Antimicrobial peptides of multicellular organisms

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Multicellular organisms live, by and large, harmoniously with microbes. The cornea of the eye of an animal is almost always free of signs of infection. The insect flourishes without lymphocytes or antibodies. A plant seed germinates successfully in the midst of soil microbes. How is this accomplished? Both animals and plants possess potent, broad-spectrum antimicrobial peptides, which they use to fend off a wide range of microbes, including bacteria, fungi, viruses and protozoa. What sorts of molecules are they? How are they employed by animals in their defence? As our need for new antibiotics becomes more pressing, could we design anti-infective drugs based on the design principles these molecules teach us?

Antimicrobial peptides are evolutionarily ancient weapons. Their widespread distribution throughout the animal and plant kingdoms suggests that antimicrobial peptides have served a fundamental role in the successful evolution of complex multicellular organisms. Despite their ancient lineage, antimicrobial peptides have remained effective defensive weapons, confounding the general belief that bacteria, fungi and viruses can and will develop resistance to any conceivable substance. Antimicrobial peptides target a previously under-appreciated 'microbial Achilles heel', a design feature of the microbial cellular membrane that distinguishes broad species of microbes from multicellular plants and animals. The insights provided by this large body of research have spawned considerable commercial effort to create new classes of anti-infective therapeutics.

A diversity of peptides

The diversity of antimicrobial peptides discovered is so great that it is difficult to categorize them except broadly on the basis of their secondary structure. (An online catalogue of all reported molecules, now about 500, can be found at http://www.bbcm.univ.trieste.it/~tossi/antimic.html). The fundamental structural principle underlying all classes is the ability of the molecule to adopt a shape in which clusters of hydrophobic and cationic amino acids are spatially organized in discrete sectors of the molecule ('amphipathic' design) (Fig. 1). Linear peptides, such as the silk moth's cecropin[1] and the African clawed frog's magainin[2], adopt this organization only when they enter a membrane, whereupon they assume an amphipathic α-helical secondary structure[3]. Frog species of the genus *Rana* modify this design by adding a single loop formed by a disulphide bond at the carboxy end[4]. Peptides such as bactenecin[5] and defensins[6] use a relatively rigid anti-parallel β-sheet constrained by disulphide bonds as the framework, around which segregated patches of cationic and hydrophobic residues are organized. A large family of linear peptides characterized by a predominance of one or two amino acids, such as the tryptophan-rich indolicidin of the cow neutrophil[7] and the proline–arginine-rich PR39 (ref. 8) of the pig neutrophil, segregate hydrophobic and hydrophilic side chains around an extended peptide scaffold in the setting of the membrane (Table 1). Most multicellular organisms express a cocktail comprising multiple peptides from several of these structural classes within their 'defensive' tissues.

All antimicrobial peptides are derived from larger precursors, including a signal sequence. Post-translational modifications include proteolytic processing, and in some cases glycosylation[9], carboxy-terminal amidation and amino-acid isomerization (reviewed in ref. 4), and halogenation[10]. A rather complex modification involves the cyclization of two short peptides leading to the fully circular θ-defensin isolated from neutrophils of *Rhesus* (macaque) monkeys[11]. Some peptides are derived by proteolysis from larger proteins, such as buforin II from histone 2A (ref. 12) and lactoferricin from lactoferrin[13].

The diversity of sequences is such that the same peptide sequence is rarely recovered from two different species of animal, even those closely related, be they insects, frogs or mammals (exceptions include peptides cleaved from highly conserved proteins, such as buforin II). However, both within the antimicrobial peptides from a single species, and even between certain classes of different peptides from diverse species (reviewed in ref. 4), significant conservation of amino-acid sequences can be recognized in the preproregion of the precursor molecules; the design suggests that constraints exist on the sequences involved in the translation, secretion or intracellular

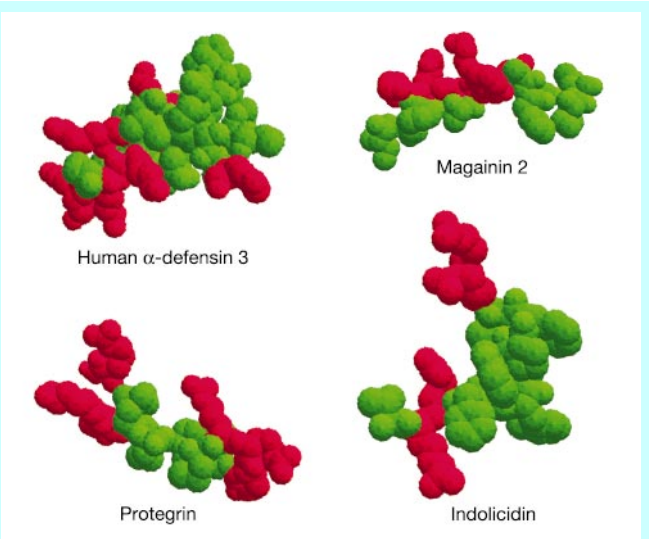

Figure 1 Clustering of cationic and hydrophobic amino acids into distinct domains in several antimicrobial peptides of different structural classes. This 'amphipathic' design is evident in many, but not all, antimicrobial peptides. Red, basic (positively charged) amino acids; green, hydrophobic ('oily') amino acids. Other amino acids are not shown. Magainin is depicted in its α-helical configuration.

