ANTIMICROBIAL PEPTIDES

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Chairman's opening remarks

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Where are the scientific roots of our present Ciba Foundation Symposium on Antimicrobial peptides? In a general way the roots can be traced back a century or more. They are to be found at the beginning of immunology (of mammals and insects), in the discovery of phagocytosis, in the beginning of chemotherapy, in the isolation of peptides from bee venom and from frog skins and in the deliberate search for microbial antibiotics. The unifying goal in the medical part of this early work was to find agents with a selective toxicity, what Ehrlich called the 'magic bullet' of chemotherapy, an immune substance, a natural product or a chemical that in a patient would effectively kill a harmful microbe without unacceptable damage to the host.

Of course, penicillin also belongs to the roots of our present meeting: after all, its nucleus is made from cysteine and D-valine. Fleming had discovered lysozyme in 1922, the first bacteriolytic enzyme. His finding of penicillin in 1929 was the result of following up a chance observation, but one might say, in Pasteur's words, that he did have a 'prepared mind'. On the other hand, the second phase of the penicillin saga, from 1939–1945, was a goal-oriented, large-scale mission fuelled by the need to find an effective treatment for infected wounds from World War II. These efforts included some 40 laboratories on both sides of the Atlantic which produced about 1200 confidential reports on the structure of penicillin, its synthesis and production by fermentation.

In a more direct way, the history of antibacterial peptides runs in parallel with the penicillin work. It starts in 1939 with Dubos' demonstration that 'an unidentified soil bacillus' produced some antibacterial compounds that could prevent pneumococcal infections in mice. The year after, Hotchkiss and Dubos reported a partial purification of the bactericidal substances produced by this soil bacterium, now identified as *Bacillus brevis*. Then, in 1941–1942, they described how, starting from a *B. brevis* culture, they had purified and crystallized tyrocidine and gramicidin and shown them to be composed of amino acids, some of them D-amino acids. Both these antibacterial peptides turned out to be more or less toxic; tyrocidine to the extent that it could never be used to treat an infection. However, before penicillin became available, gramicidin was to a limited extent used as a therapeutic agent.

In another way, gramicidin was also a forerunner to cecropins, defensins and magainins because it was the first peptide antibiotic found to induce the formation of voltage-gated anion channels in artificial membranes. As we all know, this is a property of several of the peptides to be discussed at this meeting, although it is still not clear if this is the primary mechanism by which bacteria are killed. However, there is a fundamental difference between the forerunners and the antimicrobial peptides to be discussed here: all antimicrobial peptides have their own genes. On the other hand squalamine, a recently discovered shark antibiotic, is a steroid and, as such, it cannot be made from an RNA template, but must be made stepwise just as was also found for tyrocidine and gramicidin.

Other forerunners of today's antibacterial peptides were, in the 1960s, the bee venom toxin, melittin, and the frog skin peptide, bombinin. From an early stage, these two peptides were claimed to be antibacterial, although this seems mainly to have been stated and as far as I know only once really documented for bombinin. However, bombinin at that time was most likely a mixture of peptides and the mixture was, like melittin, quite haemolytic. Thus, even if their action on membranes may have been similar to that of cecropins and magainins, bombinin and melittin did not show the same membrane specificity. Today melittin is still a toxin, but one melittin domain has been used together with a cecropin domain for the design of novel antibacterial, non-haemolytic peptides. Bombinin is now separated into a family of closely related peptides. Some of these are antibacterial and non-haemolytic, others are both antibacterial and haemolytic and they also contain a D-amino acid.

The organized search for antibiotics, which started towards the end of World War II, has now produced so many antibiotics that nobody seems to know how many there are. The number of antibiotics described in scientific journals and/or patented may be as high as 20 000, although only a few hundred of these may be in use in daily therapy. The widespread clinical use of antibiotics (and their misuse in the production of animals) has led to the spread of plasmid-borne resistance genes that are posing an increasing threat to the future medical use of these agents. The development of this phenomenon was the subject of a Ciba Foundation Symposium on drug resistance in 1957 and on penicillin resistance in 1962. However, both of these meetings seem to have been too early to consider infectious, plasmid-borne drug resistance. Up to now there has only been one more Ciba Foundation Symposium related to antibiotics and this was quite recently. In 1992, the Foundation arranged a meeting on secondary metabolites—a term used mainly for attempts to understand the biosynthesis and the production of microbial antibiotics. Thus, in a way, today's conference is the first Ciba Foundation Symposium focused directly on a new type of antibiotic, in this case gene-mediated and produced by animals, plants or microbes. For animals, these antibacterial peptides serve as immune substances and they are primary defence agents of innate immunity rather than secondary metabolites. Their potential use as drugs is motivated in part by the increasing

spread of bacterial resistance to conventional antibiotics, in part because endogenous antibiotics may offer a new avenue to the therapy of infectious diseases.

What do we mean by a peptide and what is the difference between a peptide and a protein? Often it is a matter of size and often it is rather arbitrary; in fact, the terms are partly overlapping. In practice, the term peptide is rarely used for molecules larger than 10 kDa and the word protein is rarely used for anything smaller than insulin (with 51 residues). Thus, at this meeting we should (by and large) leave out antibacterial proteins. However, it is important to stress that even the smallest peptides to be discussed here (from 12 residues and up) are all made as gene-encoded preproproteins (normally with more than 60 residues) and they are processed by defined pathways to their active peptide forms.

Cecropins and the defensins were the first antimicrobial peptides from animals to be characterized structurally, from 1981–1983. The sequences indicated that these two classes of peptides are very different: cecropins are amphipathic helices without cysteine and defensins have six cysteines involved in three intramolecular disulphide bonds. Moreover, the cecropins were discovered in insects and the defensins in mammalian phagocytes. Thus, no generalization could be made from these facts. However, the arrival of magainin in 1987 seems to have catalysed our field: in the following years quite a number of antimicrobial peptides were described and more are being found all the time. In the past, the origin of the peptide was the main criterion for classification, but subsequent findings of the insect defensins and a cecropin in pig intestine sort of shook this systematics. Today, there may be as many as 50 structures known (estimate made at a recent meeting) and I have tried to group the peptides known so far on a chemical and biochemical basis into the following five families:

(1) Linear molecules without Cys, with or without a hinge. Cecropins, PGLa and magainins are amphipathic helices. Bombinins H1–H5 are not amphipathic, they contain a D-amino acid.

(2) Peptides without Cys, and with an over-representation of one or two amino acids (so far Pro, Arg, Trp or Gly). Apidaecins, Bac5, PR-39, indolicidin, drosocin. Motifs like PRP, PP. Amidation and glycosylation occur. Gly-rich and Pro-rich domains in attacins and diptericins.

(3) Molecules with one disulphide bond. Loop structures with tail(s). Bactenecin, brevinins and esculentin.

(4) Peptides with two or more disulphide bonds. Mainly or only β-sheets. Tachyplesin, protegrins, defensins, insect defensins. N-terminal pyroglutamate in β-defensins.

(5) Molecules derived from larger peptides with other known functions by processing or alternative splicing. GIP(7–42), derived from gastric inhibitory polypeptide. DBI(32–86), derived from diazepam-binding inhibitor.

The absence (groups 1 and 2) or presence of cysteine (groups 3 and 4) is still important but each group is split on a structural basis. So far, group 4 is the largest in terms of the number of peptides found. New members of group 5 are being found and it remains to be seen if they will continue to be free of cysteine. The list of peptides given in each group is not always complete and only some of the peptides are subject to post-translational modifications. It remains to see if any peptides from plants and microorganisms will fit into this chemical taxonomy, made for the animal peptides known today. Some antibiotics with complicated ring systems (like nisin and subtilin) made by bacteria have backbones that are made as gene-encoded peptides, but the many steps of modifications that have to follow should really put them into a group separate from those made by animals. And of course, in the future, animals may very well be found to produce many antibiotics (like squalamine) that are not peptides at all.

The programme of this symposium was not intended to cover all the above peptide groups—in fact the grouping was made about half a year later than the outlines of our meeting. However, I am glad to know that we have today present —either as speakers or as discussants—people who have made important contributions to all groups of antimicrobial peptides mentioned in this introduction.

Acknowledgement

Some background reading

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This excellent textbook contains numerous short historical introductions to the early part of microbiology and immunology, covering also chemotherapy and antibiotics.
Lazarus LH, Attila M 1993 The toad, ugly and venomous, wears yet a precious jewel in his skin. Prog Neurobiol 41:473–507
The title of this review is a paraphrase from Shakespeare and it covers new and old work on frog skin peptides.
Moberg CL, Cohn ZA (eds) 1990 Launching the antibiotic era. Personal accounts of the discovery and use of the first antibiotics. Rockefeller University Press, New York
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A short review of peptide antibiotics produced by different animals.

Design and synthesis of antimicrobial peptides

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Abstract. The cecropins are a group of potent antimicrobial peptides, initially discovered in insects but later found in other animals including mammals. Synthetic peptide chemistry has played an important role in establishing their primary sequences, as well as the steps in the processing of the biosynthetic preprocecropins. Solid-phase peptide synthesis has been the method of choice. Synthetic chimeric peptides have led to more active products and a better understanding of their mode of action. The structural requirements for high activity include a basic amphipathic N-terminus, a short central flexible sequence and a hydrophobic helical C-terminus. Cecropin–melittin hybrids as small as 15 residues are highly active. In planar lipid bilayers the cecropins form pores which pass ions and carry a current under a voltage gradient. Synthetic D-enantiomers of several antibacterial peptides carry the same current as the natural all-L-peptides and are equally active against several test bacteria. Therefore, the activity is not dependent on chiral interactions between the peptides and the lipid bilayers or the bacterial membranes. Recent examination of retro and retroenantio peptides has further defined the limits of the structural requirements of these peptides. Some of the hybrid peptides are active against *Plasmodium falciparum* and *Mycobacterium smegmatis*.

1994 Antimicrobial peptides. Wiley, Chichester (Ciba Foundation Symposium 186) p 5–26

The purpose of this paper is to describe the chemical synthesis of some antibacterial peptides and to show how synthesis has been useful for establishing composition and structure of natural products, for defining details of processing of precursors, for the design of more active, more stable and broader-spectrum antibiotics, and for studies on the mechanisms of action of the peptides.

Synthetic methodology

My laboratory has focused for a long time on a method for the chemical synthesis of peptides called *solid-phase peptide synthesis* (Merrifield 1963). In 1980, we began a collaboration with Professor Hans G. Boman to apply these techniques

to the cecropins, just after they had been isolated and sequenced (Hultmark et al 1980). We succeeded in preparing cecropins A, B and D, containing 37, 35 and 36 amino acid residues, respectively (Merrifield et al 1982, Andreu et al 1983). The synthetic products helped confirm the sequences and the presence of the C-terminal amide; they were fully active compared with the natural products. The general methodology has served well for the cecropins and many of their analogues, and we and others have applied it to other linear antibiotics, including melittin (Habermann & Jentsch 1967), PYLa (Andreu et al 1985), magainins (Zasloff 1987) and alamethicin (Gisin et al 1977). The technique depends on the step-by-step assembly of peptides while they are attached to a solid support. This procedure simplifies and accelerates the synthesis, allows automation of the process and generally gives improved yields of purified peptides compared with classical solution methods.

One synthetic scheme is shown in Fig 1. It uses a cross-linked polystyrene resin support, attachment of the peptide chain as a substituted benzyl ester or amide, N^{α}-tertbutyloxycarbonyl (Boc) amino acids, coupling with dicyclohexylcarbodiimide or symmetrical anhydrides, and final deprotection and cleavage from the resin with hydrofluoric or other strong acid. The sequence of synthetic, fully protected cecropin A–resin is:

Boc–Lys (ClZ)–Trp (HCO)–Lys (ClZ)–Leu–Phe–Lys (ClZ)–Lys (ClZ)–Ile–Glu (OcHx)–Lys (ClZ)–Val–Gly–Gln–Asn–Ile–Arg (Tos)–Asp (OcHx)–Gly–Ile–Ile–Lys(ClZ)–Ala–Gly–Pro–Ala–Val–Ala–Val–Val–Gly–Gln–Ala–Thr

(Bzl)–Gln–Ile–Ala–Lys (ClZ)–NH–CH–⬡–O–$\overset{\overset{\displaystyle O}{\|}}{C}$–Ⓡ
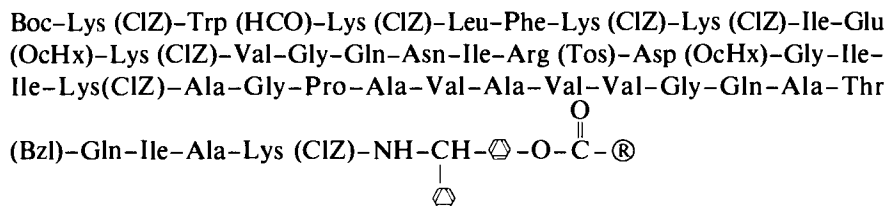
where HCO = formyl, ClZ = chlorobenzyloxycarbonyl, OcHx = O-cyclohexyl, Tos = p-toluenesulphonyl, Bzl = benzyl and R = the resin.

Any synthesis of a peptide can give rise to impurities; we go to great effort to detect and avoid or eliminate these by-products. They can be deletion peptides, addition peptides or modification peptides. High-pressure liquid chromatography (HPLC) or ion-exchange columns are commonly used for preparative and analytic purposes and mass spectrometry is now of great value for the final verification of composition, being accurate to ± 0.3 mass units for large peptides.

Many new modifications for solid-phase synthesis have been developed over the years (Merrifield 1994); for example, polyacrylamide (Sheppard & Williams 1982), polyoxyethylene (Rapp et al 1992) and carbohydrate supports, and new linkers for peptide attachment with much greater acid sensitivity. Other protecting group strategies allow cleavage by base or nucleophiles, photolysis, hydrogenolysis and recently by Pd(O) cleavage of allyl derivatives (Kunz & Dombo 1988). New coupling reagents have been introduced, including a range of phosphonium and uronium salts, urethane-protected N-carboxy
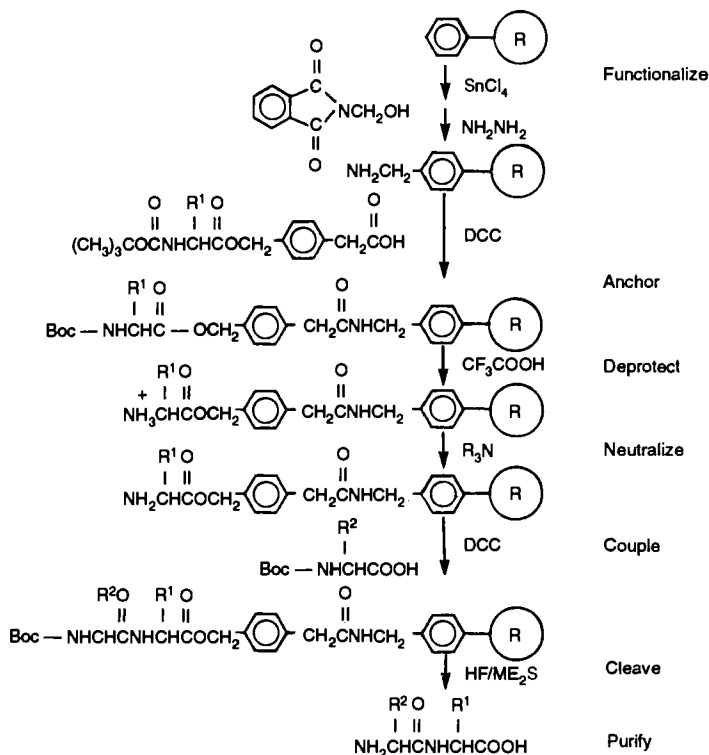
FIG. 1. A convenient scheme for the solid-phase synthesis of peptides.

anhydrides and amino acid fluorides. Comparative studies are currently underway to evaluate further the new chemistry.

Some peptide antibiotics are more complex than the cecropins or other straight chain compounds already mentioned. The defensins (Lehrer et al 1993) are an example because of their three disulphide bonds and resulting cyclic structures and especially their β-sheet conformation and basic character. For example, Rao et al (1992) made rabbit NP-1 (33 residues) using N^{α}-fluorenylmethyloxycarbonyl (Fmoc) protection, an oxymethylphenoxymethylpolystyrene support, coupling with the uronium salt, O-benzotriazolyl-N, N, N' N'-tetramethyluronium hexafluorophosphate (HBTU) (Dourtoglou et al 1984), and cleavage with trifluoroacetic acid. It was air oxidized and purified by HPLC. The overall yield was 4.5%. Human defensin (29 residues) was synthesized by Tam et al (1991) using a new, improved oxidation technique in which the disulphide bonds were formed in 20% DMSO (dimethylsulphoxide) containing 1 M guanidine hydrochloride at pH 8.2. Under these conditions, the severe difficulties with insolubility and β-sheet aggregation to give intractable precipitates were largely avoided. An overall yield, based on the first amino acid of the step-by-step synthesis was 14%.

Cecropin precursors

Chemical synthesis has not been limited to the mature cecropins, but has been used by Zong-qu Li and by David Andreu to prepare the preprocecropins A and B (64 and 61 residues) for studies on protein processing (Boman et al 1989a). $C_A(-26,38)$ and $C_B(-26,35)$ are the first preproproteins to be prepared by chemical synthesis. The synthesis of preprocecropin A was started with a C-terminal glycine needed for C-terminal amide formation as indicated by the isolated gene. Samples were withdrawn after residues 1, -2, -4, -5, -9 and -26 to allow studies on the various steps of the processing, using a dog pancreas microsomal signal peptidase, the *Escherichia coli* leader peptidase and a dipeptidyl-peptidase from *Hyalophora cecropia*. The first two enzymes could degrade the precursor beginning at residues -26, -9 or -5, but stopped at residue -4. The resulting procecropin was then cleaved in two steps by the dipeptidase, removing Ala–Pro and Glu–Pro. High antibacterial activity was limited to the mature cecropin A (1–37) and its C-terminal glycine derivative. The longer peptides were nearly devoid of activity.

Cecropin analogues

One of the goals of our research has been to design and synthesize analogues with improved antibacterial activity and a wider spectrum of susceptible microbial species. Many deletion and substitution analogues were prepared and tested on a range of Gram-positive and Gram-negative bacteria (Merrifield et al 1984). Some structural information was obtained, but none of these simple changes led to peptides more active than cecropin A. It became very clear, however, that there is considerable structural specificity for antibacterial activity and that most random or modified peptides are not active.

We then designed and synthesized several model peptides to try to answer questions about the preferred conformation, and the hydrophobic and amphipathic character of these peptides (Fink et al 1989a). The N-terminal, helical, amphipathic 1–11 sequence was retained because it was already thought to be critical. The central portion was made less or more hydrophobic and the helix-breaking Gly[23]–Pro[24] sequence was sometimes replaced with Leu–Glu. The C-terminus was made more hydrophobic and less amphipathic. From all the data, we arrived at the conclusion that the preferred conformations were: (1) a basic, amphipathic helical N-terminus; (2) a flexible hydrophilic non-helical central portion followed by a Gly–Pro sequence; and (3) a somewhat hydrophobic amphipathic C-terminus. The antibacterial activity became progressively better as these requirements were met. The best of the model peptides had the sequence:

KWKLFKKIEKV|GRNGIVKAGP|AVAKLLKEAKEIAK–NH$_2$

with a lethal concentration (LC) of $0.2 \mu M$ for *E. coli* and $0.2 \mu M$ for *Bacillus subtilis*, whereas the corresponding values for cecropin A were 0.2 and $3 \mu M$.

