependent, reversible and restricted to cryptdin-2 and -3, whereas cryptdin-1, -4, -5, and -6 lacked this activity. Thus, cryptdin-2 and -3 function as novel intestinal secretagogues [109].

In addition, there are reports identifying antimicrobial peptides such as defensins HNP-1, HNP-2 and CAP 37/azurocidin as potent chemoattractants for T cells [110]. It is likely that these peptides represent primordial antimicrobial peptides, which may have evolved over time into acute inflammatory cell-derived signals that mobilize immune-competent T cells and other inflammatory cells.

Conclusion

The original question 'why are healthy body surfaces healthy despite the high abundance of bacteria, fungi, and viruses?' may in part be answered by the presence of an innate epithelial chemical defense system, which appears to be characteristic for each surface. For mounting a fast first-line response, epithelia have the capacity to produce a high variety of different gene-encoded antimicrobial peptides. Some are constitutively released to create a steady-state situation or are inducible by proinflammatory stimuli to give a fast response when microbial density increases. The receptors on epithelial cells, which mediate induction of specific antimicrobial peptides are poorly defined or as yet unknown. Ligands on microorganisms, which mediate antimicrobial peptide synthesis are also as yet poorly defined. It will be an interesting task to identify the elicitors that induce endogenous antimicrobial peptide synthesis in epithelial cells.

Epithelial antimicrobial peptides tend to be involved as a local response to infection and have therefore been developed for therapeutic use. It has been assumed that they may be limited to treatment of topical infections, but very recent investigations revealed that antimicrobial peptides can be also used in parenteral therapy [110]. In principle, long-term clinical use of cationic antimicrobial peptides may give rise to the development of acquired resistance. Although the data are sparse due to lack of experience in the clinical use of cationic peptides, the development of acquired resistance appears to be rather unlikely since body surfaces have been exposed to endogenous antimicrobial peptides for hundreds of millions of years, and because of their rather special microbicidal mode of action, i.e., mainly by forming pores. Nevertheless, this important aspect should be addressed in future investigations, especially because there are a few reports about natural resistance to antimicrobials.

One well-studied example is the role of *Salmonella* spp. virulence regulators, PhoP and PhoQ, which activate the transcription of genes within macrophage phagosomes necessary for resistance to cationic antimicrobial peptides [111]. At a molecular level, lipid A, the host-signaling portion of LPS, was structurally modified by the addition of aminoarabinose and 2-hydroxy myristate [112], which may decrease the binding of cationic antimicrobial peptides to bacterial membranes.

Another example is the modulation of *Neisseria gonorrhoeae* susceptibility to vertebrate antimicrobial peptides by an energy-dependent efflux system, which prevents the lethal action of several cationic antimicrobial peptides in these microorganisms [113].

A third, and very recent obervation, was the discovery that inactivation of the dlt operon in *S. aureus* increased sensitivity toward HNP-1-3, protegrins, and magainin II. Wild-type *S. aureus* bearing several copies of the dlt operon (which is involved in the transfer of D-alanine into teichoic acids, a major component of the Gram-positive cell wall) tolerate high concentrations of several antimicrobial peptides, possibly by production of teichoic acids with higher amounts of D-alanine esters [114]. There is strong evidence that teichoic acids highly

esterified with D-alanine bind cationic proteins less effectively and thus Gram-positive with a high content of D-alanine-esterified teichoic acids were less sensitive to cationic antimicrobial peptides.

Nevertheless, the development of compounds that have been used for hundreds of million years by all classes of multicellular organisms for the treatment of infections or to prevent infections is a very promising task. Indeed, the first phase III clinical trials have been performed with derivatives of magainin, the antimicrobial peptide originally discovered in frog skin, to treat polymicrobic diabetic ulcers. These trials demonstrated effects similar to those of orally administered ofloxacin. Furthermore, it is conceivable that analogs of elicitors that induce epithelial antimicrobial peptide synthesis may indirectly help to protect surfaces from excessive growth of microorganisms by enhancing the chemical shield, as has been successfully used in induced plant defense [115].

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