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trafficking of this class of membrane-disruptive peptide. This feature is dramatically illustrated by the cathelicidins (reviewed in ref. 14).

Why does diversity arise? Because single mutations can dramatically alter the biological activity of each peptide, the diversity probably reflects the species' adaptation to the unique microbial environments that characterize the niche occupied, including the microbes associated with acceptable food sources (reviewed in refs 4 and 15). It seems reasonable to speculate that an individual could find itself in the midst of microbes against which the peptides of its species were ineffective; although the individual might suffer, the species itself could survive through emergence of individuals expressing beneficial mutations. Adaptive immunity, through its plasticity, permits a species to empower individuals to explore new environments and avail themselves of new food sources. However, compared with the equipment of the innate system, such as antimicrobial peptides, the effectors of adaptive immunity are more costly to maintain and slower to respond to assault[15].

With respect to the diversity created in the synthetic laboratory, almost all active molecules are composed of hydrophilic, hydrophobic and cationic amino acids arranged in a molecule that can organize into an amphipathic structure (reviewed in ref. 16). Natural peptides composed of all D-amino acids, in place of L-amino acids, typically retain full antibiotic potency while exhibiting expected resistance to enzymatic proteolysis[16]. Short linear or cyclic amphiphillic peptides that contain both L- and D-amino acids can be generated with various degrees of selectivity and antimicrobial potency[17,18]. Recently, protease-resistant antimicrobial peptides composed of β-amino acids have been constructed[19,20].

Mechanism

Antimicrobial peptides have targeted a surprising but clearly fundamental difference in the design of the membranes of microbes and multicellular animals, best understood for bacterial targets. Bacterial membranes are organized in such a way that the outermost leaflet of the bilayer, the surface exposed to the outer world, is heavily populated by lipids with negatively charged phospholipid headgroups. In contrast, the outer leaflet of the membranes of plants and animals is composed principally of lipids with no net charge; most of the lipids with negatively charged headgroups are segregated into the inner leaflet, facing the cytoplasm (Fig. 2) (reviewed in ref. 21). A model that explains the activity of most antimicrobial peptides is the Shai–Matsuzaki–Huang (SMH) model (refs 21–23; Fig. 3). The model proposes the interaction of

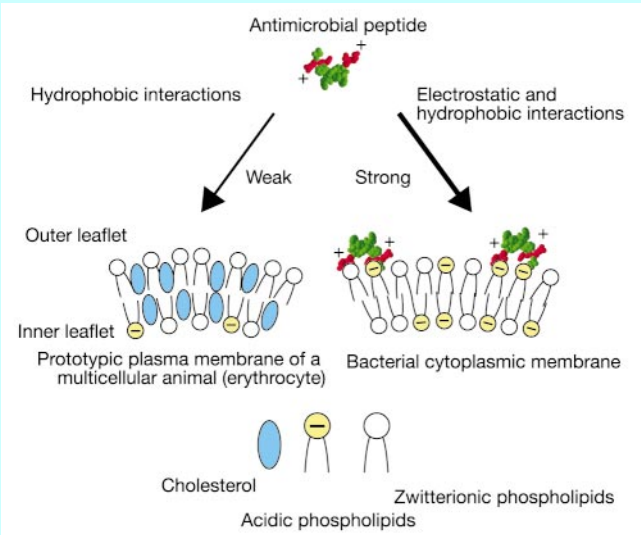

Figure 2 The membrane target of antimicrobial peptides of multicellular organisms and the basis of specificity. (Modified from ref. 21.)