Chimeric peptides

We then tested the idea of making hybrid, or chimeric, peptides using three patterns: (1) rearranging the sequence of a single natural molecule; (2) combining segments of two different cecropins; and (3) combining segments from two different classes of antibacterial peptides (Boman et al 1989b). The first positive result was obtained with cecropin A (1–11)–cecropin D (12–37) (Fink et al 1989b). It was more active than C_A against all species of test bacteria, but especially against *B. subtilis* (sixfold) and *Streptococcus faecalis* (ninefold). Dozens of hybrids of cecropins and melittin, cecropins and magainins, and cecropins and PYLa were prepared. Some of the most interesting are shown in Table 1. We considered the improvement in activity against *Streptococcus pyogenes* and *Staphylococcus aureus* to be particularly important because the cecropins and magainins have very low activity against these species. The other interesting finding was the loss of haemolytic activity when the two halves of melittin were interchanged. Inverted cecropin A was inactive.

Earlier experiments had indicated that nearly all of the cecropin molecule was required for activity. However, we found a series of hybrids where good activity was retained down as far as 15 residues (Andreu et al 1992). Not all shorter peptides were active, but some were and on a weight basis they were much more active than the native molecule. It should be mentioned that some of the hybrid molecules are active against the bloodstream form of the malaria parasite *Plasmodium falciparum* (Boman et al 1989b, Andreu et al 1992).

Mechanism of antibacterial action

The antibacterial activity of these peptides was found by Steiner et al (1988) to correlate with the lysis of lipsomes, where binding followed by slow disruption of the lipid membrane was observed. Lysis of bacteria occurs in the micromolar range, which is just sufficient peptide to form a monolayer on all cells; this may provide a mechanism of action.

We have also examined the electrical conductance of the peptides in planar lipid bilayers (Christensen et al 1988). With cecropins and several of their analogues at 10^{-7} to 10^{-6} M, and a potential of 20–100 mV across a bilayer, we observed a current in the picoamp range that increased with time until the membrane broke. The conductance was voltage dependent with a linear relationship between S and V. These were macroscopic conductances where many channels were open at the same time. It was also possible to observe single channels. To provide the observed flow of current, we calculated that pores

TABLE 1 Antibacterial activity of cecropin A, melittin and some hybrids

	Lethal concentration (μM) of peptides					
Peptide amide	Escherichia coli	Pseudomonas aeruginosa	Bacillus subtilis	Streptococcus pyogenes	Staphylococcus aureus	Red cell
Cecropin A	0.2	1	3	5	>300	>200
Melittin	0.8	3	0.2	0.5	0.2	4
$C_A(1-13)Me(1-13)$	0.5	1	0.7	1	2	>200
$C_A(1-8)Me(1-18)$	0.3	0.7	0.4	2	1	>600
Me(16–26)Me(1–13)	0.7	8	0.7	1	10	>200

from 5–40 Å in diameter were necessary and therefore that four or more peptides were required to aggregate to produce a pore (six α-helical peptides would give a 17 Å pore). Conductances of 7 pS to 2.5 nS were found, corresponding to 10^6–10^9 ions/second per channel at 100 mV and eliminating a carrier mechanism, which would give a 10^2–10^4 times smaller current.

We have suggested that the mechanism would involve the following steps (Fig. 2): (I) the positively charged peptide makes an electrostatic contact with the negative bilayer of phosphatidylserine and diphytanoylphosphatidylcholine; (II) in this apolar lipid environment, the peptide assumes an amphipathic helical conformation that inserts itself into the lipid; (III) under a voltage gradient, several of these amphipathic helices would then aggregate to form a pore, with the outside hydrophobic and the peptide in van der Waals' contact with the lipid, while the inside would be water filled and more hydrophilic and positively charged. As a consequence, ions would move through the pore and conduct a current. Cecropins and analogues as small as about 20 residues would be long enough to span the membrane as α-helices and those down to 15 residues could span it if they formed 3_{10} helices or if the hydrophobic part of the membrane was thinner at that site than the normal 30 Å. In our hands, shorter antimicrobial peptide analogues could not be shown to conduct a current unless the bilayer was formed in solvents such as squalene that were shown to give thinner membranes (E. Merrifield, unpublished results). Nuclear magnetic resonance (NMR) data showing that some active peptides are oriented parallel to the membrane surface require other explanations (Opella 1990).

The electrical conductance in bilayers caused by antibiotic peptides seems well explained by ion channels or pores and a similar mechanism may hold for certain bacterial membranes. The passage of ions would lower the proton gradient and destroy the membrane potential, stopping ATP production and all cellular metabolism, and the cell would die. The channel or pore mechanism for killing
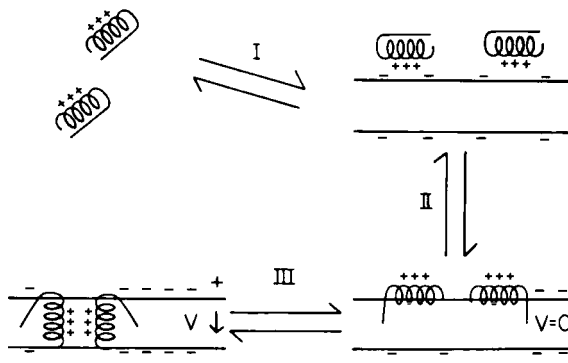
FIG. 2.   A mechanism for the formation of pores in lipid bilayers by cecropin and its analogues. See text for explanation.

bacteria is not fully proven and, although it may play a role, we believe that other mechanisms probably exist. A given organism may be killed in different ways by different peptides, even of the same structural class. Conversely, a given peptide may operate by a different mechanism on other organisms. Questions concerning concentration dependence, membrane potential, peptide amphipathicity, conformation and orientation within the membrane still need to be addressed.

We have therefore sought new structural data that might throw further light on the mechanism(s) of action of the cecropins and other antimicrobial peptides.

It occurred to one of us (RBM) that the D-enantiomers of these peptides (analogues containing all-D-amino acid residues) might provide some insight into questions of how these antibiotic peptides function. Would the peptides interact in a stereospecific way with the lipid of the artificial bilayers or with the chiral lipid or protein of bacterial membranes? If receptors or enzymes are involved in the action, we would expect these chiral proteins to undergo a stereospecific interaction with the chiral peptides. However, the peptides may simply require a hydrophobic environment to fold into a proper helical conformation to give rise to transmembrane pores. The all-D-peptides would be predicted to be mirror images of the natural all-L-peptides and would not be superimposable with them. Thus, they would not have the same topology and would not fit the same receptor or be a substrate for a normal enzyme, or make close chiral contacts with lipids.

Several D- and L-enantiomeric pairs of peptides were synthesized (Wade et al 1990), including those of cecropin A, melittin, magainin 2 amide, $C_A(1-13)Me(1-13)NH_2$ and $C_A(1-8)Me(1-18)NH_2$. All the D–L pairs were exact mirror images as shown by circular dichroism measurements in water and in the presence of several concentrations of organic solvents. Thus, the D-enantiomers are left-handed helices. The D-peptides were not substrates for trypsin and were at least 15 times more stable in rabbit serum than the L-isomers.

Conductivity measurements in lipid bilayers showed that the D-enantiomers of all five pairs produced the same current flow as the corresponding L-isomers (Fig. 3). This means that these peptides do not depend on a close fit with the chiral centres of the lipids, but probably depend only on the apolar environment to cause the peptides to assume pore-forming conformations.

The D–L pairs of peptides were assayed against several representative Gram-positive and Gram-negative bacteria. The members of each pair gave almost exactly equivalent killing of each of the five bacterial species. The error in such assays is about a factor of two. The ratios of LC values for the L–D-isomers were between 0.7 and 2, with an average of 1.4 ± 0.5, and the isomers are considered to be equally active. The two cecropin–melittin hybrids in either L or D configuration are about 100 times as active as cecropin A or magainin against *Staph. aureus*. Therefore, the improved potency of the L-isomers was retained in the D-isomers. The resistance of red cells to lysis was
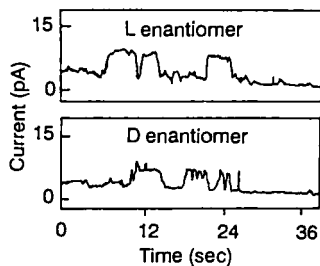
FIG. 3. Single-channel conductances produced by the L- and D-enantiomers of cecropin A in lipid bilayers. Membrane composition of 2:1 phosphotidylserine: diphytanoylphosphatidylethanolamine. Peptide concentration 90 nM, applied voltage −60 mV.

also retained in the D-enantiomers. The D- and L-enantiomeric pairs for five of the shorter 15-residue C_A–Me hybrids were also shown to be equally active against the five test organisms (R.B. Merrifield, S. Mitchell, H.G. Boman & D. Andreu, unpublished results). Representative data are shown in Table 2.

Killing of mycobacteria by cecropin analogues

The recent emergence of resistant strains of the tubercle bacillus is posing a severe health problem. New antibiotic agents are clearly needed. As a first step, cecropin A and some of its hybrids have been studied for their effects on the non-pathogenic strain *Mycobacterium smegmatis* 655 (E. Merrifield, unpublished results). The peptides were tested in LC and MIC (minimum inhibitory concentration) assays and compared with the standard tuberculosis antibiotic, rifampin (Table 3). All samples were active in the micromolar range and the D-enantiomers were significantly more active than the L-isomers. Thus, the 15-residue peptide D-C_A(1–7)Me(2–9)NH$_2$ was as active as rifampin and D-C_A(1–8)Me(1–18)NH$_2$ was four times more active suggesting that the D-isomers were more stable during these rather extended incubations. On continued incubation of both LC and MIC cultures of the rifampin-treated organisms, small, uniformly distributed colonies appeared in the previously clear areas, suggesting only a bacteriostatic action by this antibiotic. The cultures containing cecropin peptides remained clear, supporting a bactericidal action. The data justify further studies on virulent strains of *Mycobacterium tuberculosis*, where the D-enantiomers may be especially beneficial against these very slow-growing organisms.

Retro and retroenantio peptides

We wanted to know whether or not reversing the direction of the peptide sequence or inverting the direction of the amide bond in a normal sequence

TABLE 2 Antibacterial activity of some L- and D-enantiomeric pairs

Peptide amide	Lethal concentration of peptide (μM)						Average ratio L:D
	Escherichia coli	Pseudomonas aeruginosa	Bacillus subtilis	Streptococcus pyogenes	Staphylococcus aureus		
L-C$_A$(1–37)	0.2	1	3	5	>300		
D-C$_A$(1–37)	0.3	0.8	3	2	>300		1.3±0.4
L-Mg2(1–23)	4	30	3	4	300		
D-Mg2(1–23)	1	30	3	3	100		2.0±0.9
L-C$_A$(1–13)Me(1–13)	0.5	1	0.7	1	2		
D-C$_A$(1–13)Me(1–13)	0.8	2	1	0.8	8		0.7±0.2
L-C$_A$(1–7)Me(5–12)	0.5	2	0.3	0.4	8		
D-C$_A$(1–7)Me(5–12)	0.7	2	0.5	0.8	4		1.0±0.4

Average ratio L:D = 1.2±0.4.

TABLE 3   Activity of cecropin analogues against *Mycobacterium smegmatis* 655

Peptide	Lethal concentration (μM)	Minimum inhibitory concentration (μM)	Relative activity (average) (%)
Rifampin	6.6	6.3	100
L-Cecropin A	39	38	20
D-Cecropin A	5.2	ND	130
L-C$_A$(1–13)Me(1–13)NH$_2$	11	9.4	60
D-C$_A$(1–13)Me(1–13)NH$_2$	3.4	8.4	130
L-C$_A$(1–8)Me(1–18)NH$_2$	4.5	1.3	310
D-C$_A$(1–8)Me(1–18)NH$_2$	1.7	1.1	490
L-C$_A$(1–7)Me(2–9)NH$_2$	ND	9.4	70
D-C$_A$(1–7)Me(2–9)NH$_2$	8.9	4.7	100

ND, not done.

would affect the antibacterial activity of the cecropin–melittin hybrids and thereby say something more about their mechanism of action. We have therefore prepared some retro and retroenantio hybrids of C$_A$(1–13)Me(1–13)NH$_2$ and C$_A$(1–7)Me(2–9)NH$_2$ (D. Andreu, P. Juvvadi, H.G. Boman, R.B. Merrifield, unpublished results). A retro peptide has a reversed sequence, with residue 1 of the parent peptide attached to the resin and a sequence growing towards the former C-terminal residue. The result is a normal L-peptide reading in reverse sequence. If the peptide is read in the other direction it will have the original sequence but the –CONH– amide bonds will be reversed, giving –NHCO– bonds. In addition, when reading the retro derivative in the direction of the parent peptide, the side-chain R groups will have the configuration of D-amino acids relative to the peptide chain backbone. The latter can be corrected by synthesizing the retro peptide with D-amino acids, giving a retroenantio peptide. To avoid differences in the charges on the end groups of the peptides when read in the direction of the normal sequence, we have used C-terminal amides, as in native cecropin and melittin, and have acetylated the N-termini, so that both ends in all samples will contain uncharged amide structures. These structures are illustrated in Fig. 4.

Some predictions about the activity we expected to find in the new analogues could be made (Table 4). If only sequence or only amide direction are important, both the retro and the retroenantio peptides should be active, but if both sequence and amide direction are important, neither would be expected to be active. Data were obtained for four analogues of C$_A$(1–13)Me(1–13)NH$_2$: (1) the normal L-peptide; (2) the enantio peptide (all-D-amino acids); (3) the retro peptide (with reversed sequence and all-L-amino acids); and (4) the retroenantio

L- Peptide

Retro-enantio Peptide

**Retro-enantio Peptide
(inverted)**
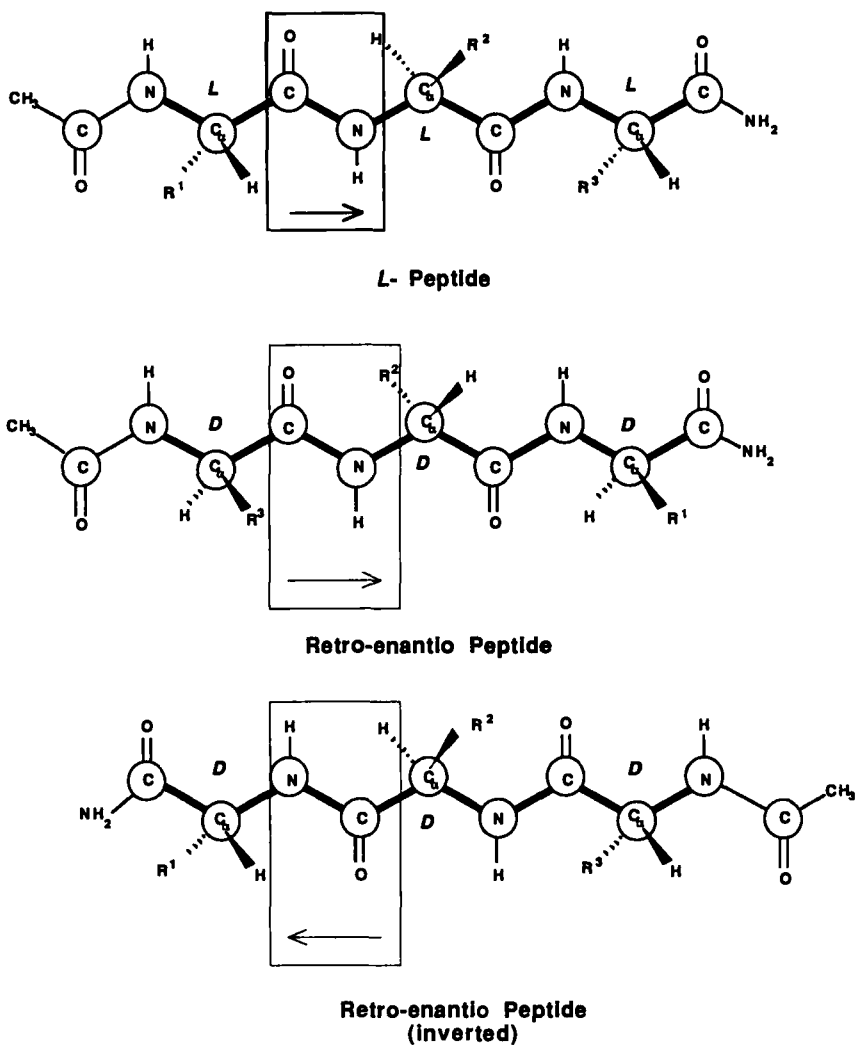
FIG. 4.   Structure of an acetylated natural L-peptide amide and its retroenantio analogue in the N→C and C→N orientation. Only one internal amino acid residue is shown.

peptide (with reversed sequence and all-D-amino acids) as shown in Table 5. For five of the bacteria all four analogues were essentially equally active, in which case none or only one of the structural features (sequence, direction of amide bond or chirality) could be important (not two or three features), but we cannot tell which. For *B. subtilis*, however, the retro and retroenantio peptides were each 10-fold less active. Therefore, sequence and amide direction are important. If we use our earlier data to conclude that chirality is not an

TABLE 4 Predicted activity of cecropin–melittin analogues

Assumed critical features	Structure			
	Normal	Enantio	Retro	Retroenantio
Sequence	+	+	+	+
Amide direction	+	+	+	+
Chirality	+	+	+	+
Sequence + amide	+	+	−	−
Sequence + chirality	+	−	−	+
Amide + chirality	+	−	+	−
Sequence + amide + chirality	+	−	−	−

+, active; −, inactive or lowered activity.

important structural feature determining activity and also consider the many examples of peptides with modified sequences showing decreased or lost activity, we can conclude that the direction of the peptide bond is not generally a critical structural feature for determining antibacterial activity of these hybrid peptides. However, for some more demanding organisms, e.g. *B. subtilis*, it appears that peptide bond direction can be important. Whether this is due to a change in mechanism is not yet clear. An analysis of the acetylated $C_A(1-13)Me(1-13)NH_2$ peptides gave the same conclusions as the unacetylated forms having a free N-terminal amino group. The assay data on the unacetylated $C_A(1-7)Me(2-9)NH_2$ series were similar to the unacetylated $C_A(1-13)Me(1-13)NH_2$ series except this time, the resistant species were *Pseudomonas aeruginosa* and *Staph. aureus*, showing either sequence, amide bond or end group charge to be important. For *Staph. aureus*, masking the N^α amine by acetylation, thereby eliminating the positive charge, nearly returned the low activities of the retro and retroenantio peptides to normal levels. Thus, it appears that for the shorter peptides the end group effect can be more critical. For all peptides examined, the sheep erythrocytes were very resistant to lysis and the introduction of D-amino acids, reversed sequence or inverted amide bonds did not make the cells significantly more susceptible to the peptides.

The overall conclusions about requirements for activity of the cecropins and related peptides are that sequence composition and charge are important, but peptide length, chirality and direction of the amide bond are not. The peptides probably function as ion channels or pores in membranes, but are not involved in tight chiral interactions with a receptor enzyme or chiral lipid components. More than one mechanism may be operative for different antimicrobial peptides and in different organisms.

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TABLE 5 Lethal concentration (μM) of $C_A(1–13)Me(1–13)$ analogues against different test bacteria

Peptide structure	Escherichia coli	Pseudomonas aeruginosa	Bacillus subtilis	Bacillus megaterium	Streptococcus pyogenes	Staphylococcus aureus	Red cell
Normal	0.7	4	0.9	0.5	0.7	2	320
Enantio	0.8	2	1	0.5	0.8	0.8	430
Retro	1	6	10	0.5	0.7	0.5	100
Retroenantio	1	7	13	0.3	1	3	100

Summary

The areas of value of chemical synthesis of peptides in the study of the cecropins can be summarized as follows:

(1) Correcting and confirming sequences of the natural peptides, for establishing their C-terminal amide structures and for eliminating the possibility that activity of isolated products is due to highly active impurities.
(2) Examining the processing of precursors of the mature peptides.
(3) Relating peptide composition and structure to function.
(4) Preparation of chimeric (hybrid) peptides with increased activity.
(5) Deducing mechanisms of action.
(6) Demonstrating the formation of ion channels in lipid bilayers.
(7) Determining the effect of chirality on activity and ion channels via synthesis of D-enantiomers.
(8) Determining the effects of peptide sequence and amide bond direction on activity via synthesis of retro and retroenantio isomers.

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DISCUSSION

Lehrer: Have you done any studies with mixtures of the D- and L-peptides?

Merrifield: Just a few, but they didn't come out the way we expected them to.

Lehrer: How did you expect them to come out and how did they come out?

Merrifield: I thought that for pore formation to occur, six all-D-peptides or all-L-peptides would be needed to give a conducting pore, and that if one or two peptides competed for a position in the pore, they would minimize the amount of homopeptide bundles, so you wouldn't get as much channel-forming activity. However, when we used mixtures of all-D- and all-L-peptides, we actually got the same activity as we did with either component singly. It may be that the peptides of the same chirality are selected for a pore and that we had mixtures of all-L pores and all-D pores, which conducted independently. We did do one experiment where we used a peptide that was half-L and half-D (i.e. the N-terminal end contained D-amino acids and the C-terminal end contained L-amino acids). The activity was not distinguishable from the all-L-peptide.

Sahl: In your planar membrane work, what was the ion selectivity of the channels? Was there a preference for anions?

Merrifield: Yes, but there was only about a twofold selectivity for Cl^-. This is not very much and we would have expected to see a much higher value than that. There was also the problem of the concentration effect, which was not as great as expected—selectivity should go up exponentially, with the exponent equal to the number of chains per pore. Aggregation in solution was offered as an explanation for the observed result.

Flajnik: You showed that some amino acid residues in the cecropin structure are critical for its function. Presumably it is possible to identify the amino acids that differ between the insect and the pig cecropin sequences. What happens if you change these amino acids? Are they critical for function?

Merrifield: We don't have any data on this at the moment.

Boman: The single residue that appears to be the most important in the cecropin structure is the tryptophan. We have substituted the tryptophan residue with phenylalanine, which cut down activity by two- to ten-fold. Then we put in glutamic acid, which was a rather drastic change, and this cancelled all the activity. The tryptophan is important; it's conserved in all the cecropins isolated so far, either at base 1 or base 2.

Merrifield: I don't think it's very clear why the tryptophan is important except for its hydrophobic aromatic character. This is one of the things that needs to be studied in more detail.

Kreil: About 20 years ago, we showed that upon addition of melittin to phospholipids, the indole ring of its sole tryptophan residue is immersed into the hydrophobic region of the lipid bilayer (Mollay & Kreil 1973). This leads to a blue shift of the fluorescence maximum. Have you observed this with cecropin as well?

Merrifield: We haven't measured this, but it is what I assume the tryptophan must be doing, since you can replace it with another aromatic amino acid, phenylalanine, and the cecropin analogue still has appreciable activity. It probably

has to do with the hydrophobic aromatic character of the residue and it's likely that it does interact with the lipid.

Boman: Günther Kreil, you are hitting on something essential, because the whole idea of trying to get melittin–cecropin hybrids was derived from the fact that the melittin sequence was like an inverted cecropin sequence: there is a tryptophan residue ahead of a number of basic residues in both of these peptides.

Kreil: Procecropin with the additional tetrapeptide APEP at the N-terminal end is much less active: does it still bind to membranes? I did an experiment many years ago (unpublished) where I mixed [^{3}H] promelittin and ^{14}C-labelled melittin with lecithin. After centrifugation, more than 95% of the melittin floated with the lipid layer, while the promelittin remained in solution. Is this also the case with procecropin and cecropin?

Merrifield: I don't know. Yours is an interesting experiment, which we should repeat with our peptides. I had assumed that activity depended upon the ability of the peptide to form helical bundles. It may be that the bundle aggregates are much more soluble in the lipid than is a single helical chain, because I would not expect such a dramatic difference in partitioning of procecropin.

Boman: It's a matter of scale. In the original paper (Boman et al 1989), the most dramatic effect is seen on *Micrococcus*, where only the mature peptide was active. For *Escherichia coli* and the other two bacteria, there was a scale of different activities, but the mature cecropin A was clearly the most active.

We (Hugosson et al 1994) wondered how it was possible to synthesize a membrane-active antibacterial peptide in cells which themselves contain a number of membranes. We have followed up work which was initiated by Michael Zasloff looking at the activity of peptides on mitochondria (Westerhoff et al 1989). It is probable that mitochondria are endosymbionts of bacterial origin and consequently the effect of these antibacterial peptides on mitochondria might tell us something interesting about the early action of peptides, because mitochondria are not lysed by cecropin, while bacteria are quickly lysed. This is important, because in mitochondria you can study a reaction without lysis occurring.

Ham: I wanted to ask about the activity of cecropins against *Plasmodium falciparum*. I know the activity is very low, but there appeared to be a difference between the effects of cecropin A and B. Is that significant? Do you have any explanation for this?

Merrifield: Cecropins A and D, as well as magainin, all have very little activity, but the cecropin–melittin hybrids are very active. Just as with the bacteria, you can make some correlation between activity and peptide structure, but a real understanding is still uncertain.

Elsbach: It surprised me that it is still so difficult to discern a direct correlation between activity and certain modifications of the overall structure of these antimicrobial peptides. But in that same framework of taking a given compound and then testing it with different types of microorganisms, it's quite striking

that if you compare, for instance, *E. coli* and *Pseudomonas aeruginosa*, there are clearly major differences in activity. In other words, it appears that the particular substrate or target that you present to your peptide makes a big difference to its activity. I wonder how much information one can draw from these differences in terms of what the peptide needs in order to bind to its target and then exert an effect on the envelope, which is obviously related to its biological effect?

Merrifield: On the basis of the activity of the D-enantiomers, and now of the retro and retroenantio analogues, I continue to believe that these peptides do not bind to specific receptors like the peptide hormones do. I still prefer the idea of effects on the membrane channels or the induction of pores in the membrane as an explanation for the relative activities of these peptides. However, it is possible that there are a number of different mechanisms of action and that one peptide will behave against different organisms in different ways. So I don't think it's likely that the lysis effect is going to be by identical mechanisms in all cases.

Elsbach: But in analysis of the structural requirement for bioactivity, have you paid much attention to distribution of the charge and the net charge of the peptide in relation to its target affinity?

Merrifield: There is some correlation with charge, but the location of the charge obviously can be different. Most of the positive charges of the cecropins are near the N-terminus and for melittin they are near the C-terminus. The magainins are not as charged as the cecropins, for instance, but they are still active. It remains a puzzle.

Zasloff: From the work that we've been doing over the last few years, we still don't know the determinants of the specificity of peptides. One of the most important experiments was done by Shunji Natori, on sapecin. It was his work that showed that, at least in part, the activity of sapecin against *E. coli* is dependent upon the affinity of that molecule for cardiolipin, which is an anionic phospholipid displayed on the outer leaflet of the cytoplasmic membrane of *E. coli*.

Natori: We did two experiments. The first was that we tested the binding of sapecin to various phospholipids present in the bacterial membrane. We found that sapecin preferentially binds to cardiolipin, which is a major phospholipid of Gram-positive bacteria. In the second, we tested the susceptibility to sapecin of an *E. coli* mutant with a defect in cardiolipin synthesis. We found that the cardiolipin-deficient mutant was much less sensitive to sapecin than wild-type *E. coli*, suggesting that cardiolipin is a target of sapecin (Matsuyama & Natori 1990).

Zasloff: I think that some of the subtleties of activity reflect still very poorly described interactions between our peptides and lipids. This is a very difficult area to study.

Elsbach: And not necessarily just lipids. There are also many non-lipids, especially in Gram-negative bacteria, that one also has to take into consideration as determinants of interactive events.

Merrifield: On the basis of the D-analogue and the lack of a tight chiral interaction, it seems to me that the non-polar environment of the lipid is what induces the peptide to form its own structure.

Elsbach: I couldn't agree more; it's especially true for the larger antimicrobial proteins and peptides, or any membrane-interactive molecule where you have these induced alterations in structure the moment there is a hydrophobic interaction.

Merrifield: The helicity of these peptides in solution depends on the polarity of the medium, and this agrees with Peter Elsbach's comment.

Hultmark: You find that when you change single amino acid residues in the cecropin molecule, the activity is sometimes increased against one species of bacteria and decreased against another. At first sight this is confusing, but this is a complex situation and it's not only the specific interaction of the peptide with the cell membrane that you are testing, but there are also other factors that might affect the activity. For instance, interaction with the outer membrane may effectively lower the real concentration interacting with the inner membrane. Sensitivity of the cecropin to proteases might also affect its activity: proteases are made more by some bacteria and less by others. So when you test the effect of cecropin on living bacteria, you should perhaps expect to get complex results, because there are other aspects than just the interaction with the cell membrane which you are looking at.

Rees: How do you standardize the amount of biological material in these bioassays when you are comparing the relative inhibitory concentration against, for example, different bacteria? Because if these peptides are membrane active, then a small bacterium will have a greater area of membrane proportional to its mass than a larger one.

Merrifield: Steiner et al (1988) found with different bacteria of different size and shape that the amount of peptide required for lysis was roughly equivalent to the amount needed for forming a monolayer around that bacterium, so for different species it would take different amounts.

Boman: I think this may hold for liposomes. However, some of the Gram-positive bacteria, like *Bacillus*, are considerably larger than the Gram-negative bacteria, and yet the lethal concentration (LC) and minimum inhibitory concentration (MIC) values are of the same order. There may be something in this, but it's not a universal rule. That, again, may go back to the fact that the peptides may have several mechanisms of action.

Elsbach: In this context, are all the assays that you referred to radial diffusion assays in agar plates?

Merrifield: We used two assays: one is a measure of the LC by the radial diffusion plate assay in agarose and the other is a measure of the MIC in liquid culture medium in the test-tube. The relative activities for a series of peptides correlate well, but there's an absolute difference, with the MIC about fourfold higher than the LC. However, you can correct for that by changing the inoculum

size, which makes it possible to get the same numerical answer from both assays.

Ganz: Have you ever tried testing the peptides in solid phase when they are attached to beads; are they still active?

Merrifield: I've never done this sort of assay, although we have discussed it several times.

Ganz: Because that has to do with the question of whether they need to enter into the bacterium itself to be active, or whether it is enough for them to penetrate the membrane without entering the cell.

Merrifield: The main problem with this kind of experiment is the fact that only an incredibly small proportion (about 0.003%) of the total peptide is on the surface of the beads, the rest is inside. I don't think the experiment would work.

Rees: You might find that concentration is a limitation: that with the peptides on the beads you can't get a high enough concentration in solution to do the experiment.

Lehrer: Do you have any information about the stability of the D-enantiomers of these peptides, either in mammalian cells or in whole animals? Are they degraded?

Merrifield: In serum they were quite stable. Hans Boman's lab did some experiments and found that in rabbit serum, D-cecropin was at least 15 times more stable than L-cecropin.

Zasloff: They're chemically stable in the body fluids of an animal.

Merrifield: That was what we hoped, of course, at the beginning. We thought D-peptides would be really nice because they were fully active and should persist *in vivo* for a long time.

Lehrer: Yes, one would predict that they would be stable in body fluids. I wonder if they get into the reticuloendothelial system (using an old terminology). Do you have much information about that?

Zasloff: We have insufficient evidence.

Elsbach: But when you're talking about stability, is it also biological stability in body fluids?

Zasloff: They, like polymyxin, find tight binding sites which haven't yet been characterized.

Merrifield: The stability study in serum was based on antibacterial activity after treatment.

Kreil: Are these peptides containing only D-amino acids antigenic?

Merrifield: In an experiment that I know about they were not immunogenic.

Boman: I have had lengthy discussions with several immunologists about that; I think it depends on the mechanism by which the D-peptides are going to be presented. I don't think they can be presented by the major histocompatibility complex. An immune response, if there is one, has to be started from a pre-existing B cell.

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Gene-encoded antibiotics made in bacteria

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Abstract. Production of antimicrobial peptides and proteins is very common among bacteria and a variety of such substances has been described. In general Gram-negative bacteria produce protein bacteriocins (e.g. colicins) with narrow action spectra based on receptor-mediated activity. They produce comparatively few peptides, such as the post-translationally modified microcin B17. In contrast Gram-positive bacteria tend to produce peptide bacteriocins smaller than 10 kDa and of wider activity spectra. These show particular potential for application. They can be divided into unmodified peptides (e.g. lactococcins, lactacins, pediocins) and lanthionine-containing peptides (lantibiotics, e.g. nisin, epidermin, Pep5). The unmodified peptides are mostly hydrophobic or amphiphilic and act by disturbing the function of the cytoplasmic membrane. They are synthesized as prepeptides with a characteristic N-terminal leader peptide. In some cases genes for immunity peptides were found in close proximity to structural genes; furthermore, two-component response regulators seem to be involved in the regulation of their synthesis. The biosynthetic genes for lantibiotics are also organized in operons. Lantibiotic gene clusters include genes encoding the unique enzymes which dehydrate serine and threonine and form the characteristic thioether-bridged lanthionines. Three types of lantibiotics are currently distinguished on the basis of structural features and functional aspects: type A, which include elongated, amphiphilic, pore-forming peptides (e.g. nisin); type B, which are of globular shape and inhibit phospholipases (e.g. duramycins); and type C (e.g. actagardine) with intermediate features which act by inhibiting bacterial cell wall biosynthesis.

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Production of antagonistic substances is a highly important factor in microbial ecology. Among the many different substances known to play a role in bacterial interactions, bacteriocins are the most specific and efficient antagonists. The term 'bacteriocins' designates bacterial peptides and protein antibiotics which are ribosomally made or derive from gene-encoded precursor peptides and towards which the producing strain posseses a specific self-protection mechanism ('immunity'). Such bacteriocins have been found in all bacterial species in which they have been looked for; it has been assumed that they will be found to occur in

all bacteria, provided enough indicator strains are tested and growth conditions resemble those found *in vivo*, i.e. not in pure culture, not on rich media and not at optimum growth conditions (Tagg 1992). Although bacteriocin production was recognized long ago and since then has been studied intensively, detailed knowledge of this phenomenon is limited to some groups of bacteriocins from bacteria which are easy to grow and study, such as Enterobacteriaceae (colicins), *Pseudomonas* (pyocins), lactic acid bacteria (e.g. lactococcins, lantibiotics), and streptococci and staphylococci (e.g. lantibiotics). In general, it seems that as a result of the different cell wall architecture, bacteriocins of Gram-positive and Gram-negative bacteria have evolved differently with respect to size and specificity (Table 1). In Gram-negative bacteria the outer membrane necessitates receptor-mediated antagonistic activities and very specific proteins are produced with domains for receptor binding, translocation and activity. In contrast, Gram-positive bacteria possess a multilayered peptidoglycan wall without an outer membrane. This favours small peptides reduced to the minimum size for activity which can penetrate the murein network without receptor binding and specific translocation. Consequently, the activity spectra of these bacteriocins are generally wider than those of, for example, the colicins. This paper gives an overview on bacteriocins with the focus on lantibiotics, of which nisin has gained considerable importance as a food preservative (Hurst 1981).

Bacteriocins of Gram-negative bacteria

Antagonism between strains of *Escherichia coli* was observed as early as 1925 (Gratia 1925) and the putative active substances were designated colicins. They are the prototype bacteriocins and have been studied intensively as model systems. Colicins either form voltage-dependent channels in the cytoplasmic membrane (e.g. colicins A, B, E1, I, N) or act as nucleases (colicin E2, E3). Colicin M is unique as it acts by arresting murein biosynthesis and is considerably smaller (29 kDa) than the others (60–90 kDa). Colicin action takes place in three successive steps.

(1) Binding to specific receptor proteins (e.g. BtuB, OmpF) in the outer membrane of target bacteria.

(2) Unfolding and translocation through the outer membrane and the periplasmic space to the cytoplasmic membrane which is the target site for the channel-forming colicins and colicin M; the nucleases are further translocated through the cytoplasmic membrane to the cytoplasm.

(3) Killing activity, i.e. channel formation which results in depletion of ions and collapses the electrochemical proton gradient, binding to C_{55}-polyisoprenol pyrophosphate which blocks murein precursor transfer, or cleavage of RNA and DNA.

This sequence of events is reflected in the domain structure of colicins: the N-terminus is necessary for translocation, the central part of the protein

TABLE 1   Gene-encoded antimicrobial peptides and proteins from bacteria (bacteriocins)

	Bacteriocin	Structural properties	Targets/mode of action
Gram-negative bacteria			
Common	Protein bacteriocins e.g. colicins A, E1 colicins E2, E3	>20 000 Da unmodified	Channel formation, RNase, DNase
	pyocins	Phage particles	Channel formation
Less frequent	Peptide bacteriocins e.g. microcin B17	<10 000 Da modified (thiazoles, oxazoles)	Gyrase inhibitor
Gram-positve bacteria			
Common	Peptide bacteriocins e.g. lactococcins lactacin	<10 000 Da unmodified	Membrane pertubation, pore formation
	e.g. lantibiotics type A,B,C	Modified (thioethers)	Pore formation, phospholipase inhibition, cell wall biosynthesis inhibition
Less frequent	Protein bacteriocins	>10 000 Da	Enzymic activities (e.g. cell wall degradative enzymes, phospholipases)

recognizes the receptor and the C-terminus forms the channel or acts as a nuclease; in the latter case the C-terminal domain is cleaved off and then enters the cytoplasm.

The synthesis of colicins is regulated by the LexA repressor and induced under so-called SOS conditions. The structural gene is organized in an operon together with a gene for a bacteriocin release protein (lysis protein; colicins are not exported but released from cells after disruption from inside, 'lethal synthesis') and an immunity protein which protects non-producing cells of a colicinogenic strain. Those immunity proteins that antagonize channel-forming colicins reside in the cytoplasmic membrane and interact with the channel domain, while the nucleases are released as an inactive stoichiometric complex with the immunity protein; the complex dissociates upon binding to receptors of a target cell.