Table 1 Overview of antimicrobial peptides from plants and animals

Representative peptides		Origin	Tissue*
α-helical			
Cecropin A	KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK*a*	Silk moth	E, BC, H
Magainin 2	GIGKFLHSAKKFGKAFVGEIMNS	Frog	E
Pexiganan	GIGKFLKKAKKFGKAFVKILKK*a*	Synthetic	
Dermaseptin 1	ALWKTMLKKLGTMALHAGKAALGAAADTISQGTQ	Frog	E
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	Human	E, BC
Buforin II	TRSSRAGLQFPVGRVHRLLRK	Vertebrate	E
One disulphide bond			
Bactenecin 1	RLCRIVVIRVCR	Cow	BC
Thanatin	GSKKPVPIIYCNRRTGKCQRM	Insect	BC
Brevinin 1T	VNPIILGVLPKVCLITKKC	*Rana* frogs	E
Ranalexin	FLGGLIKIVPAMICAVTKKC	*Rana* frogs	E
Ranateurin 1	SMLSVLKNLGKVGLGFVACKINKQC	*Rana* frogs	E
Esculentin 1	GIFSKLGRKKIKNLLISGLKNVGKEVGMDVVRTGIDIAGCKIKGEC	*Rana* frogs	E
Two disulphide bonds			
Tachyplesin	RWC$_1$FRVC$_2$YRGIC$_2$YRKC$_1$R*a*	Horseshoe crab	BC
Androctonin	RSVC$_1$RQIKIC$_2$RRRGGC$_2$YYKC$_1$TNRPY	Scorpion	H
Protegrin 1	RGGRLC$_1$YC$_2$RRRFC$_2$VC$_1$VGR*a*	Pig	BC
Three disulphide bonds			
α-defensin (HNP3)	DC$_1$YC$_2$RIPAC$_3$IAGERRYGTC$_2$IYQGRLWAFC$_3$C$_1$	Human	BC, E
β-defensin (TAP)	NPVSC$_1$VRNKGIC$_2$VPIRC$_3$PGSMKQIGTC$_2$VGRAVKC$_1$C$_3$RKK	Cow	E, BC
θ-defensin	GFC$_1$RC$_2$LC$_3$RRGVC$_3$RC$_2$IC$_1$TR	Monkey	BC
Defensin (sapecinA)	ATC$_1$DLLSGTGINHSAC$_2$AAHC$_3$LLRGNRGGYC$_2$NGKAVC$_3$VC$_1$RN	Insect	E, BC, H
Thionin (crambin)	TTC$_1$C$_2$PSIVARSNFNVC$_3$RIPGTPEAIC$_3$ATYTGC$_2$IIPGATC$_1$PGDYAN	Plant	E
Four disulphide bonds			
Defensin	QKLC$_1$QRPSGTWSGVC$_2$GNNNAC$_3$KNQC$_4$IRLEKARHGSC$_2$NYVFPAHC$_3$IC$_4$YFPC$_1$	Radish	Seeds, E
Drosomycin	DC$_1$LSGRYKGPC$_2$AVWDNETC$_3$RRVC$_4$KEEGRSSGHC$_2$SPSLKC$_3$WC$_4$EGC$_1$	*Drosophila*	H
Hepcidin	DTHFPIC$_1$IFC$_2$C$_3$GC$_4$C$_1$HRSKC$_2$GMC$_3$C$_4$KT	Human	Liver
Linear, not α-helical			
Bac 5	RFRPPIRRPPIRPPFYPPFRPPIRPPIFPPIRPPFRPPLGRPFP*a*	Cow	BC
PR-39	RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFPPRFPPRFP*a*	Pig	BC
Indolicidin	ILPWKWPWWPWRR*a*	Cow	BC
Apidaecin	GNNRPVYIPQPRPPHPRI	Honeybee	H
Pyrrhocoricin	VDKGSYLPRPTPPRPIYNRN	Insect	H
Histatin 5	DSHAKRHHGYKRKFHEKHHSHRGY	Human	Saliva

Cysteines paired in disulphide linkages are noted by common numerical subscripts. C-terminal amides are noted by *a*. In θ-defensin, the first and last residues are joined in a peptide bond.
*BC, blood cell; H, haemolymph; E, epithelial tissue.

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the peptide with the membrane, followed by displacement of lipids, alteration of membrane structure, and in certain cases entry of the peptide into the interior of the target cell. The presence of cholesterol in the target membrane in general reduces the activity of antimicrobial peptides, due either to stabilization of the lipid bilayer or to interactions between cholesterol and the peptide[21]. Similarly, it is believed that increasing ionic strength, which in general reduces the activity of most antimicrobial peptides, does so in part by weakening the electrostatic charge interactions required for the initial interaction.

In general, peptides operating by the SMH mechanism kill microbes at micromolar concentrations. In contrast, the peptide nisin, a 14-amino-acid amphipathic molecule produced by *Lactococci*, operates at nanomolar concentrations. Nisin binds with high affinity to Lipid II, the fatty acyl proteoglycan anchor in the bacterial membrane, from which it subsequently diffuses into the surrounding membrane[24]. Certain plant defensins use a similar strategy[25].

How do antimicrobial peptides actually kill microbes? Many hypotheses have been presented, which include: fatal depolarization of the normally energized bacterial membrane[26]; the creation of physical holes that cause cellular contents to leak out[22]; the activation of deadly processes such as induction of hydrolases that degrade the cell wall[27]; the scrambling of the usual distribution of lipids between the leaflets of the bilayer, resulting in disturbance of membrane functions[21]; and the damaging of critical intracellular targets after internalization of the peptide, as suggested by the example of the peptide pyrrhocoricin[28].