Colicin V, the first colicin to be discovered, turned out to be a small peptide (6 kDa) which for several reasons has to be grouped with the microcins (Kolter & Moreno 1992). The latter are a heterogeneous group of peptide antibiotics from Enterobacteriaceae of which microcin B17 is most interesting because it is a potent inhibitor of bacterial gyrase. It has much in common with the lantibiotics (see below) in that it is derived from a precursor peptide which is post-translationally modified. Modifications take place at the peptide backbone and

include serine, cysteine and glycine residues, resulting in thiazole and oxazole rings (Bayer et al 1993). The peptide is activated by removal of an N-terminal leader peptide. Biosynthesis genes are organized in an operon (Kolter & Moreno 1992).

Bacteriocins of Gram-positive bacteria

The need for better food preservation and the successful use of the lantibiotic nisin in dairy products (Hurst 1981) has recently stimulated a remarkable revival of interest in bacteriocins of lactic acid bacteria (Klaenhammer 1993). Application of modern purification protocols and molecular genetics has provided a solid body of information and research is still increasing in quantity and quality. Thus, *Lactobacillus*, *Lactococcus*, *Pediococcus* and other genera of lactic acid bacteria are those best studied, but there is also information on bacteriocins from staphylococci, streptococci and bacilli which allows us to draw the following picture.

In Gram-positive bacteria, production of antibiotic peptides is the rule; proteins of the size of colicins are hardly ever encountered. Many of the numerous large bacteriocin complexes reported in the early literature (Tagg et al 1976) may be small peptides, because of insufficient purification procedures. This was exemplified for staphylococcin 1580 which was described as a bacteriocin of several hundred kilodaltons but is identical to the lantibiotic epidermin (Sahl 1994). In some cases, antagonistic proteins turned out to be exoenzymes with cell wall-degrading or phospholipase activity (e.g. megacin A). Helveticin J is a 37 kDa bacteriocin from *Lactobacillus helveticus* which has been cloned and sequenced (Klaenhammer 1993); its mode of action is unknown.

The majority of the peptide bacteriocins are relatively thermostable, have molecular masses of 2–6 kDa and are currently subdivided into lantibiotics (see below) and unmodified peptides (non-lantibiotics). The latter share several characteristic features.

(1) The structural genes from which the peptides are derived code for prepeptides with an N-terminal extension (leader peptide); selected peptides are shown in Table 2. The leader peptides show some sequence homology, particularly at the proteolytic processing site where two glycine residues are at positions −1 and −2. Leader peptides are predicted to be amphiphilic; a hydrophobic stretch typical of signal peptides of export proteins is missing.

(2) Flanking regions upstream and downstream of the structural gene contain additional open reading frames for which a function in bacteriocin production was either demonstrated or postulated on the basis of sequence homologies (Table 2). Such genes code for immunity peptides, for two-component regulatory proteins consisting of a histidine kinase and the respective regulator, as well as proteins with significant homology to the ABC-transporter protein family. There are also indications of an operon-like organization of such genes, although

there is currently no example of a complete operon where all these functions have been identified and clearly attributed to the production of one of these bacteriocins. Additionally, no proteases have been identified in any gene cluster, suggesting that there is no need for a specific protease to cleave this otherwise unusual processing site (Table 2).

(3) The processed, active bacteriocins contain a substantial amount of hydrophobic amino acids or, on the basis of prediction plots, have the potential to form amphiphilic helices. This suggests that the plasma membrane could be the target for the antimicrobial activity of such peptides, as has been shown for lactococcin A (van Belkum et al 1991). This peptide dissipates the membrane potential of sensitive bacterial cells; insensitive strains and the immune producer strain are not affected at comparable bacteriocin concentrations. Lactococcin A also induced amino acid efflux from cells which had been de-energized by valinomycin and nigericin before bacteriocin treatment. It was concluded that lactococcin A depolarizes the energy-transducing cytoplasmic membrane in a potential-independent fashion and that for activity it requires a specific receptor in the membrane. Other peptides, however, require a membrane potential for activity (Bruno & Montville 1993).

Lanthionine-containing antibiotic peptides (lantibiotics)

The designation 'lantibiotics' was proposed with reference to the most prominent features of these peptides, the antibiotic activity and the content of the thioether-forming amino acid lanthionine (Schnell et al 1988). Besides lanthionine and its analogue 3-methyllanthionine all lantibiotics contain didehydroalanine and/or didehydrobutyrine. Individual lantibiotics may contain further unusual residues such as S-aminovinyl-cysteine or lysinoalanine (Jung 1991). A list of the lantibiotics described to date is compiled in Table 3. On the basis of structural features, Jung (1991) distinguished type A (e.g. nisin, subtilin, epidermin, Pep5) and type B (cinnamycin, duramycin, ancovenin) lantibiotics with mersacidin and actagardine sharing properties of both groups. Nisin and epidermin were isolated from different strains of *Lactococcus lactis* and *Staphylococcus epidermidis*, respectively. In addition, structural variants were described such as nisinZ ([^{27}Asn]-nisin), gallidermin ([^{6}Leu]-epidermin) and [^{1}Val, ^{6}Leu]-epidermin. Recently, several lantibiotics from streptococci and lactic acid bacteria were identified on the basis of lanthionine detection in peptide hydrolysates (carnocin U149, Stoffels et al 1992; lactocin S, Mørtvedt et al 1991). In the case of lacticin 481 (Piard et al 1993), streptococcin A-FF22 (Hynes et al 1993) and salivaricin A (Ross et al 1993), partial amino acid sequences allowed the synthesis of DNA probes and therefore detection and sequencing of the structural genes. However, proposals for structures on the basis of nuclear magnetic resonance (NMR) data only were given and thioether bridges could not be assigned to defined positions.

TABLE 2 Selected unmodified peptide bacteriocins from Gram-positive bacteria

Peptide	Amino acid sequence: leader peptide/mature bacteriocin	Additional genes identified (function)
lactococcin A	MKNQLNFNIVSDEELSEANGG/ KLTFIQSTAAGDLYYNTNTHKYVYQQTQNAFGAAANTIVNGWMGGAAGGFGLHH	lciA (immunity) lcnD (transport)
lactacin F	MKQFNYLSHKDLAVVVGG/ RNNWQTNVGGAVGSAMIGATVGGTICGPACAVAGAHYLPILWTGVTAATGGFGKIRK	orfs x, y, z
pediocin PA-1	MKKIEKLTEKEMANIIGG/ KYYGNGVTCGKHSCSVDWGKATTCIINNGAMAWTCGGHQGNHKC	pedB, pedC, pedD (transport)
leucocin A	MMNMKPTFSYEQLDNSALEQVVGG/ KYYGNGVHCTKSGCSVNWGEAFSAGVHRLANGGNGFW	orf 2 (immunity?)
plantaricin A	MKIQIKGMKQLSNKEMQKIVGG/ KSSAYSLQMGATAIKQVKKLFKKWGW	plnB, plnC, plnD (response regulator)

For references, see Klaenhammer (1993).

TABLE 3 Lantibiotics identified to January 1994

Lantibiotic	M_r	Charge	Producer strain	Structure	References
Pep5	3488	+7	Staphylococcus epidermidis	Established by chemical and spectroscopical methods	Jung (1991)
Nisin	3353	+3	Lactococcus lactis		
Subtilin	3317	+2	Bacillus subtilis		
Epidermin	2164	+3	Staphylococcus epidermidis		
Cinnamycin	2041	+1	Streptomyces cinnamoneus		
Duramycin	2012	+1	Streptomyces cinnamoneus		
Ancovenin	1959	0	Streptomyces sp.		
Mersacidin	1825	−1	Bacillus subtilis	Established by 2D NMR	
Actagardine	1890	0	Actinoplanes sp.		
Lacticin 481	2901	0	Lactococcus lactis	Lanthionine present, primary structure deduced from gene, thioether pattern not proven	Piard et al (1993)
Streptococcin AFF 22	2795	+1	Streptococcus pyogenes		Hynes et al (1993)
Salivaricin P 20	2315	0	Streptococcus salivarius		Ross et al (1993)
Lactocin S	3769	?	Lactobacillus sake	Lanthionine present	Mørvedt et al (1991)
Carnocin IU 49	4635	?	Carnobacterium piscicola		Stoffels et al (1992)

33

A

Pep5

Nisin

Epidermin
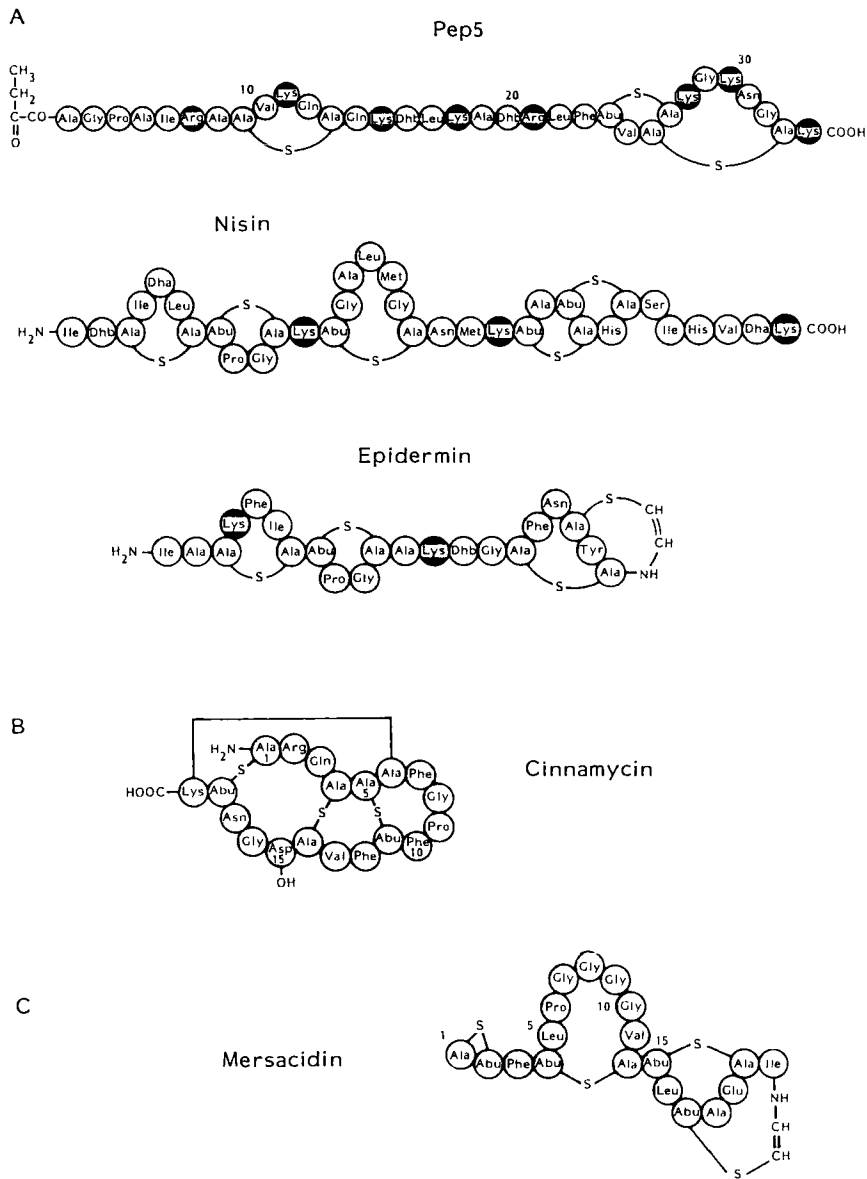

B

Cinnamycin

C

Mersacidin

FIG. 1. Primary structures of selected representatives of type A, B and C lantibiotics. The amino acids derived from post-translational modifications of serine, threonine and cysteine are: Dha, dehydroalanine; Dhb, dehydrobutyrine; Ala–S–Ala, lanthionine; Abu-–S–Ala, 3-methyllanthionine. For further details refer to Jung (1991).

Primary and spatial structures

Representative peptides of each subgroup of lantibiotics are given in Fig. 1. Type A peptides are elongated, rod shaped and positively charged. The bridging pattern shows similarities between the nisins, subtilin and epidermins, while Pep5 is rather different. However, rings are always formed from a C-terminal cysteine to an N-terminally located dehydroamino acid. Circular dichroism spectra and two-dimensional NMR experiments indicated that in aqueous solution the peptides are without a defined spatial structure and are very flexible, especially in the central region of the molecule. Only the small rings have less conformational freedom and are somewhat rigid. This is particularly the case with Pep5, which has only three lanthionine rings. In trifluoroethanol or detergent micelles, the peptides adopt screw-like, helical shapes which result in separation of polar and non-polar amino acids on opposite sides of the molecule. The overall dimensions range from 3 nm long and 1 nm in diameter for gallidermin to 5 nm long and 2 nm diameter for nisin. Dipole moments of 50 Debye for nisin and 80 Debye for gallidermin were calculated (e.g. van de Ven et al 1991, Freund & Jung 1992, Jung & Sahl 1991).

In contrast, type B lantibiotics have a typical head-to-tail bridging pattern with an additional lysinoalanine bridge in duramycins (Jung 1991). This results in clearly defined, highly rigid solution structures and a globular shape of the peptides (Freund & Jung 1992). Type B lantibiotics have net charges of + 1 or 0; lanthionine bridges are formed in both directions, i.e. from N-terminal cysteines to C-terminal dehydro residues and vice versa.

Type C lantibiotics so far include only two peptides, mersacidin and actagardine, which, in spite of serious differences, were first included in type A lantibiotics mainly because they do not fit into the clear sequence homology and ring pattern of type B peptides (Jung 1991). Because recent studies showed that they also have different modes of action, here I propose they form a separate group. These peptides are more globular than type A peptides but lack the head-to-tail bridge of type B lantibiotics; solution structures have not been reported so far. Rings are also formed from both directions.

Modes of action

The different structural properties of the subgroups are reflected in three different modes of action. The primary activity of type A lantibiotics is based upon formation of voltage-dependent, short-lived pores in the cytoplasmic membrane. This model is derived from a series of experiments with intact cells, membrane vesicles and planar lipid bilayers (Sahl 1991, Benz et al 1991). These studies showed that the peptides rapidly induce leakage of ions and small metabolites from bacterial cells and a collapse of the electrochemical proton gradient. Loss of precursors and energy leads to the cessation of biosynthetic

processes and eventually to cell death. Type A lantibiotics require a driving force for pore formation which is the proton motive force; one of its components, the electrical potential $\Delta\psi$ or the chemical proton gradient ΔpH, can promote activity alone provided it is of sufficient magnitude; e.g. the threshold value for the membrane potential at neutral pH is 50–100 mV, depending on the individual peptide and the bacterial cells used as indicators (Sahl 1991). Similar values were obtained with black lipid bilayers. Epidermin needed only 50 mV and formed rather long-lived (up to 30 s) and well-defined pores with diameters of 0.5 nm, whereas nisin, subtilin and Pep5 induced membrane conductance only at more than 80–100 mV; pores were unstable with rapidly changing conductance levels (pore lifetimes in the millisecond range) but with considerably larger diameters (approximately 1–2 nm). Pores were assumed to be formed by a transiently associated peptide oligomer in a transmembrane orientation (barrel stave model) as suggested for alamethicin (Sahl 1991).

The susceptibility of different bacterial species, or even of strains within one species, towards a particular peptide varies much more than one would expect on the basis of the pore-formation model for which the only prerequisite is sufficient energization of the target membrane. This indicates that *in vivo* pore formation or pore stability may be positively or negatively influenced by factors such as phospholipid composition of the membrane, interaction of the peptides with integral membrane components, the presence of surface layers, etc. In addition, the peptides could exert secondary effects which contribute to the bactericidal activity. Such an effect was observed with staphylococci, which are particularly sensitive to Pep5 and nisin. In this case the lantibiotics induce autolysis of cells by activating the cell wall-hydrolysing enzymes of the target cell. The cationic peptides displace the autolytic enzymes from their cell wall-intrinsic inhibitors, the strongly anionic polymers teichoic and lipoteichoic acids, which results in an apparent enzyme activation (Bierbaum & Sahl 1987). This effect may also be relevant for the activity of other cationic peptides such as the defensins and cecropins.

The antibacterial effect of type B lantibiotics is rather weak and their action on eukaryotic cells may be more interesting for potential applications. They bind to the head group of phospholipids, preferentially to phosphoethanolamine and thereby inhibit in a non-competitive way phospholipase A_2 (Märki et al 1991). Ancovenin inhibits the angiotensin converting enzyme and cinnamycin displays some immunostimulatory effect which is based on a partial structural similarity to thymopoietin.

Actagardine, first described as gardimycin (Jung 1991), was reported to inhibit cell wall biosynthesis and to cause accumulation of membrane-bound cell wall precursors (Somma et al 1977). Mersacidin is particularly active against staphylococci, and is as effective as vancomycin *in vivo* (Limbert et al 1991). Recently, we obtained evidence that it also blocks peptidoglycan biosynthesis; although the molecular target of mersacidin is not yet characterized, it is likely

processing site

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PepA   M - - KNNKNLFDLEIKKETSQNTD-ELLEPQ   TAGPAIRA-SVKQCQKT--LKATRLFTVSCKGK-NGCK
NisA   MST-KD----FNLDLVSVSKK--DSGASPR      ITSISL---CTPGC-KTGALMGCNMKTATCHCSIHVSK
SpaA   MS--KFDD--FDLDVVKVSKQ--DSKITPQ      WKSESL---CTPGCV-TGALQTCFLQTLTCNCKII--SK
EpiA   MEAVKEKNDLFNLDVKVNAKESNDSGAEPR      IASKFII--CTPGCAKTGS----FNSYCC
LctA   M---KEQNS-FNLLQEVTESEL-D-LILGA      KGGSGVIHTISHECNMNSWQ----FVFTCCS
ScnA   ME--KNNEV-INSIQEVSLEEL-D-QIIGA      GKNGVFK-TISHECHLNTWA----FLATCCS
SalA   MNAMKNSKD--ILNNAIEEVSEKELME--VAGG   KRGSGWIATITDDCPNS-V-----EV--CC
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FIG. 2.   Alignment of the prepeptides of the lantibiotics Pep5 (PepA), nisin (NisA), subtilin (SpaA), epidermin (EpiA), lacticin 481 (LctA), streptococcin A-FF22 (ScnA) and salivaricin A (SalA). For references see: LctA (Piard et al 1993), ScnA (Hynes et al 1993), SalA (Ross et al 1993). Alignment was done manually based on the Clustal V software.

Subtilin

spaB   spaT spaC spaS   spaR spaK

Nisin

nisA   nisB   nisT   nisC nisI   nisP   nisR

Epidermin

epiT˙˙ epiT˙ epiA   epiB   epiC epiD epiQ epiP

Pep5

pepT   pepI   pepP   pepB   pepC

pepA
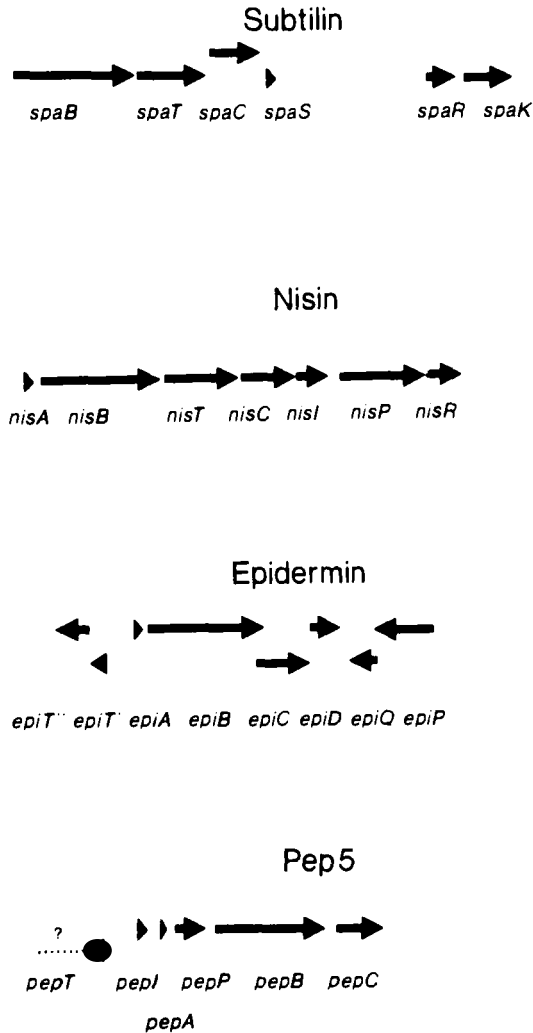
FIG. 3. Genes involved in biosynthesis of lantibiotics. Designation of genes follows the recommendations given at the First International Workshop on Lantibiotics (Jung & Sahl 1991): A, structural genes (except *spaS*); B, C, code for proteins of unknown function; D, codes for an enzyme which oxidizes lanthionine to aminovinyl-cysteine at the C-terminus of epidermin; I, genes for immunity; P, genes for processing proteases; R, genes for regulatory proteins (except *epiQ*) and K for corresponding histidine kinases; T, genes for transporters of the ABC transporter family. The gene sizes are drawn to scale. For references see Klein et al (1992), Bierbaum & Sahl (1993), Hansen (1993), van der Meer et al (1993), Kuipers et al (1993a); sequences of the *Pep5* operon are unpublished (C. Meyer, C. Heidrich, M. Reis, G. Bierbaum & H.G. Sahl 1993).

that its action differs from that of antibiotics such as β-lactams and vancomycin which are currently in use as chemotherapeutics for staphylococcal diseases (H. Brötz, G. Bierbaum & H.G. Sahl, unpublished results 1993).

Biosynthesis of lantibiotics

Almost all the information available on the biosynthesis of lantibiotics derives from work with subtype A peptides. Like the unmodified bacteriocins of lactic acid bacteria, lantibiotics are synthesized from prepeptides with characteristic N-terminal leader peptides. These leader peptides are able to form amphiphilic helices (Jung 1991) and have a net negative charge, while the propeptide parts are positively charged. Sequence alignment of the prepeptides as deduced from structural genes (Fig. 2) does not yield strictly conserved motifs throughout all peptides although there is significant homology within two groups, the nisin group (nisin, subtilin, epidermin) and the lacticin 481 group (lacticin 481, streptococcin A-FF 22, salivaricin A); Pep5 differs from both groups but has more similarity to the nisin group, particularly in the leader peptide.

In the case of lantibiotics, the prepeptides are modified before processing and secretion. Modification starts with specific dehydration of serine and threonine residues in the propeptide parts, yielding dehydroamino acids with an $\alpha-\beta$ double bond; cysteine thiol groups are then added to some of the double bonds to form the lanthionine or methyllanthionine rings. This postulated sequence of events was verified in the case of Pep5 by isolation of biosynthetic intermediates (Weil et al 1990). As with the unmodified bacteriocins from lactic acid bacteria, genes for biosynthetic enzymes were found in an operon-like organization with the lantibiotic structural genes. Sequence data are available for nisin, subtilin, epidermin and Pep5 (Fig. 3). On the basis of sequence comparisons of putative proteins and on functional analysis in some cases (e.g. NisI, NisP, EpiD, EpiQ, PepI), the following picture is emerging (Klein et al 1992, Peschel et al 1993, van der Meer et al 1993, Kuipers et al 1993a, C. Meyer, C. Heidrich, M. Reis, G. Bierbaum & H.G. Sahl, unpublished results 1993).
(1) Biosynthesis of lantibiotics is regulated by two-component regulatory proteins (SpaR, NisR and EpiQ were identified as regulators, the corresponding histidine kinase SpaK has so far only been found for subtilin).
(2) Serine proteases which could function as leader peptidases were identified (NisP, EpiP, PepP).
(3) Peptide secretion could be accomplished by ABC transporters (SpaT, NisT, EpiT, PepT) although in the case of epidermin and Pep5 these genes are not essential for production of the active lantibiotic.
(4) Immunity peptides were identified for Pep5 (PepI) and nisin (NisI).
(5) Proteins designated with B and C (e.g. NisB, NisC) were found in all four gene clusters (Fig. 3) and seem to be unique for lantibiotics; they are not found with the unmodified bacteriocins and do not have significant homology to known

proteins. It is tempting to speculate that these are involved in dehydration and lanthionine ring formation.

It is not known which factors direct pre-lantibiotics towards modification. It has been assumed that a specific signal is present in the leader peptide. The lack of a common sequence motif (Fig. 2) makes this rather unlikely; the leader peptide of SalA resembles those of the non-lantibiotic bacteriocins even more than those of the nisin group (Ross et al 1993). It is, however, possible that different signals are recognized within the nisin and the lacticin 481 groups. Differences between the type A and B lantibiotics may even be more serious; the cinnamycin prepeptide CinA, the only non-type-A lantibiotic studied so far, does not have any obvious sequence similarity to type A prepeptides (Entian & Kaletta 1991). Once the fundamental questions of the biosynthesis of lantibiotics are solved this unique biosynthetic machinery could be used to construct peptides with novel structural and functional properties; in that respect, recent success with manipulations in the nisin and subtilin structural genes (e.g. Hansen 1993, Kuipers et al 1993b) is very promising.

Acknowledgements

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(Restriction of the number of references made it necessary for me to cite reviews whenever possible. Please refer to e.g. Jung 1991, Jung & Sahl 1991, Kolter & Moreno 1992, Bierbaum & Sahl 1993, Klaenhammer 1993 and Hansen 1993 for detailed references.)

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DISCUSSION

Elsbach: It is a fascinating story that you have told us. I'm particularly intrigued by your ability to distinguish channel formation, activation of degradative enzymes and biosynthesis. How can you distinguish between an early event that involves energy metabolism *per se* and something that works, for example, on a proton pump? In other words, what comes first? How can you distinguish between the direct effect of the degradative enzyme that subsequently affects the structure of the membrane where the biosynthetic machinery is, and a process that first affects an energy-dependent biosynthetic event?

Sahl: I think that the channel formation happens first, as soon as the peptides reach the cytoplasmic membrane. This happens so rapidly that, under favourable conditions, efflux of e.g. amino acids is completed after 30 seconds. But you never see molecules bigger than nucleotides leaving the cell. This correlates nicely with the size of the pores that we see in planar membrane experiments.

Elsbach: What about amino acid uptake and transport?

Sahl: Once lantibiotics have been added to cells, there is no longer any uptake because the cells are de-energized—they do not have the membrane potential necessary for taking up amino acids any more. Amino acids which had been taken up by the bacteria but not incorporated (experiments are usually done

in the presence of chloramphenicol!) rapidly efflux from the cells; this happens within seconds of addition of the peptide.

The lysis is what I would call a secondary effect. Lysis is not a consequence of de-energization of the cells, at least not in the case of staphylococci. Others have shown that bacilli start to lyse after de-energization with a protonophore, but this does not happen with staphylococci. In staphylococci, enzymic activity is necessary to cause the bacteria to lyse. We isolated cell walls and autolytic enzymes, and we did kinetic studies in the presence of the peptides. This is not trivial, because you have an insoluble substrate, soluble enzymes and a soluble effector. The result was that the peptides activate the enzymes, but not in the sense that they are direct enzyme effectors; they do not interact directly with the enzymes, they displace them from their cell wall-intrinsic inhibitors, the teichoic acids, and then lysis starts. We have only done these experiments with staphylococci; it takes about 30 min for 50% of the cells to be lysed. This is why I propose that autolysis occurs much more slowly and therefore is a secondary effect.

Elsbach: I'm very intrigued by these observations, because it's very similar to what bactericidal permeability-increasing protein (BPI) does in activation of certain autodigestive enzymes (Elsbach & Weiss 1992, 1993).

Sahl: This is probably also a highly relevant effect in understanding the mechanism of action of the defensins. When the phagocytic cells have taken up bacteria, they do not necessarily need enzymes of their own to digest them, they can activate the autolytic enzymes of the bacteria themselves.

Boman: Which is fastest: lysis by an autolytic enzyme or lysis as a secondary phenomenon of channel formation?

Sahl: When you activate the enzymes, after half an hour, more than 50% of the staphylococcal cells are lysed. When you just depolarize the cells by a protonophore and then look for lysis, there is no significant effect, even after a few hours. In the case of *Bacillus*, autolysis triggered by de-energization sets in faster (Jolliffe et al 1981).

Boman: Cecropins lyse bacteria within minutes. If you look at a highly sensitive strain of bacteria, like an *envA* mutant, you can reduce viable counts from 10^8 to 10^4 CFU/ml in 1 min.

Lehrer: A problem here is that people are referring to different things by the term 'lysis'.

Sahl: To me, lysis means the disintegration of the cell envelope to such an extent that the cell explodes. It's not the same as killing.

Boman: By lysis, I meant decrease of optical density. You can record a significant decrease in optical density within a few minutes of treatment.

Sahl: Yes, but the optical density doesn't really tell you how many bacteria you have lysed.

Boman: No, I think that fairly large fragments are actually left. But if you have a decrease in optical density to 25% of the original value, then substantial lysis has occurred.

Andreu: How stable are type A lantibiotics in biological fluids?

Sahl: Their stability is variable. Pep5 is so highly charged that it is extremely labile to serine protease. But nisin and epidermin are quite stable, as are the globular type B and C molecules.

Andreu: How efficient is their biotechnological production, in comparison to their chemical synthesis, which is very difficult?

Sahl: There are two lantibiotics which are very promising with respect to their ease of biotechnological production. The first is nisin, of which yields of several hundred milligrams from one litre of culture can be obtained. One company produces nisin in 30 000 litre fermentors. The second is epidermin, which has also been produced at yields of 1 g of peptide per litre of culture (Ungermann et al 1991). But in both cases you have to use very rich media for getting these production yields, and this makes purification quite complicated.

Hansen: One very clear difference between these peptides and the eukaryotic antimicrobial peptides is that they have so many unusual amino acids: about a third of the amino acids are unusual ones. The double bonds of dehydroalanine have electrophilic activity and the thioether cross-linkages are rather stable. These give the peptides access to properties, chemistry and, potentially, mechanisms of action that aren't really accessible to peptides that don't have these features. It's somewhat ironic that at this point, perhaps, some of the mechanisms of action of attacking membranes appear superficially to be similar to what's happening with the eukaryotic peptides, but I think there's an underlying difference that may turn out to be quite profound.

Elsbach: Does that imply that the evolutionary aspects of these peptides are also related to what targets they are meant ultimately to hit? In other words, I would expect that these are non-toxic towards eukaryotic cells.

Sahl: I would think that depends on how you apply them. When you consume nisin, for example, it has about the same toxicity as NaCl. But if you injected it intravenously (i.e. if you want to use nisin as a systemic drug), it could be more toxic. Nisin is to some extent immunogenic, while it was not possible to find antibodies against epidermin and type B and C lantibiotics. One has to look at each lantibiotic individually with respect to its toxicity or applicability as a drug. There may be some which are very helpful and can be used as antibiotics (e.g. mersacidin); others, such as nisin, should be kept in the food area.

Lehrer: Do you have any information about the reversibility of the lantibiotic hit on your target bacteria? If you expose them to lantibiotics, allow them to depolarize and then wash away the lantibiotics, do they recover?

Sahl: They can do, but it depends completely on the conditions under which you do the tests. When you do an experiment in buffer of very low ionic strength without any nutrients present, killing occurs very fast, and you really have to wash quickly to restore viability. If you do it at 200 μM NaCl in nutrient medium, then killing happens much more slowly. It's obvious that the efflux of molecules

from the cells via diffusion is much slower in this case, but we have also shown that binding of the peptides to the cell wall is reduced at this salt concentration, resulting in lower antibacterial activity.

Zasloff: How much work has gone into the development of bacterial antibiotics as chemotherapeutic agents? As you point out, they have a spectrum that includes some medically important organisms.

Sahl: Nisin has been used as a food preservative, especially in dairy products; it has been so beneficial that I think it shouldn't be used as a chemotherapeutic agent because that would preclude its use in food. Some work has been done to develop mersacidin. It is particularly active against streptococci and staphylococci. We know that mersacidin inhibits cell wall biosynthesis, but it does it in a different way to vancomycin. And this is certainly something that we would like to pursue further in terms of developing it into a chemotherapeutic agent.

Natori: You mentioned that lantibiotics have no solution conformation but they have a conformation in SDS micelles or in trifluoroethanol. How soluble are these peptides in water?

Sahl: Again, it depends on the individual peptide. Pep5 is perfectly soluble in milligram amounts per millilitre. Nisin is also soluble, but only at low pH. Epidermin is not so soluble at neutral pH but prefers acidic pH. Some of the type B and C lantibiotics require at least 50% alcohol to be soluble.

Natori: Did you look at their hydropathy profiles?

Sahl: They're amphipathic peptides. They have up to seven or eight positive charges and the rest are hydrophobic amino acids. Type B lantibiotics are more hydrophobic—they have fewer positively charged residues.

Natori: You mentioned that these peptides disrupt membrane potential and that glutamic acid or glutamine leaks out from bacteria treated with them. Is this specific for these amino acids or do other substances leak out from these treated cells?

Sahl: We did experiments with potassium ions, rubidium ions and several amino acids, looking at the efflux of nucleotides such as ATP; all these compounds leaked out of treated cells. ATP does not come out with epidermin, which indicates that this pore is smaller. This correlates with the planar membrane results where pore diameters of up to 0.5 nm were found. ATP comes out when bacteria are treated with nisin and Pep5; this again correlates with the planar membrane work where you find pores with diameters of 1–2 nm, but which are very transient.

Boman: Can you make any of these modified peptides *in vitro*, in a cell-free system?

Sahl: Do you mean by chemical synthesis?

Boman: No, by *in vitro* translation.

Sahl: No one has tried that, and I'm pretty sure that at the moment it will not work, because we do not know all the enzymes involved in their biosynthesis.

I mentioned these B and C enzymes, which seem to be important in the case of lantibiotics. There are indications from hydrophobicity profiles that these proteins may be membrane bound. This means that one would have to have at least membrane vesicles present in *in vitro* translation experiments.

Boman: In the biosynthesis of these molecules, how long does it take to complete the peptide backbone, compared to the time required for closing all the rings, dehydrating the hydroxyamino acids and forming the final antibiotic molecule?

Sahl: I'm afraid I can't distinguish that. When we tried to isolate the primary translation product of the structural gene of these lantibiotics, we did not find any unmodified peptide, we found a mixture of dehydrated peptides—from sixfold to roughly onefold dehydrated. This indicates that the half-life of the primary translation product is fairly short and that they're readily turned into the dehydrated pre forms, but I don't know what happens afterwards. I don't know how fast the thioether bridge forms, or how fast transport and cleavage of the leader peptide occur. So far, we have not been able to do any experiments addressing these questions.

Andreu: Have you tried making synthetic analogues of Pep5 to test the effect on activity of replacing different residues?

Sahl: No, but we have replaced one of the residues by proline by site-directed mutagenesis in the bacterium. Site-directed mutagenesis with lantibiotics is not trivial, because you have to deal with the whole operon. Nonetheless, a few research groups are doing this sort of work at the moment. Some groups do it with nisin, there's one group working with epidermin and we're doing it with Pep5. So far there is only a little improvement in activity in just a few cases; not surprisingly, most of the mutations have turned out to have negative effects.

Chemical synthesis of lantibiotics is possible: Wakamiya et al (1991) have completely synthesized nisin by chemical synthesis, including the rings and the dehydroamino acids. But this took an enormous amount of time, manpower and chemicals, and cannot be used as an economical alternative to biological synthesis.

Kreil: Are all the serine and threonine residues dehydrated or is this reaction somehow influenced by adjacent amino acids?

Sahl: No, in the case of Pep5, all serines and threonines were dehydrated, but only in the propeptide part. In the leader peptide there are serines and threonines that have not been touched. But in the case of nisin, there is one serine present in the active molecule which is obviously not seen by the dehydrating enzyme. One can speculate about why is it so. One could think about a conformational property of the prepeptide which somehow results in this one serine residue being hidden away from the enzyme.

Kreil: Subsequently, upon formation of the thioether by the addition of a sulphhydryl group to the dehydroamino acid, is the latter always converted to the D-isomer?

Sahl: So far, yes. Additionally, in one case at least, we have an indication that one of these dehydroamino acids is rehydrated to yield alanine which is in the D-configuration.

Kreil: How are the lantibiotic precursors transported out of the cell? They do not have a signal or leader peptide sequence characteristic of other secreted polypeptides, so there must be a special transport mechanism for them.

Sahl: Yes. In the four biosynthetic operons which have been characterized so far, transport proteins were found that belong to the large family of ABC transporters. These are membrane-bound proteins with an ATP-binding cassette which are involved in many transport processes; they are also found with the unmodified peptide bacteriocins from Gram-positive bacteria. It seems that these are involved in transport of either the prepeptide, which undergoes cleavage outside the cells, or the modified peptide. There are no experiments that have been done so far which could clearly distinguish between these two possibilities. It would be intriguing to postulate that cleavage takes place outside the cell, for self protection of the producing cell. In addition, there's also an 'immunity' mechanism taking place which involves either bigger proteins of 280 amino acids, like nisin, or very small peptides, as in the case of Pep5, which is just a 69 amino acid peptide that is membrane associated and which somehow makes the producer strain 'immune'. We don't know how this works at the molecular level.

Boman: It's interesting that you have found conserved parts of the signal peptides; this has been observed for the cecropins, frog peptides and the defensins. The only rational explanation for this is that the conserved part of the signal is a packing label for special transport vesicles, or something like that. If they exist, these vesicles must have their own signal peptidases, otherwise there is no point in having conserved information in the signal sequences. Although nobody has any evidence for this, I see no other way of explaining why there are four or five peptide classes where a large part of the signal sequence is conserved.

Kreil: But here we must be careful. We must distinguish between 'classical' signal or leader peptides which have a characteristic hydrophobic sequence and the type of 'leader' peptide (which is actually not a good name in this context) found in these precursors of lantibiotics. The latter are quite rich in acidic and basic amino acids and do not have the features typical of signal peptides of secreted polypeptides.