Resistance

Unlike conventional antibiotics such as penicillin, which microbes readily circumvent, acquisition of resistance by a sensitive microbial strain against antimicrobial peptides is surprisingly improbable. Resistant species of genera such as *Morganella* and *Serratia* express an outer membrane that lacks the appropriate density of acidic lipids to provide peptide-binding sites. Other resistant species, such as *Porphyromonas gingivalis*, secrete digestive proteases that destroy peptides. Published studies of 'acquired resistance' against antimicrobial peptides, by and large, have identified genes that, when disrupted, make sensitive organisms more susceptible to a particular antimicrobial peptide; indeed, these genes usually appear to have a role in virulence.

A recent report of a survey of thousands of clinical isolates against the synthetic magainin analogue pexiganan illustrates the general picture that has emerged over the past decade regarding the issues of resistance[29]. Bacterial species exhibit a wide range of susceptibilities, with some, such as anaerobes in the case of pexiganan, among the most sensitive. The basis for the different susceptibilities of bacterial and fungal species against particular peptides remains unexplained. Attempts at inducing pexiganan resistance in *Escherichia coli* and *Staphylococcus aureus* by chemical mutagenesis have been unsuccessful. As expected, no evidence of cross-resistance between pexiganan and any antibiotic in clinical use has been documented.

Gram-negative bacteria possess an outer membrane composed of lipopolysaccharide (LPS), which is held together by magnesium and calcium ions that bridge negatively charged phosphosugars. Addition of cationic peptides results in displacement of metal, damaging the outer membrane, and facilitates entry of additional molecules from the exterior (as reviewed in ref. 30). Peptides, having gained access to the periplasmic space, can now integrate into the cytoplasmic membrane. In many species of Gram-negative bacteria, the charge on the outer membrane is modulated by the PhoPQ regulon, a two-component system that uses a sensor (PhoQ) and an intracellular effector (PhoP)[31]. The PhoP/PhoQ regulon affects antimicrobial peptide sensitivity through modulation of the PmrA regulon, which controls a bank of genes that mediate decoration of the outer membrane with the positively charged moieties ethanolamine and 4-aminoarabinose[32].

Why have microbes not been more successful in resisting the

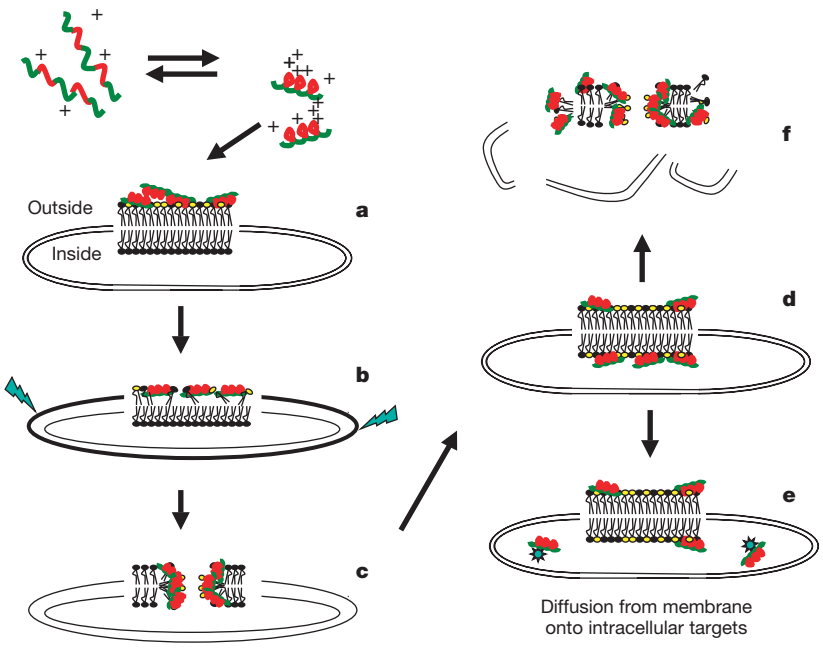

Figure 3 The Shai–Matsuzaki–Huang model of the mechanism of action of an antimicrobial peptide. An α-helical peptide is depicted. **a**, Carpeting of the outer leaflet with peptides. **b**, Integration of the peptide into the membrane and thinning of the outer leaflet. The surface area of the outer leaflet expands relative to the inner leaflet, resulting in strain within the bilayer (jagged arrows). **c**, Phase transition and 'wormhole' formation. Transient pores form at this stage. **d**, Transport of lipids and peptides into the inner leaflet. **e**, Diffusion of peptides onto intracellular targets (in some cases). **f**, Collapse of the membrane into fragments and physical disruption of the target cell's membrane. Lipids with yellow headgroups are acidic, or negatively charged. Lipids with black headgroups have no net charge.