Sahl: We want to avoid using the term 'signal peptide', because that would somehow imply that the leader peptide has a function in transport; we are not sure of this. In contrast, when the first structural genes were sequenced, we had some suspicion that a common FDLE or FNLD motif in the leader peptide might be a signal for the dehydrating enzyme to recognize and modify the peptide. There must be some signal that tells the modifying enzymes that this is a peptide that it can work on. After all, if these enzymes worked on the wrong proteins, it would be disastrous for the cell. But, later on, additional prepeptide

sequences were worked out which lacked this motif and did not have much sequence homology to the previous ones. So, we do not know whether there really is a signal in the leader peptide that is recognized by the modifying enzymes.

Boman: The signal peptides in the insect and frog antimicrobial peptides are all hydrophobic. The N-terminal parts of the signal peptides are clearly more conserved than the rest of the signal sequence. I don't know whether you can guess that vesicles for a separate transport mechanism are going to be different from the normal ER–Golgi pathway. It could still be right that the information which is present in the conserved signal is telling you something about an event that takes place before the cleavage.

Zasloff: Although, Hans, in the case of the frog antibiotic peptide family, precursors are not secreted. The precursor is processed intracellularly and the mature active peptides are stored in the granules.

Sahl: We don't really know that the precursor isn't secreted. There's no evidence one way or the other.

Zasloff: But you were suggesting that there was a transporter, so I was assuming that the precursor was processed, molecularly modified, and then the peptide antibiotic transported out by a transporter of some type, as in the case of the colicins.

Sahl: Right, but we don't know whether the transporter transports the modified but unprocessed prepeptide or the processed and active lantibiotic. There's no evidence concerning this at the moment.

Boman: What it all boils down to is that we cannot yet settle how large the conceptual differences are between the animal antibiotics and the microbial ones. The chemistry and the biosynthesis of these two classes of antibiotics may differ more than we realize at the moment.

Ganz: With the eukaryotic antibiotics, we can answer the question experimentally; we can switch the signal peptides and find out if this has any effect on what happens. For defensins, they are the most conserved part of the molecule.

Kreil: I predict it will not have any effect. A standard type of experiment which has been done in many laboratories with many different precursors is the following. An mRNA for a secreted polypeptide is translated in a cell-free system in the presence of dog pancreas microsomes. Precursors from many different sources (animals, plants, bacteria) will be transported into the lumen of the microsomes with concomitant cleavage of the signal peptide. If you do this experiment with one of the precursors of these lantibiotics, I would expect that this transfer will not take place.

Sahl: In the case of the lantibiotics, it has been shown by Kuipers et al (1993) that the subtilin leader can direct nisin biosynthesis. But exchanging leader peptides between lantibiotics which are not so closely related does not work.

Zasloff: If you were to replace the signal sequence, in the case of the frog family, it is not obvious that the protein concerned would be trafficked into a granular gland granule.

Kreil: I'm not aware of any experiments on this, but I would assume that just exchanging signal sequences in some of these frog skin peptides wouldn't make any difference. Signal sequences are highly variable: all you need is a positively charged amino acid close to the N-terminus, a central core composed of hydrophobic amino acids and a suitable sequence recognized by the signal peptidase complex (Gierasch 1989). On the other hand, if one exchanged pro regions of the precursors of the frog skin peptides with pro regions from other secreted polypeptides, I'm not so sure it would work. This might make a difference in the intracellular sorting of the precursor polypeptides.

Boman: Signal peptides are normally quite variable in the hydrophobic part and we have von Heine's statistics showing to what extent they vary. On the other hand, there are cases—the cecropins, defensins and some frog peptides— where there are far more regions conserved than you would expect from von Heine's predictions. We think that they have something to do with transport, but there is to my knowledge not yet any experimental evidence related to such a function.

Ganz: The problem, of course, is that the leader peptides are almost instantly co-translationally removed. So you then have to speculate that there is a specialized region of the endoplasmic reticulum where the ribosome attaches. This is something that we will have to look at.

Kreil: Yes, by the time the peptide gets into the lumen of the endoplasmic reticulum, the signal peptides are gone. All the subsequent steps of the secretory pathway, i.e. transport through the Golgi stacks into the trans-Golgi network and the sorting between constitutive and regulated pathways, happen after cleavage.

Boman: My guess is that the signal peptide has a packing or a sorting function. It follows that the transport vesicle (or endoplasmic reticulum compartment) must have its own signal peptidase. Whatever is happening has to happen before the removal of the signal sequence. The question is: should we believe that everything goes through the same endoplasmic reticulum–Golgi pathway or should we believe there is an alternative pathway of export for small peptides?

Kreil: There is an alternative pathway of secretion; the α-factor peptide of yeast is probably the best-known example of a peptide secreted this way (for a recent review see Kuchler 1993). But the peptides we are talking about are, as far as one knows, all secreted via the secretory pathway involving endoplasmic reticulum, Golgi stacks, etc. There is one difference, however, for very small prepropeptides consisting of less than ca. 80 amino acids. Contrary to the typical co-translational, vectorial transport across the membrane of the endoplasmic reticulum, it has been shown by Zimmermann and colleagues that small precursors, like those for melittin, PGLa and cecropin, can be transported

post-translationally and independently of the signal recognition particle (Zimmermann & Mollay 1986, Schlenstedt & Zimmermann 1987, Boman et al 1989). Evidence presented by the same group shows that this is not due to particular signal sequences; it only seems to be a question of size (Müller & Zimmermann 1987, Schlenstedt & Zimmermann 1987). What you have speculated upon—that there may be a different transport mechanism for these peptide precursors—would be radically different from our present concepts and I don't think there is any experimental evidence for such a hypothesis.

Elsbach: Are there not modifications of this relatively simple scheme when you are dealing with a protein that is destined to be located in a particular subcellular particle, such as a granule? Isn't there an additional role for these signal peptides to help in the translocation there?

Kreil: This is very unlikely, since the signal peptide has already been removed and has probably also been degraded by this time. The propeptides are transported through the Golgi stacks and subsequently, in the trans-Golgi network, these enter into either the regulated or the constitutive pathway of secretion. How this choice is made is currently not known. Most likely, polypeptides that are transported into storage granules are sorted away from the 'bulk flow' of constitutive secretion. At present, we don't know which peptide sequences, structural motifs and so on are essential for this routing into the regulated pathway, where products are stored and released only upon the arrival of a suitable stimulus from outside the cell.

We had an interesting case which could be mentioned in this context. Expression of preprodermorphin, a precursor for one of the frog skin peptides, in mouse pituitary cells (AtT-20), yielded prodermorphin, which was not transported to the secretory granules. Some of this precursor was secreted via the constitutive pathway, while the bulk was degraded inside the cells (Seethaler et al 1991). If one constructs a hybrid precursor containing part of the pro region of rat proenkephalin in front of the prodermorphin, then correct transport, processing and storage in secretory granules occur in these cells.

Zasloff: You were responsible for making this extraordinary observation, at least in the frog field, of the conservation of the signal sequence, and I took that to heart. From my view, it speaks only of our ignorance in not being able to find the right experimental system to explore the purpose of the signal sequence. I would argue that until you have constructed a transgenic animal, where you have actually introduced a hybrid molecule lacking the sequence and shown that the molecule is trafficked identically, you really can't answer it.

I would like to make one other point: if you take one of the *Xenopus* signal sequences, such as the one you described, and the signal sequence of caerulein (which is the frog version of cholecystokinin) and scan the database, what you pick out from the whole universe is mammalian cholecystokinin along with the frog antibiotic peptides.

Ganz: The same holds for the defensins. You can take just the signal sequence of defensins and pick out only defensins from the database using it.

Hultmark: But couldn't this mean that they're just closely related evolutionarily? Do we know that they are more conserved than other signal peptides? What we've seen is just that they are surprisingly conserved compared to the mature peptide, but this may just reflect that the mature peptides are evolving unusually rapidly, and in comparison the signal sequences seem to be conserved.

Kreil: When one compares homologous precursors from different species, for example preproinsulins, one usually finds that the sequence of the final product, i.e. the A and B chain in our example, is more highly conserved than signal peptides and pro parts. Two types of exception to this rule have turned up in studies on frog skin as well as in other peptides. In one case, precursors of very different end products contain signal peptide and parts of the pro region which are quite similar. We have encountered this in studies on the precursors of caerulein, PGLa and xenopsin from the skin of *Xenopus laevis* (Kuchler et al 1989). The genes for these precursors have a common 'export exon' encoding a signal peptide and part of the pro region, while the rest of these genes show no discernible homology. The same is true for the precursors of deltorphins and the antimicrobial peptide adenoregulin from *Phyllomedusa bicolor* (Richter et al 1990, Amiche et al 1993). We have speculated that this situation may be due to exon shuffling where a particular exon was inserted into different regions of the genome.

The second case is more puzzling. When we compared precursors for related peptides, we found that in some cases the signal peptides and the pro regions are more closely related than the end products, exactly the opposite to the preproinsulin case. Dermorphin and deltorphins are heptapeptides present in the skin of two related species of *Phyllomedusa*. One needs 9–13 point mutations to convert the genetic code for dermorphin to one or other of the peptides (Richter et al 1990). On the other hand, the spacer peptides of 18–19 amino acids are more similar (difference = five point mutations and deletion or insertion of one amino acid). Similar situations have been encountered with the precursors of conotoxins from Conus snails (Woodward et al 1990) and for cecropin and defensin precursors. It looks like the segments encoding the biologically active peptides are 'mutational hotspots' while the rest of the molecule is more conserved. The simple explanation that the pro parts are biologically even more important than the peptides we are interested in is not supported by any experimental evidence. I find this situation really very puzzling and I do not have any idea why these precursors evolve in this peculiar way.

Ganz: But if you look at the rate of evolution of the signal sequence, there are fourfold fewer mutations than you would expect in the signal sequence, compared to the average rate of evolution of the signal sequences. So there are fewer mutations in the signal sequence than you would expect, going back to

mammalian radiation (which I think is about 60 million years), compared to signal peptides of other proteins.

Bevins: If you examine the many families of antimicrobial peptides, conservation is seen in the propeptides, but conservation is also quite striking at the nucleotide level. Of particular note, some of that conservation is seen in the 5' untranslated region of the mRNA. It's possible that the conservation that we see reflects a selective pressure on a specific *nucleotide* sequence, either at the gene level or possibly at the mRNA level. The conservation that we see in the signal peptide itself may be merely a consequence of that conservation of nucleotide sequence. The conservation at the nucleotide level is high enough that we can readily clone, at the genomic level, new members of the various families that have been discovered (Jones & Bevins 1992). For example, that was our strategy to identify defensin 5 and defensin 6 in human. Also, when we first isolated tracheal antimicrobial peptide (TAP), we used the 5' cDNA sequence in a similar way, and we found evidence that there was a large family of TAP-related genes. One of those TAP-related genes encodes a β-defensin which has now been characterized by Mike Selsted as a neutrophil β-defensin (Selsted et al 1993).

Ganz: One other point is that even if we do the switching experiments and find it makes no difference, there may be one variable such an experiment doesn't include, which is that many of these peptides have to be produced at very high rates. The switch may affect the translation rate or the rate of some other step that we're not measuring that may be evolutionarily important, but we would not pick that up in the experiment.

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General discussion I

Assaying antimicrobial activity

Boman: Assays for the activity of antimicrobial peptides have to be adjusted according to the experience, the manpower, the economic constraints and the machinery available to each laboratory: we will never be able to standardize assays to the extent that we all use just one assay. The question is: can we find a way of making the different assays people use somewhat more comparable?

The following list summarizes the activities that have so far been demonstrated with the cecropins.

(1) Inhibition zones on a thin bacterial plate/lawn.
(2) Bacterial killing as a loss of viable counts.
(3) Lysis of bacteria recorded with a photometer.
(4) Lysis of liposomes as a release of dye.
(5) Channel formation in artificial bilayers.
(6) Uncoupling of respiration in mitochondria.
(7) Inhibition of protein import into mitochondria.
(8) Different ELISA reactions with mitochondria.

Each one of these activities can exhibit proportionality under certain conditions, but there are many conditions in which they don't, when they cannot be used as assays.

In the beginning, when we didn't have any alternative, we incubated bacteria with our crude peptide fractions and looked at the rate of decrease in viable counts. We soon found out that this was very time consuming. There is also quite a limited peptide concentration range over which this assay works. Of course, it happens that people just record the number of colonies, but what you should record is really the rate of killing. In order to get an accurate value for this, you have to check that twice the amount of substance gives twice the rate, and not twice the number of colonies. The way we assayed the cecropins in the last phase of their isolation was by recording lysis as the turbidity of a liquid culture. This reaction is very fast, so we had all our results within a day. On the other hand, the lysis assay used fairly large amounts of material. Later on, we began to use the inhibition zone assay. Dan Hultmark's contribution here was that he realized that you can calculate a minimum lethal concentration (LC value) from a dilution series; he also devised the mathematics for calculating this. This is really a modification of the old penicillin assay, developed by the large team that worked in Oxford during World War II.

TABLE 1 *(Boman)* **Antibacterial activity of cecropins A, B and P1 and tetracycline (TC)**

Bacterial species	Strain	Lethal concentration (μM) for cecropins and TC			
		A	B	P1	TC
Escherichia coli	D21	0.2	0.3	0.3	0.9
Escherichia coli	ATCC 25922	0.3	0.2	0.7	1.2
Salmonella typhimurium	LT2	0.8	0.6	1.7	5.8
Pseudomonas aeruginosa	OT97	1.4	1.1	13	12
Acinetobacter calcoaceticus	Ac11	0.2	0.3	0.5	1.9
Bacillus megaterium	Bm11	0.5	0.3	4.5	0.6
Streptococcus pyogenes	—	5.1	12	44	0.9

Lethal concentration determined according to Hultmark et al (1983).

The important thing to realize is that, for this assay, you should have a standard curve where you plot the area of the inhibition zone against the log of the antibiotic concentration. Some people record only the zone diameter; then they subtract the diameter of the well, which means that they subtract a constant from a logarithmic function. If you do this you may still end up with a reasonable result, but intellectually it's a very strange way of handling the data. The really nice thing with the zone assay is that it gives values over a very large concentration range: you can have about a 1000-fold difference in the amount of peptide.

I would say that this is the best method. It uses very little material and it has a wide variety of applications. Bob Lehrer and Mike Zasloff have both used variations of this assay.

How should one compare the results from different laboratories? There are three things to be said concerning this. First, always use a reference strain and always include a reference peptide. In addition, try to use a reference antibiotic which is not the peptide but is easily available. In Table 1 *(Boman)*, I have put together antibacterial data for cecropins A and B from the cecropia moth, the porcine cecropin P1 and tetracycline. You can see that there's no difference between the two strains of *Escherichia coli*; D21 is our normal assay organism and ATCC 25922 is a clinical reference strain that is widely used. One should try to include reference peptides that are readily obtainable. I propose we should agree on a reference strain, a reference peptide (all three cecropins are sold by Sigma and Peninsula) and a reference antibiotic that we can use in our assays, in order to obtain results that are more easily comparable.

Lehrer: May I respectfully disagree with almost everything you have said? I think the question of how to test antimicrobial activity is complex and yet— at first glance—it seems so straightforward. All of us want to get the testing done so that we can go on to the interesting stuff. But how you test often

determines what you are going to find out. Many 'interesting' technical factors can exert critical effects on outcome, including the strain of organism, the incubation conditions, ionic strengths, specific ions and presence of extrinsic substances or contaminants which might inhibit or potentiate the antimicrobial effect. In the agar diffusion system (which I also favour), incubation temperatures and agar types are important variables, as is the molecular size of the antimicrobial molecule, since large proteins do not migrate as far in the well as small proteins. The use of tetracycline, which is a microbistatic agent, as a control for peptides which in most cases are microbicidal, is probably not optimal. I think that the most rigorous way to assay antimicrobial activity is by doing colony counts. But doing these correctly poses a formidable logistical challenge, because we need to test a range of concentrations, incubation times and incubation conditions. Basically, this is too laborious for routine use. Colony count analyses (CFU per ml versus concentration of peptide) typically show a threshold concentration, below which no effect is seen, followed by a log linear zone, which reaches a plateau. Usually, colony counts have a blind spot and are insensitive to fewer than a hundred colonies per ml or so, because of the way we perform them. Other peptides typically have dose–response curves that are parallel but are displaced either to the left or the right depending on their relative potency. Only the analysis of dose–response curves provides information on relative potency—not the analysis of a single concentration at a single time. There are many other things one could say about testing, but they would inevitably lead to an outbreak of glazed eyes. I agree that it is important to standardize testing and that at least we should try to test reference peptides and microbes in our system. From this we can get some idea of relative activities under the varied conditions of testing we tend to use.

Boman: You mentioned that one variable was the type of agar you use in the inhibition zone assay; it is true, this makes quite a difference. It is best to use high-quality agarose. I would say that the main disadvantage with the inhibition zone assay is that you can have interference from the solidifying medium. However, the reference peptides I suggested in Table 1 are relatively insensitive to the type of medium, the composition of the buffer and ionic strength.

Curves for viable count versus dose were, of course, used in bacterial genetics long ago and you can define wild-type and resistant mutants by the highest concentration that gives 100% viability. What happens now is that people sometimes do not make a complete curve but just take a point here and then another there; this does not tell you anything. I have been a referee on this type of data, trying in vain to explain how you can run off in conclusions.

Ganz: There are assays that you haven't even mentioned. I was just remembering a beautiful assay recently carried out with the luciferase system (Jacobs et al 1993). A luciferase-containing phage or plasmid is introduced into the bacterium, and since light production by the luciferase requires ATP, you

can show the effects of an agent on the bacterial ATP level. It is an extremely sensitive assay that works well with very slowly growing bacteria like *Mycobacterium*. So there are new assays that may make it much easier to work with these systems, and which are much more sensitive than the ones we have now.

Elsbach: One thing that many assays don't distinguish between is a bacteriostatic and a bactericidal effect. The luciferase assay is a way in which you can determine an actual killing event. We have looked for many years at the ability of the target cell to carry out any kind of biosynthetic activity as a measure of true bactericidal effect as opposed to a merely bacteriostatic effect.

With respect to the diffusion assay, I'm very concerned about the different diffusion rates of different peptides that have subtly different, not only charge but also conformational properties. In addition, the effect of ions, for example, may influence the diffusion rates in the agar. All these raise serious questions about the comparative aspects of the assay that you advocate.

Boman: Another interesting point is that the zone-inhibition assay can sometimes distinguish different mechanisms. If you take dead *Micrococcus* and make a turbid plate with this material, lysozyme zones will continue to grow for quite some time. Also melittin on red cell plates will give zones that continue to grow.

Zasloff: We also, of course, use assays for different purposes. When we quantify the potency of the peptide, it's done as an MIC. In this assay, a fixed inoculum is introduced into a volume of full-strength medium, then one determines whether or not the well is sterilized 24 h later. Generally, 10^5 organisms have to be eliminated over 24 h. But for discovery, we do what we can to increase the sensitivity of the assay—add Triton, reduce ionic strength and so on.

Boman: Of course, if you want to discover new antimicrobial agents, it's advantageous to have an assay that picks up both bacteriostatic and bactericidal activity.