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activity of antimicrobial peptides, considering the time span over which such mechanisms could have evolved? Because the target of antimicrobial peptides is the bacterial membrane, a microbe would have to redesign its membrane, changing the composition and/or organization of its lipids, probably a 'costly' solution for most microbial species. Destruction of the antimicrobial peptide poses several problems. Most peptides are created from nondescript sequences of amino acids lacking unique epitopes that could serve as the recognition site of a protease required for selective destruction of the antibiotic in the presence of cellular protein constituents. In addition, multicellular organisms attack microbes with multiple peptides of different structural classes, hence destruction of one peptide might not suffice to ward off the lethal assault. Perhaps the virulence genes that bacteria now express which affect susceptibility towards antimicrobial peptides represent the best defences that most microbes can mount without suffering a loss of viability.

Regulation

Insects

After the discovery of inducible antimicrobial peptides in silk moths[1] and *Drosophila*[33], the corresponding genes were cloned and sequenced. The genes are expressed in blood cells, epithelia and the insects' fat body, an organ resembling the vertebrate liver that secretes proteins and peptides directly into the animals' haemolymph. The 5′-flanking regions harboured sequences that bound 'Rel' transcription factors, analogues of the nuclear factor κB (NFκB)-binding motifs of mammals[34,35]. These putative regulatory regions had previously been implicated in early embryonic development of *Drosophila* (as reviewed in ref. 36). Examination of the intracellular pathway involved in the regulation of antimicrobial peptides revealed that much of the early embryonic circuit was co-opted for a defensive function. A recent view of this pathway (Fig. 4) shows it to involve initiation of the signal through proteolytic generation of the protein Spaetzle from its precursor; the presumed interaction of Spaetzle with a receptor called Toll; subsequent communication through a series of intracellular proteins resulting in the chemical modification of cytoplasmic Cactus, which is then released from its physical union with the Dorsal-related protein Dif; the translocation of Dif into the nucleus, where it binds to a DNA sequence in the vicinity of the antimicrobial gene, activating transcription. Drosomycin, an important antifungal peptide in *Drosophila*, appears to be regulated by this circuit[36].

As predicted, mutant flies that have lost the function of certain genes within this pathway can no longer express Drosomycin after a fungal challenge and succumb to overwhelming fungal infection. Conversely, mutants that are created to overexpress Spaetzle, the Toll ligand, produce Drosomycin in the absence of fungal challenge. Mutants defective in the Toll–Drosomycin circuit can still express antibacterial peptides such as cecropin and diptericin[36]. The residual circuit is called the *imd* pathway, regulated by a pathway that is activated by a Dif-related protein called relish[37]. Like Dif, relish resides in the cytoplasm, but, in contrast, must be proteolytically cleaved by upstream events[37]. The actual receptor that turns on the *imd* intracellular pathway has not yet been identified.

Certain general features of the antimicrobial systems of animals are illustrated by the *Drosophila* story. First, multiple 'hard-wired' circuits exist in an animal linking microbial assault to the expression of the genes of the corresponding antimicrobial peptide. Second, different circuits are wired to different banks of defensive peptides. The Toll pathway seems to have a role in fungal defence, whereas the *imd* pathway engages a different array of antimicrobial weapons to fend off bacteria, suggesting that these two pathways are intrinsically designed to respond to different physiological insults.

Microbes themselves have not been implicated as ligands of the insect Toll receptor. A recent report implicates a proteoglycan binding protein circulating in *Drosophila* haemolymph (seml) as the proximal receptor that initiates signals in the Toll pathway when the animal is invaded by Gram-positive bacteria (but not fungi)[38]. It is not yet clear how the binding of this 'pattern recognition' receptor to proteoglycan turns on the proteolytic cascade that results in the generation of mature, processed Spaetzle from its precursor protein.

Vertebrates

Expression of the antimicrobial β-defensins TAP[39] in epithelial cells of the bovine respiratory tract and LAP in the tongue[40] are