Lehrer: I don't think there is such a thing as a perfect assay. In our work, we're always challenged by the fact that we never have enough material. The advantages of the radial diffusion assay are that it uses miniscule amounts of material and it is quantitative. It's not without fault, though. We actually have increased the sensitivity somewhat over the assay that Dan Hultmark developed by having a double layer of agar and making the underlayer, which has the organisms, highly permissive for a wide variety of antimicrobial peptides (Lehrer et al 1991). If there were defensins in the system, you probably wouldn't see them with your assay, because they're not active against Gram-negative bacteria under your high-salt assay conditions. Our low ionic strength underlay gel is highly permissive for peptide-mediated antimicrobial activity, allowing us to conserve material. We have found an occasional peptide which is more active in high-salt than in low-salt conditions and some of yours may be similar.

Hultmark: Of course, which assay you should use depends on the kind of questions you are asking. But if you choose to work with the inhibition zone assay you should be aware of some of its quantitative aspects. What you're asking is actually: what is the concentration of the antimicrobial agent at the margin of the inhibition zone at the time when bacteria have just reached a colony size at which they are no longer affected? The size of the inhibition zone depends, in a complex way, not only on how sensitive the bacteria are, but also on how fast the peptides diffuse and how fast the bacteria saturate the plate. You can take care of these complexities simply by repeating the assay with a couple of dilutions of your substance. From the concentration dependence of the diameter of the inhibition zone, you can calculate a 'critical concentration', which is the concentration just needed to kill the bacterium. It turns out that this calculated critical concentration fits very nicely with MIC values, if the latter are measured at relatively low bacterial concentrations, similar to the initial conditions of the plate assay. If you do MIC assays with high starting inocula, you might get different results.

Ganz: Sometimes we're interested in showing inactivity. For example, in comparing defensins and prodefensins, we're interested in showing that prodefensins are inactive. In this case, it is important to show that they have diffused, so we label them radioactively, dry the agar and use autoradiography to show this.

Sahl: We have used two types of assay; one is the inhibition zone assay, the other is the MIC value. One disadvantage with the MIC value is that there is a problem with defining the time at which you read the result. When you read the assay after 18 h you will come up with one MIC value; if you do it after two days or more, the MIC may be shifting because of surviving bacteria which can start regrowth. The reason for the cationic peptides becoming ineffective may be the development of surface layers around the bacterial cell—we have seen this happening. In a diffusion assay these cells show up as individual colonies. Standardization of the reading time is also a problem when you want to compare different organisms. Very often, *Micrococcus luteus* is used because it's very sensitive to many of these basic peptides. This species grows best at 30 °C and grows much more slowly than *Staphylococcus*, for example. With *Micrococcus*, you cannot read the plates before two days because you won't see much growth. How do you compare these with staphylococci which are growing fast enough that you can read the plates after 18 h?

Elsbach: There is an important paper by Cross et al (1993), where they made the point that we all have a tendency to focus heavily on laboratory strains that we are familiar with and that are easy to work with. But in terms of the biological relevance (which ultimately is what counts), we often avoid the really pathogenic strains, where the situation becomes very different from what we generally observe with the simple laboratory strains.

Boman: This is relevant from the medical point of view. Many of the peptides isolated here come from animals other than human and we don't know yet what survival value the antibacterial mechanisms may have in many hosts. However, in insects, plants and marine organisms they serve as immune substances.

The concept of 'pathogens' is one that has been invented by doctors—it doesn't exist in Nature. Pathogens have rarely evolved as obligate parasites and many virulence factors are plasmid borne. Resistance to host defence often creates a pathogen.

Thus one should see an infection as an occasional competition between a microbial population and the host.

Elsbach: But we are living in an environment in which people feel the need to look at these various antimicrobial agents as potential therapeutic agents. In order to judge them as therapeutic agents, you have to see them in a context where they would be used as therapeutic agents. Nobody has a need for dealing with *E. coli* J5, because the host deals very effectively with this under almost any circumstances. You can't consider the concept of pathogen as merely a theoretical issue.

Hansen: Do some of these antimicrobial peptides have activities that we don't even realize? For example, many of the lantibiotics are extremely effective against bacterial spore outgrowth. They are actually much more effective at this than they are in killing bacteria: they work at very low concentrations to prevent transition from a dormant spore to a vegetative cell, whereas virtually all these assays look at the effects of antimicrobial agents on growing cells. An agent that would prevent the germination of a spore could be as valuable (if not more valuable) than something that would kill bacteria after they have started growing.

Zasloff: Then you must look at it from the other side: in many cases, the organisms that are being studied in these *in vitro* assays are not phenotypically the same as those which are growing robustly in a compromised animal. One could ask whether or not we are assaying the correct organisms. In other words, an antibiotic that exhibited certain potency when assayed against an organism growing *in vitro* might have a very different potency against an organism that has just been recovered, in a sense, from a living thing.

Sahl: There is another disadvantage of testing just a few standard strains: the bacterial peptides, at least, show extreme variation in their ability to kill different species and even strains within a species. By testing just a few standard strains, you may completely over- or underestimate the potency of your peptide, which can be very misleading in terms of judging the significance of these peptides in Nature or with respect to analysis of structure–function relationships.

Lehrer: I want to comment on an issue that both you and Michael Zasloff are dealing with slightly differently; the very same organism is not metabolically and structurally the same when grown up in a rich medium versus poor medium and, as a result, it can have very different susceptibilities to the same peptide.

Also, there are three different general milieus that antimicrobial peptides can work in. They can work extracellularly, which is easiest for us to deal with, because we have a very accurate understanding of the composition of the extracellular milieu. We are much less certain how to test peptides that might act on mucosal surfaces, and we don't have a clue about what conditions to use when we test peptides that function intracellularly. Most intracellular antimicrobial peptides are designed to work within phagolysosomes, and although we have a little information about phagolysosomal pH, we don't know anything about its ionic composition. Nor are we able to simulate the concentrations of antimicrobial peptides that are obtained inside phagolysosomes, which for defensins, for example, are probably between 10 and 100 mg/ml.

Boman: I think probably the tolerance to peptides that are delivered outside an animal is largest because the skin is relatively tolerant in a number of respects. You may consider the intestinal system in the same way—for frogs, the same peptides are used on the outside and in the inside.

Lehrer: Michael Zasloff and I were chatting earlier about the frog skin peptides. It seems quite possible—at least to me—that these glands are designed to deliver the peptides onto the frog's skin and that they are consequently absorbed to provide effective systemic concentrations.

Ham: Most of these assays work by looking at peptides in isolation, yet synergy must be occurring *in vivo*. In insects, at least, there is a whole range of antimicrobial agents being induced, or maybe constitutively present, in addition to the one that you are perhaps interested in. Surely the assays have to take into account the fact that biologically functional synergism may be occurring.

Boman: This is something we have overlooked by and large because it's too complicated to assay for. In Nature, synergism probably plays an important role.

I have often wondered about the function of lysozyme, why it is so often present in the defence response. One explanation could be that it works in consonance with many of the other components. Another possible function of lysozyme is to degrade the murein skeletons that are floating around after the lysis of bacterial membranes. In certain cases, immune genes are turned on by microbial products like lipopolysaccharide or murein fragments. It could be harmful for the host to have bacterial cell wall components floating around for long periods. Perhaps the role of lysozyme is to remove a signal for danger when the danger is over. But gene activation is very complicated and this is probably an oversimplification of the situation in an infected living creature.

Ham: It is important to know as much as you can about the basic biology of the system that you are using and to feed that information into the assay. This is why it is very difficult to standardize on a fixed number of bioassays because there may be extra constituents you want to put into your assay that suit the system you are working with.

Sahl: It's a classical dilemma: on the one hand there's an urgent need for standardization and for comparison with other peptides, which requires simplification; on the other hand biology is complex, and if we want to have relevant results we have to make the test system as complex as possible.

Elsbach: There is a way out of this dilemma: by comparing the functional properties of the isolated protein under simple laboratory conditions and in a much more complicated biological environment. One cannot really take the results of experiments with isolated protein or peptide in an artificial setting and arrive at conclusions about biological significance.

Bevins: Sometimes, we are faced with very limited amounts of isolated peptide, and yet we wish to report some data on antimicrobial activity. It sounds as though, on the basis of sensitivity, the zone assay may be considered a reasonable approach.

Boman: I don't promise that the zone assay will work in every case, but I would say that at present it appears to be the best alternative.

Lehrer: I agree largely with what Hans Boman says, but would add that the zone assay is worthless for microbistatic proteins, which are also potentially quite important.

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Biosynthesis of defensins and other antimicrobial peptides

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Abstract. Defensins are small (about 30 amino acid residues) cationic antimicrobial peptides with a conserved framework of six disulphide-linked cysteines. Human defensin HNP-1 and the closely related HNP-3 are amphiphilic dimers that act in part by permeabilizing cell membranes. Defensin mRNAs, abundant in neutrophilic promyelocytes, certain non-human macrophages and Paneth cells, encode 94–100 amino acid prepropeptides. PreproHNP-1 is post-translationally processed to inactive proHNP-1 then to mature HNP-1 stored in granules. Bactenecin Bac-5 and perhaps other related neutrophil peptides are also synthesized as prepropeptides but are stored in granules as inactive propeptides. Their conserved cathelin-like propiece inhibits the cysteine protease, cathepsin L, and is removed only during granule release. Charge neutralization of mature peptide by the propiece is seen in both probactenecins and prodefensins. In contrast the propiece of cecropins is very short and procecropins are microbicidal. The pathways that convert myeloid preprodefensins to defensins are specific to myeloid cells but the signal for targeting to granules also functions in non-myeloid granulated cells. The truncation of the anionic propiece by deletion mutagenesis dramatically reduces defensin synthesis, suggesting that the propiece may assist in peptide stabilization, folding or subcellular transport. Despite some similarities in the mechanism of action of the various families of antimicrobial peptides, their precursors differ greatly, presumably owing to differing functions of the propieces.

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Structure of defensin peptides

Defensins are a family of mammalian antimicrobial peptides that are 29–35 amino acid residues long, variably cationic, and contain a conserved motif of six disulphide-linked cysteines. The six known human defensins (Fig. 1) include four peptides (human neutrophil peptides HNP-1–4) that were purified from polymorphonuclear leukocytes (Ganz et al 1985, Selsted et al 1985, Singh et al 1988, Wilde et al 1989) and two (human defensins HD-5 and 6) whose production in the intestinal Paneth cells was deduced from gene/cDNA cloning and *in situ* hybridization (Jones & Bevins 1992, 1993). Other mammalian defensins include rabbit, mouse, rat and guinea pig peptides of
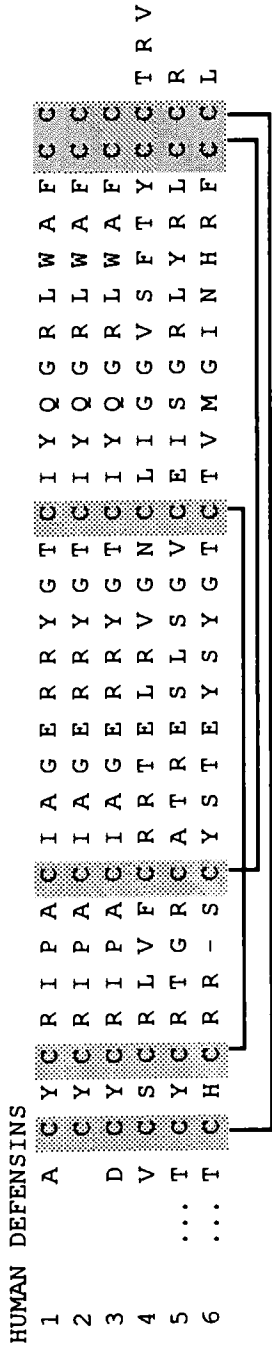
HUMAN DEFENSINS

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1     A C Y C R I P A C I A G E R R Y G T C I Y Q G R L W A F C C
2       C Y C R I P A C I A G E R R Y G T C I Y Q G R L W A F C C
3     D C Y C R I P A C I A G E R R Y G T C I Y Q G R L W A F C C
4     V C S C R L V F C R R T E L R V G N C L I G G V S F T Y C C
5 ... T C Y C R G R C A T R E S L S G V C E I S G R L Y R L C C T R V
6 ... T C H C R R - S C Y S T E Y S Y G T C T V M G I N H R F C C R L
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FIG. 1.   Amino acid sequences of human defensins. The conserved cysteines are highlighted and their disulphide bonding pattern is shown. The positions of the N-termini of defensins HD-5 and HD-6 are uncertain.

both myeloid (polymorphonuclear leukocyte or macrophage) and intestinal (Paneth cell) origin (Lehrer et al 1993). Functionally similar three-disulphide peptides that differ from defensins in their core structure (Selsted & Harwig 1989) include bovine β-defensins (Selsted et al 1993) and tracheal antimicrobial peptide (Diamond et al 1991) and insect defensins (Hoffmann & Hetru 1992). It is not known whether any of these peptides are evolutionarily related to defensins. The three-dimensional structures of two closely related defensins, HNP-1 and HNP-3, have been determined by two-dimension nuclear magnetic resonance and X-ray crystallography, respectively (Zhang et al 1992, Pardi et al 1992, Hill et al 1991). The peptides form amphiphilic dimers that consist mostly of antiparallel β-sheets with several tight turns. Defensins and possibly other three-disulphide microbicidal peptides thus differ structurally from amphiphilic helical peptides such as cecropins and magainins.

Mechanism of action, release and clearance of defensins

Purified defensins permeabilize phospholipid bilayer membranes with a voltage dependence that suggests that the peptides are driven into the bilayer by electromotive force and that there they multimerize into channels (Kagan et al 1990). When purified defensins act on prokaryotic or eukaryotic targets, disruption and permeabilization of plasma membranes is an early step in the sequence of events leading to cell death (Lichtenstein et al 1988, Lichtenstein 1991, Lehrer et al 1989). *In vitro*, defensins are toxic not only to microbes but also to mammalian cells (Lichtenstein et al 1986, Okrent et al 1990).

Because the intrinsic biocidal activity of defensins is not restricted to microbial targets, selectivity of action is largely a result of regulated delivery by degranulation of preformed defensin into phagocytic vacuoles (Joiner et al 1989). Extracellular release of defensins from activated polymorphonuclear leukocytes is very modest (Ganz 1987). Moreover, the cytotoxicity of extracellular defensins is ablated by plasma proteins, especially α_2-macroglobulin, which avidly binds human defensins HNP-1–3 (Panyutich & Ganz 1991). Interaction of defensins with plasma proteins may regulate the extracellular activity of defensins and facilitate their clearance from inflammatory sites.

Biosynthesis of defensins compared with other antimicrobial peptides

Defensins are initially synthesized as 94–100 amino acid preprodefensins (Fig. 2) that share at the N-terminus a highly conserved 19 amino acid signal sequence, followed by an anionic propiece, a short cationic region and the 29–34 amino acid mature peptide at the C-terminus (Daher et al 1988, Palfree et al 1993, Jones & Bevins 1992, 1993). Pulse–chase studies of defensin (HNP-1 and 3) synthesis and processing in HL-60 myeloid leukaemia cells (Valore & Ganz 1992) identified a defensin-processing pathway that removes the signal sequence (probably

co-translationally), then over several hours cleaves the propiece in two or more steps, leaving the mature defensin. The process is highly efficient, generating only small amounts of residual processing intermediates or variants (Harwig et al 1992). Studies of preprodefensins processing in murine cell lines engineered to express HNP-1 cDNA revealed that this processing pathway is specific to myeloid cells (Ganz et al 1993) and lymphocytes (L. Liu & T. Ganz, unpublished results). Other cells transduced with HNP-1 cDNA (NIH/3T3 embryonic fibroblasts, AtT-20 pituitary adenoma and Sf9 insect cells) remove the signal sequence only, leaving a 75 amino acid prodefensin. The prodefensin contains a motif that targets the transgenic protein into cytoplasmic storage compartments (granules) in those transduced cells that contain such organelles, including not only granulated myeloid cells but also the pituitary adenoma cell line AtT-20 (Ganz et al 1993) and granulated lymphocytes (L. Liu & T. Ganz, unpublished results). Since AtT-20 cells do not process preprodefensins past the removal of the signal sequence, the targeting and processing mechanisms act independently.

The properties of the 75 amino acid proHNP-1 have been investigated by overproducing it in baculovirus-infected insect cells that secrete the prodefensin (E. V. Valore, E. Martin & T. Ganz, unpublished results). Unlike the corresponding mature defensin HNP-1, the prodefensin lacks microbicidal activity, presumably because the anionic propiece keeps the mature cationic peptide latent (Michaelson et al 1992). The anionic propiece is likely to have additional functions during defensin synthesis since its total (or near total) deletion dramatically impaired defensin production in NIH/3T3 cells or 32D cl3 myeloid cells transduced with defensin cDNA (L. Liu & T. Ganz, unpublished results). It is possible that the anionic propiece functions as an intramolecular chaperone that assists the folding of the mature defensin. If so, the misfolded defensin formed in the absence of the propiece may be rapidly degraded. On the basis of our observation that the 75 amino acid prodefensin fails to interact with α_2-macroglobulin and other defensin-binding proteins (Panyutich & Ganz 1991) in plasma or serum, it is also possible that the propiece blocks aberrant interactions of prodefensins with cellular proteins. Despite the variation in the charge of mature mammalian defensins from $+8$ to $+2$, most prodefensins have near neutral charge at pH 7, suggesting the importance of ionic interactions between the anionic propiece and the cationic defensin (Michaelson et al 1992).

Other antimicrobial peptides of similar size to defensins are generated from structurally and functionally distinct precursors (Fig 3). The family of mammalian neutrophil antimicrobial peptides that includes the bovine Bac-5, indolicidin and cyclic dodecapeptide, porcine PR-39 and protegrins, and rabbit CAP18, contains a highly conserved propiece that is homologous to the cysteine protease inhibitor, cathelin. The best studied of these peptides, Bac-5, is initially synthesized as a prepropeptide but is processed only by removal of the signal

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              signal sequence                    anionic propiece

HNP1   MRTLAILAAILLVALQAQA   EPLQARADEVAA------APEQIAADIPEVVVSLAWDE   51
HNP3   MRTLAILAAILLVALQAQA   EPLQARADEVAA------APEQIAADIPEVVVSLAWDE   51
HNP4   MRIIALLAAILLVALQVRA   GPLQARGDE-AP------GQEQRGPEDQDISISFAWDK   50
HD5    MRTIAILAAILLVALQAQA   ESLQERADE-AT------TQKQSGEDNQDLAISFAGNG   50
HD6    MRTLTLTAVLLVALQAKA    EPLQAEDDPLQAKAYEADAQEQRGANDQDFAVSFAEDA   57
       **..**.*.*.*******..*   ..**.*.  .     ...*  .  . ...  .*.. *  .


              cleavage sites?                    mature defensin

HNP1   SLAPKHPGSRKNM   ACYCRIPACIAGERRYGTCIYQGRLWAFCC           94
HNP3   SLAPKHPGSRKNM   DCYCRIPACIAGERRYGTCIYQGRLWAFCC           94
HNP4   SSALQVSGSTRGM   VCSCRLVFCRRTELRVGNCLIGGVSFTYCCTRVD       97
HD5    LSALRTSGSQARA   TCYCRTGRCATRESLSGVCEISGRLYRLCCR          94
HD6    SSSLRALGSTRAF   TCHCR-RSCYSTEYSYGTCTVMGINHRFCCL         100
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FIG. 2. Amino acid sequences of human preprodefensins. Charged residues (anionic:: aspartate = D and glutamate = E, cationic: lysine = K and arginine = R) are in bold. The positions of the N-termini of mature HD-5 and HD-6 are uncertain. Conserved residues are marked with asterisks, partially conserved ones with dots.