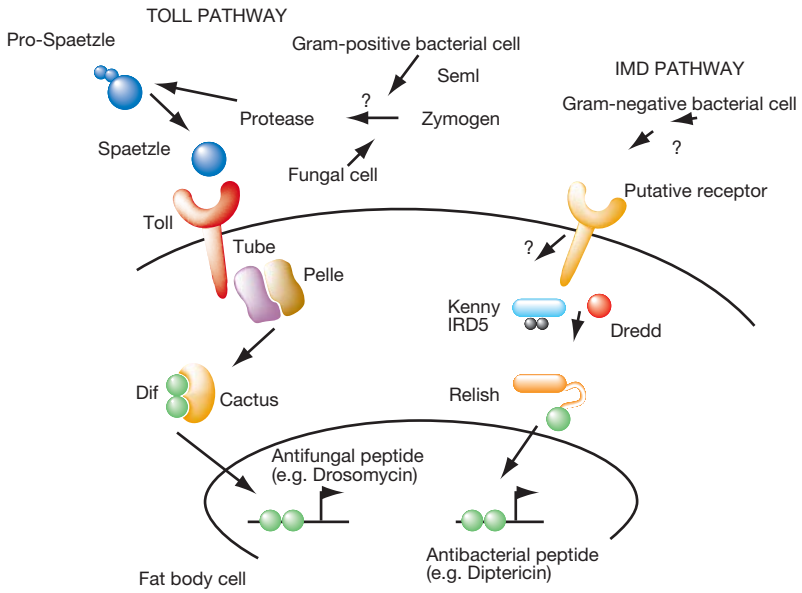

Figure 4 Signalling cascades that activate antimicrobial peptide genes in the fat body of *Drosophila*. The pathways have been deduced, by and large, through genetic analysis of immune function in *Drosophila*, and proteins encoded by genetic loci are noted. The proteolytic system that results in the cleavage of pro-Spaetzle is not known. The existence of a receptor in the Imd circuit is depicted, but remains speculative.

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stimulated by LPS, interleukin-1β (IL-1β) and tumour necrosis factor (TNF)[41], and were upregulated *in vivo* in the setting of inflammation and after acute bacterial challenge. Reports followed describing induction of β-defensins in various epithelia including the human gastrointestinal[42] and respiratory tracts[43,44] and skin[45]. Analysis of the 5′-flanking regions of several inducible genes of epithelial antimicrobial peptides from mammals[46,47] and frogs (reviewed in ref. 4), like insects, revealed NFκB-binding sites. Exposure to the appropriate stimulus resulted in increase in intracellular levels of NFκB, its translocation into the nucleus, and activation of the corresponding antimicrobial gene.

The inducing activity of IL-1β implicated a role for the IL-1 receptor (IL-1R) in the regulation of epithelial defensin production. The IL-1R is a close structural relative of Toll, and both respond to a protein ligand, which must be proteolytically processed. Furthermore, the intracellular pathways involved in IL-1 signalling converge on NFκB like the corresponding Toll pathway in insects. In mammals, IL-1 can be liberated from monocytes, macrophages, dendritic cells, or from injured epithelial cells (reviewed in ref. 48).

The discovery that Toll and Toll-like receptors (TLRs) function in expression of genes for antimicrobial peptides in insects and mammals led to a search for additional human homologues, driven by the hypothesis that the innate immune system used specially tuned receptors to recognize specific and unique patterns of microbial chemical constituents that are presented when microbes attack a multicellular organism (reviewed in ref. 49). Subsequent studies have demonstrated that at least ten TLR genes exist in humans[49]. Studies linking several of these TLRs with antimicrobial peptide gene expression in vertebrate systems have very recently been reported by several laboratories, but as yet are unpublished. The current model suggests that microbial products such as LPS bind directly to these TLRs, in some cases in association with specific binding proteins that might enhance specificity (such as the LPS-binding protein CD14), but the full story regarding ligand–receptor interactions as well the intracellular circuitry used to modulate antimicrobial peptide gene expression is still incomplete.

Plants

Like animals, plants express antimicrobial peptides such as defensins and thionins both constitutively and in response to microbial assault. The initial insult is transmitted by highly specific pattern-recognition receptors such as the nucleotide-binding site plus leucine-rich repeat with Toll and IL-1 receptor homology (TIR–ND–LRR) receptors, analogues of the mammalian TLRs; they in turn activate a relatively specific hard-wired response against the specific organisms to which they are tuned, a concept termed the 'gene-for-gene' defensive strategy (reviewed in ref. 50).

Roles in health and disease

Epithelial surfaces

Antimicrobial peptides are expressed by the epithelial cells within the barrier and delivered to the site by circulating white cells. This generalization is valid for all vertebrates studied, and probably every invertebrate, including insects[51]. In humans and other mammals, fully processed active peptides can be isolated from keratinized epithelial sheets of dry skin and tongue, where they probably act as epithelial 'preservatives'. In other sites in humans, such as the columnar epithelium of the airway, antimicrobial peptides are secreted into a micrometre-thick biofilm directly overlying the epithelium. The concentration and composition of electrolytes within this antimicrobial-rich unstirred layer are regulated by a variety of pumps and channels, which maintain conditions that maximize the antimicrobial activity of these defences. In cystic fibrosis, this homeostasis is disturbed by the genetic defect in the cystic fibrosis transmembrane-conductance regulator (CFTR); it has been suggested that, in this setting, antimicrobial peptide defences fail to provide an effective barrier, resulting in bacterial overgrowth and chronic, tissue-destructive inflammation[52,53].

The skin of an animal is under constant microbial assault, and a recent study highlights the defensive contribution to this barrier provided by antimicrobial peptides. The mouse has only a single member of the cathelicidin gene family, called Cramp, which it expresses in both leukocytes and epithelial tissues. Knockout mutants of Cramp exhibit hypersusceptibility to group A *Streptococcus*, and succumb to destructive necrotic ulceration after inoculation of a dose of bacteria that causes only a mild self-limited reaction in wild-type mice. Similarly, mutants of group A streptococci selected for reduced susceptibility to Cramp (which, curiously, seem to grow more slowly than the wild type), exhibit an enhanced virulence when introduced into the dermis of wild-type mice[54].

The gastrointestinal tract of mammals is covered by a continuous sheet of epithelial cells that is folded into villus projections and crypts. Within the base of the crypts in humans and mice, where the stem cells of the GI tract can be found, are specialized, granular cells called Paneth cells. Both the enterocytes and the Paneth cells produce antimicrobial peptides (as reviewed in ref. 55). The enterocytes synthesize and secrete antimicrobial peptides both constitutively and on induction, and either secrete them onto their surface, as in the respiratory tract, or retain them in a cell-associated fashion in the superficial non-viable sheets of epithelium, as in the rectum. In contrast, after a microbial stimulus the Paneth cells at the base of the intestinal crypts secrete α-defensins into the cryptal well, at concentrations estimated at the level of grams per litre, which eventually flush into the gut lumen[56].

Both systems contribute to bowel health. In children and adults suffering from diarrhoea caused by *Shigella*, synthesis of the colonic enterocyte β-defensin HBD-1 and the cathelicidin LL37 is markedly depressed; expression recovers in time during resolution of the illness[57]. Similarly, mice lacking the proteolytic enzyme required for processing cryptdins, which are the α-defensins of murine Paneth cells, and consequently lacking functional cryptdins, exhibit increased susceptibility to orally administered *Salmonella*[58].

A rather surprising pattern has been discovered operating in the gastric mucosa of humans, mice and other vertebrates[12]. It appears that histone 2A is synthesized in excess of the needs required for DNA packaging in the gastric mucosal cell, and accumulates within cytoplasmic secretory granules. On secretion, the histone is processed by pepsin to the potent antimicrobial peptide buforin II, which remains adherent to the mucous biofilm coating the stomach surface, thus providing the stomach with a protective antimicrobial coat.

The healthy human is inhabited by a population of bacteria called 'commensals', which include organisms such as *Fusobacterium nucleatum* in the mouth and *Lactobacillus* species in the gut. These bacteria are relatively resistant to the action of endogenous antimicrobial peptides[29]. Commensal bacteria are generally regarded as beneficial to the host. They can suppress pathogens by displacing them from a microbial niche or by secreting antimicrobial substances. Recent data suggest that commensals also provide protection by chronically stimulating epithelial surfaces to express antimicrobial peptides at levels that kill pathogen microbes[15]. In the gingival epithelium, *F. nucleatum* stimulates the inducible defensin HBD-2, whereas *P. gingivalis*, the anaerobe that destroys gum tissue, does not, behaving as a silent invader[59]. Frogs that have been pharmacologically depleted of skin antimicrobial peptides will not re-accumulate skin antimicrobial peptides unless the animals are gradually exposed to bacteria in their environment; in their depleted state these frogs will succumb to overwhelming infection if suddenly exposed to otherwise innocuous microbes[60].

Sentinels of adaptive immunity

Antimicrobial peptides, released from circulating cells or induced in epithelia, can alert the adaptive immune system to trouble brewing.

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Table 2 Commercial development of antimicrobial peptides of animal origin

Mode of use	Peptide	Company	Application	Stage
Topical	Pexiganan (MSI-78)	Magainin (Genaera)	Infected diabetic foot ulcers	Completed Phase III; not approved by FDA, pending additional studies
Topical	MBI-226	Micrologix	Catheter infection	Phase III
Topical	MBI-594	Micrologix	Acne	Phase II
Oral	Protegrin analogue (IB-367)	Intrabiotics	Mucositis	Phase III
Oral	Histatin analogue (P-113)	Demegen	Gingivitis	Phase II
Systemic	Heliomycin	Entomed	Antifungal	Preclinical
Systemic	Human lactoferricin	AM Pharma	Antibacterial	Preclinical
Systemic	BPI*	Xoma	Meningococcal meningitis	Phase III

*BPI, bactericidal permeability increasing protein.

The α-defensins of the human neutrophil directly attract human peripheral blood T cells that express CD4/CD45RA (naive) and CD8 antigens; the α-defensins also attract immature dendritic cells both *in vitro* and after injection under the skin of mammals (as reviewed in ref. 61). When administered simultaneously with antigens, these neutrophil defensins enhance antigen-specific immune responses. The cathelicidin LL37 attracts neutrophils along with monocytes and certain peripheral T cells. LL37 seems to act through a receptor for formyl peptide receptor-like 1 (FPRL1), a G-protein-coupled receptor that also recognizes ligands such as the bacterial formyl peptides[62]. LL37 is induced within epithelial cells of skin and lung in states of inflammation by some 10–50-fold, and would be expected to attract neutrophils, monocytes and T cells to the site of damage. The epithelial β-defensins, constitutively expressed HBD-1, and the inducible HBD-2 and HBD-3 are also chemoattractants. HBD-2 selectively attracts the memory subset of peripheral T cells ($CD4^+/CD45RO^+$), along with immature dendritic cells. The receptor used in this case is chemokine (C-C) receptor 6 (CCR6), which also recognizes the selective dendritic cell attractant macrophage inflammatory protein (MIP)-3α[61]. What is striking about the chemokine role ascribed to antimicrobial peptides is their relatively low affinity (10^{-6} to 10^{-5} M) compared with optimal concentrations observed for classical chemotactic factors (10^{-8} to 10^{-7} M). A cell relying solely on high-affinity ligands for directing movement might be stunned as it approaches the source of the chemokine. However, low-affinity interactions will still operate, and because of the presence of a large concentration gradient, cellular traffic will be directed with greater precision to specific sites of injury.

Applications

The growing problem of resistance to conventional antibiotics and the need for new antibiotics has stimulated interest in the development of antimicrobial peptides as human therapeutics. Most pharmaceutical effort has been devoted to the development of topically applied agents, such as the magainin analogue pexiganan[63], largely because of the relative safety of topical therapy and the uncertainty surrounding the long-term toxicology of any new class of drug administered systemically. The main hurdles that have impeded the development of antimicrobial peptides as systemic therapy include the unpublished experience that many of the naturally occurring peptides (such as magainin), although active *in vitro*, are effective in animal models of infection only at very high doses, often close to the toxic doses of the peptide[64], reflecting an unacceptable margin of safety. Antimicrobial peptides in pharmaceutical development are shown in Table 2.

Diverse applications have been demonstrated for antimicrobial peptides as anti-infective agents. The broad antimicrobial spectrum of antimicrobial peptides positions them for consideration as 'chemical condoms' to limit the spread of sexually transmitted diseases, including *Neisseria*, *Chlamydia*, human immunodeficiency virus (HIV) and *Herpes simplex* virus (HSV)[65]. The affinity of antimicrobial peptides for microbial membranes has encouraged their evaluation as imaging probes for bacterial and fungal infections[66]. Antimicrobial peptides can enhance the potency of existing antibiotics *in vivo*, probably by facilitating access of antibiotics into the bacterial cell[64,67], a phenomenon previously recognized for the cationic peptide component of polymyxin.

Microbial colonization and growth on the surfaces of synthetic polymeric materials is a problem that complicates the use of medical devices such as intravenous catheters. One solution has been suggested by the successful demonstration that magainin peptides, covalently bound to insoluble polymeric beads, retain antimicrobial activity[68].

Introduction of antimicrobial genes into both plants and animals has been successful in transferring some benefit against disease. Agricultural uses have progressed most extensively, as demonstrated in tobacco[69] and potatoes[70]. The possibility of alleviating the pulmonary bacterial infections associated with cystic fibrosis by transferring in a genetic construct capable of expressing super-physiological levels of LL37 has been demonstrated in an animal model[71]. The potential of re-engineering human macrophages to express β-defensins to enhance their efficacy against *Mycobacterium tuberculosis* has recently been proposed[72].

Lastly, the discovery that the essential amino acid isoleucine can pharmacologically stimulate expression of β-defensin genes in isolated enteric cells suggests that new classes of therapeutics could be developed on the basis of their ability to turn on endogenous antimicrobial peptides[73].

Conclusion

The discovery of the widespread distribution of antimicrobial peptides over the past 20 years has provided insights into the innate defensive systems that permit multicellular organisms, including humans, to live in harmony with microbes. It is hard to imagine (especially after reading a classic immunology text) that most animals now alive, including insects and creatures like octopus and starfish, rely heavily on antimicrobial peptides for defence against microbes, and do so quite effectively without the help of lymphocytes, a thymus, or antibodies. If the story regarding diversity continues to unfold based on our current views, we might well discover that every species harbours a unique, specific collection of antimicrobial peptides, tuned to defend the organism against microorganisms that it will predictably encounter. Newly characterized molecules have inspired molecular designs for the creation of therapeutics, and will continue to do so as more are discovered, because these are based on antimicrobial strategies that have proven efficacious over millennia. Studies both in the laboratory and in the clinic confirm that emergence of resistance against antimicrobial peptides is less probable than observed for conventional antibiotics, and provides the impetus to develop antimicrobial peptides, both natural and laboratory conceived, into therapeutically useful agents.

Note added in proof: Bacterial lipoprotein has been shown to induce the expression of HBD-2 in human epithelial cell lines through a TLR-2/NFκB-dependent pathway[74]. □

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