PRE   PRO DEFENSIN

in granules

PRE   PRO   BAC5
(cathelin)

? in granules

amidation
tripeptide

PRE PRO CECROPIN

PRE(PRO)TACHYPLESIN

PREPOLYPROPOLYMAGAININ
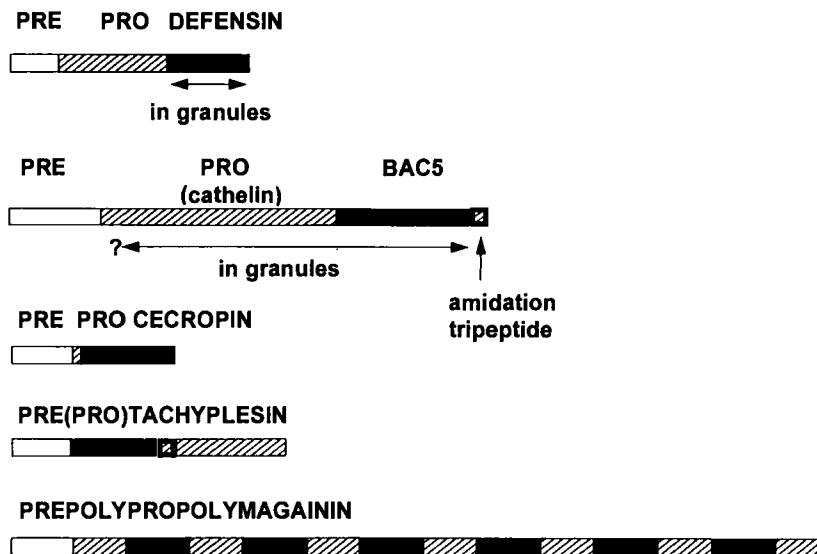
FIG. 3.   Diagrams of prepropeptides for five families of antimicrobial peptides. Despite
the similar sizes of mature peptides (23–45 amino acid residues), the precursors vary
substantially, mainly because of differing structure and function of propieces.
Prosegments (signal sequences) are in white, anionic prosegments are hatched and mature
peptides in black. Amidation sites are marked by squares.

sequence to probactenecin before storage in granules (Scocchi et al 1992, Zanetti
et al 1990, 1991, 1993). The propiece is cleaved off after release from granules,
probably by neutrophil elastase. The propiece is likely to contain a granule-
targeting motif but may also function independently as a protease inhibitor.
Like prodefensins, probactenecins lack microbicidal activity and exhibit charge
neutralization of the mature peptide by the propiece. In contrast, the insect
procecropins have only a very short propiece and are bactericidal (Boman et
al 1991). Perhaps defensins and bactenecins would be cytotoxic at the high
concentrations at which they are produced and must be maintained in a latent
state by their propieces, but cecropins are less cytotoxic so this constraint is
absent. Unlike defensins, cecropins and several members of the cathelin-like
family of microbicidal peptides are amidated on the C-terminal residue. The
amino group originates from an adjoining glycine residue that is removed by
a specific processing enzyme. Although the function of the C-terminal amidation
is not known, this modification neutralizes the negative carboxyl charge that
could interfere with the activity of some cationic peptides. The relative positions
of mature cationic peptides and anionic propieces in prepropeptides also vary.
In preprotachyplesins, synthesized in the haemocytes of horseshoe crabs, the

anionic propiece is C-terminal to the cationic mature protein (Shigenaga et al 1990). Magainins, frog skin antimicrobial peptides stored in secretory granules, are synthesized as polyproteins with alternating anionic propieces and cationic mature peptides (Terry et al 1988). Notwithstanding these variations, it has become apparent that charge neutralization of cationic mature peptides by anionic propieces is a common theme in the synthetic pathways of antimicrobial peptides.

Defensin genes

Human defensin genes encoding HNP-1 (*DEF-1*) and HNP-3 are located on chromosome 8, band p23 (Sparkes et al 1989). Although its band location has not yet been pinpointed, the gene for HNP-4 is also on chromosome 8 (Palfree et al 1993). The gene for HNP-2, a peptide that lacks the N-terminal alanine of HNP-1 and the N-terminal aspartic acid of HNP-3, has not been found. It is likely that HNP-2 is generated by differential post-translational processing from preproHNP-1 and/or preproHNP-3. In the course of the cloning of the various human defensin genes, we and others (Jones & Bevins 1992) encountered one or more pseudogenes as well; evidence that the human defensin family contains additional as yet uncharacterized genes in the region contiguous to the gene for HNP-1. The mouse intestinal defensins (cryptdins) form a similar cluster in the homologous region of the mouse chromosome 8.

Each of the human, rabbit and guinea pig myeloid defensin genes spans about 3 kilobases and contains 2 introns and 3 exons that correspond roughly to 5′ untranslated region, defensin signal peptide/propiece and mature peptide (Linzmeier et al 1993). In contrast, the human Paneth cell defensin gene *HD-5* and all known mouse Paneth cell defensin genes lack the first exon (Jones & Bevins 1992). The Paneth cell defensin gene *HD-5* is transcribed from a promoter whose homologue is preserved at the 5′ end of the first intron of *HNP-1* and *HNP-3* (Linzmeier et al 1993), suggesting that the myeloid and Paneth cell genes may have evolved from a common ancestral gene that contained two separate tissue-specific promoters. Human defensin mRNAs are among the most abundant mRNAs in immature myeloid cells, which may have led to their random cloning (Mars et al 1987) and original designation as 'the myeloid-related sequence'. However, in mature polymorphonuclear leukocytes, defensin mRNAs are undetectable by Northern blotting (Daher et al 1988). The elements of defensin genes responsible for their developmental and tissue-specific regulation and massive selective transcription have not yet been functionally characterized.

The expression of *HNP-3* shows clinically silent variability among the blood donor population of the USA, with many donors lacking the peptide completely, while others have only half of the expected amount, consistent with the presence of a null or defective *HNP-3* allele in the population. The rare patients with

'specific granule deficiency' have only 10% of the normal defensin concentration in their neutrophils (Ganz et al 1988). In this disorder, mRNAs for several specific granule proteins and the azurophil granule peptides HNP-1 and HNP-3 (Johnston et al 1992, Lomax et al 1989) are severely diminished or absent, owing to a defect in transcriptional activation of these genes during the maturation of myeloid precursors in the bone marrow. The patients have recurrent bacterial infections and *in vitro* phagocytic killing by their polymorphonuclear leukocytes is delayed.

Conclusions

Defensins and similar antimicrobial peptides are ancient components of host defence in mucosal surfaces and phagocytes. Their broad spectrum of activity is mediated in part by permeabilization of biological membranes. Specificity of action is ensured by molecular mechanisms that regulate their localized production, storage, delivery to microbial targets and eventual clearance from infected tissues. Despite some similarities in the mechanism of action of defensins and other antimicrobial peptides, each class of peptides is generated from structurally and functionally distinct precursors.

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DISCUSSION

Kreil: Is anything known about the processing enzymes that convert prodefensins into defensins? It's obviously not the usual prohormone–hormone type of cleavage that is taking place.

Ganz: No, the processing sites are different from those of the usual prohormone processing enzymes of the KEX-2 type. This is a yeast processing system; there is a mammalian or vertebrate homologue of that system which processes various peptide hormones. The processing enzymes for the defensins seem to be different and I know nothing about them yet.

Zasloff: At what point in this processing pathway do you think the disulphide array is generated?

Ganz: It's probably generated co-translationally. I can't say that the disulphide bonds are formed right at that point, but we know that the general shape of the molecule is established soon after or simultaneously with translation, because our polyclonal or monoclonal antibodies would not recognize the material unless it were properly folded.

Boman: When we looked at the precursor for the cecropins and we saw the Ala–Pro–Glu–Pro motif, we realized immediately that the sequence was the same as the beginning of the melittin pro sequence; this was of great help to us. In fact, I even remember Günther Kreil saying to me that it was not difficult to purify the dipeptidyl-dipeptidase (dipeptidyl aminopeptidase) processing enzyme and test whether it's right. So here is a pro structure from a toxin that is also present in an immune substance. This is to some extent the same for the frog peptides, where the processing structures are like those for hormones. I think that in the case of the processing of the prodefensins in myeloid cells, these molecules need to have information not only for processing, but also for targeting. Thus they may require a larger propeptide than otherwise would be needed.

Can you distinguish the targeting part of the sequence?

Ganz: The true test of a targeting segment is whether it can cause a protein which is normally constitutively secreted to be transported into granules. We still have to do the experiments where we take a putative targeting region, attach it to a peptide which would normally be constitutively secreted, and see it going to the granules. Without having done these experiments I really cannot say much about the targeting peptide yet.

Kreil: I think one should make a distinction here. Peptides like cecropin, melittin and apidaecin are probably constitutively secreted, then processed outside the cell. That would be my guess, and in the case of melittin I think we know this is so. So you don't have any targeting information there; the role of the propiece may just be in keeping the peptide inactive as long as it is inside the cell.

These propeptides, which turn up in many different contexts, are usually acidic (perhaps always acidic). Even in a case such as preprothyrotropin-releasing hormone, which is a simple tripeptide, we do have these basal peptides that are acidic, which are required for something—probably, as you mentioned, for targeting. But in this instance it's certainly not the case that you have acidic residues that neutralize something that is in the mature product—it's just an acidic part that is there, with whatever function it has. From the initial studies from different laboratories it is clear that there is some information in these acidic segments, but nobody can put their finger on a particular residue or domain and say 'this is essential'. Usually, one makes deletions and then targeting or secretion is slowly lost: it is not an 'all-or-nothing' situation, which is what makes it so difficult to study.

Ganz: That's right. We have not yet identified a particular region of the propeptide that can do a particular job. It just seems that we lose some of the functions that we ascribed to the propeptide as we delete more and more of it. It's possible that some deletions will give better localizations, but that has not been the experience in the hormone field where they have not been able to locate particular targeting peptides with great success.

Bevins: Have you tried simply hooking your prepropeptide to some unrelated cationic peptide that would not ordinarily be localized to a granule and seeing if that peptide was packaged into a granule?

Ganz: I don't have such data. One experiment of Nature that indicates that this could happen is the cathelin system of peptides—Bac5, the protegrins and a variety of the bovine peptides targeted into a particular granule subtype in these neutrophils. The propiece seems to be relatively well conserved and attached to it are peptides that seem to have nothing in common. My guess would be that there is a region which is targeted to the granule, and added on to it are various and sundry peptides that are dragged along to the granule. I think this one system is going to turn out to work like that, but the data are not yet there. We need to attach another peptide that does not occur naturally in that combination, and see whether it will still be targeted to the granule.

Bevins: Many members of the β-defensin family are targeted to granules of the cow neutrophil, as you know. In this case, the putative propeptide segment is extremely short; the full prepropeptide is about 63 amino acid residues and the mature peptide is about 40 residues long. If you suppose that the putative signal peptide is the first 20 residues, that leaves you about five residues unique for the propeptide. Do you have any thoughts about similarities or differences with the propeptides you have studied?

Ganz: Maybe in that situation the targeting information is in the mature peptide; it is even possible that this will turn out to be the case for defensins, and that what we're seeing with our deletions is interference with normal targeting mechanisms. So when we delete portions of the prodefensin, we may be interfering with the normal targeting mechanism that is actually not located in the propiece, but is located in the mature peptide. That is a potential explanation. It would be unpleasant if that turned out to be the case, because it makes the problem doubly difficult.

Lehrer: A biological point: when we talk about targeting to granules, it's important to remember that in the neutrophil there are several classes of granule, and they're not all made at the same time. This temporal dissociation can also influence which granule populations are targeted at any given time in a cell.

Tomas Ganz, were your deletion experiments done in such a way that the experiments that showed an effect on secretion and processing also established that the cells remained healthy, and that other things that should have been going on at that time were happening as they should have been?

Ganz: The cells remained healthy as judged by overall protein synthesis (the cysteine incorporation), total RNA content and the level of mRNA for the construct that we made. These cells had comparable amounts of mRNA for the product as the control cells, or as the cells that had less than the full deletions. They made protein, but for some reason they either didn't make as much defensin or degraded whatever they made. It could be that the material was misfolded and rapidly degraded.

Boman: Are the constructs that you used stable? Can you keep them in culture or is the phase when they express the defensins transitional?

Ganz: These are stable constructs where the retrovirus is incorporated into the cellular DNA; they can be used indefinitely.

Boman: Have you also done that for a T cell line?

Ganz: Yes.

Boman: Have you tried to grow up T cells and see if you can isolate the peptide from the medium? Is enough peptide produced, or do you need to use Northern blots to detect its synthesis in the cells?

Ganz: We are able to isolate the peptide from the medium, and we are also able to isolate the peptide from the cells. But the T cells produce awfully small amounts of peptides: the expression we get in the retroviral system is probably about at least 20–100-fold less than the natural level of expression in the neutrophil, so we have to use radioactive tracers to be able to follow the peptide.

Zasloff: How does a cell protect itself from destruction as it begins to store high concentrations of a membrane-active substance within a membrane-enclosed structure?

Ganz: The granules, in addition to defensins and other microbicidal proteins, also contain a polyanionic matrix consisting mostly of chondroitin sulphate. It is very likely that there is electrostatic interaction between the chondroitin sulphate and the defensins and the other cationic peptides. My guess is that the chondroitin sulphate can act as a neutralizing agent. One of the most interesting features of these peptides is that, for some reason, arginines are favoured in granule proteins. Granule proteins that associate with the matrix have a lot of arginine, but versions of those proteins that are in other parts of the body may have much less arginine. This is very clear in the case of lysozyme. Lysozyme that goes into neutrophil granules has a lot of arginine, but in species where there is a type of lysozyme that is secreted instead, that lysozyme doesn't have as much arginine. So there may be something special about arginine and its interaction with the granule matrix.

Rees: Are you saying that the forms that are generally secreted don't have such a high cationic charge because they don't have so much arginine?

Ganz: No, they may have more lysine than arginine. There is a lot of arginine and little lysine in the neutrophil granule peptides, and it's the other way round in the forms that don't go to granules.

Elsbach: But that 'rule' doesn't really apply across the board. Bactericidal permeability-increasing protein (BPI) is a very lysine-rich protein—I appreciate that it is distinctively different from most of the granule proteins—but it does not contain arginine at all, and it's clearly a primary-granule-associated protein. In addition, the relative arginine content or basic content in your defensin family seems to correlate well with bioactivity. How the protein is held in the granule, and held there possibly in an inactive form, may also be closely related to its hydrophobic properties and how it's linking things other than negatively charged agents like heparin sulphate and chondroitin sulphate. With BPI, it appears that hydrophobic properties of the portion of the molecule may play a role in holding the protein there, therefore making it less capable of freely interacting with membranes other than the ones it is associated with already.

Ganz: But you extract your protein with high acid. It seems to me that you are trying to dissociate charge interactions.

Elsbach: That's true. The various sulphated polysaccharides may play an important role in terms of anchoring. But there may be multiple forces that prevent the protein from getting out before time; once the granule starts to lyse, there are obviously multiple disturbances of multiple structures of that intracellular particle which may have a lot to do with what happens to the constituents. This may vary for the different constituents as well, so that you get sequential release, for example.

Natori: We are studying the processing of sapecin, which is an insect defensin. Sapecin is synthesized by NIH-Sape-4 cells, as preprosapecin, and the signal sequence and the pro segment are removed from the preprosapecin sequentially to form mature sapecin. We raised antibodies to sapecin and its pro segment and examined the fate of these peptides by immunoassay or immunoblotting. We found that the pro segment is very unstable and is rapidly degraded as soon as it is separated from sapecin (Homma et al 1992).

Boman: Concerning the biosynthesis of the cecropins, their dipeptidyl-peptidase is present in the haemolymph and is quite active before the onset of an infection. From haemolymph, Dan Hultmark isolated small amounts of cecropin peptides extended with glycine. So I think we can conclude that the amidation reaction is probably the last processing step. Judging from the amount of enzyme, we would expect that the removal of the propeptide occurs as soon as the precursor is delivered into the haemolymph. This would explain how the peptide is kept inactive and prevented from acting on the membranes inside the cell. Marie Hugosson and Elzbieta Glaser in Stockholm have studied the effect of cecropin precursors on mitochondria (Hugosson et al 1994). There, procecropin is the least active; it is almost inert. The prepro form is relatively active against mitochondria but, according to what we agree, it should never be found free and the signal is probably removed co-translationally. The amidated peptide is the most active form, while the C-terminal glycine clearly removes some of the intracellular activity.

Lehrer: One of the protective devices has to be the compartmentalization of the cell; the peptide may not have direct access to the target structure, which may be the cell membrane. Another is that many of these peptides become cytotoxic when there's a permissive transmembrane potential. When they're in the cytoplasm, they're looking at the transmembrane voltage from the wrong side of the membrane. They're designed to work from the outside in rather than from the inside out.

Sahl: With the lantibiotics, we see that some operate with both orientations of the membrane voltage, i.e. can act from both sides of the membrane, and some can act only from the outside of a bacterial cell. Is this true for the defensins?

Lehrer: No, they're one-way peptides.

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Antimicrobial peptides from amphibian skin: an overview

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Abstract. Over the past three decades, numerous peptides have been isolated from amphibian skin secretions. Many of these peptides were shown to be homologous to hormones and neurotransmitters of mammals. In recent years it has been shown that these secretions also contain a multitude of antimicrobial peptides. Most of these peptides are positively charged and have a propensity for forming an amphipathic helix. Other types of peptides have been detected as well, including one group which contain D-allo-isoleucine in their sequences. This work has mainly been done with three species from different families, *Xenopus laevis*, *Bombina variegata* and *Rana esculenta*. Each of these frogs produces distinct sets of peptides which are not related to those of other species. It can therefore be expected that many additional peptides with antimicrobial activity are present in amphibian species from other families.

Extracts of the skin of frogs and toads have for centuries been used in folk medicine and witchcraft. With the advent of modern science, however, organic chemists, pharmacologists and physiologists began to investigate the structure and biological function of the many compounds that are present in amphibian skin. Starting with the investigations of Wieland in 1930, numerous biogenic amines, mostly derivatives of serotonin, have been isolated from this source (Erspamer 1971). About thirty years ago, it was independently demonstrated by two groups that frog skin also contains peptides. From the skin secretion of *Bombina variegata*, a peptide with the sequence Glu–His–Phe–Ala–Asp.amide was isolated (Kiss & Michl 1962); in the same year, bradykinin and physalaemin were shown to be present in the skin of other frogs (Erspamer et al 1962a,b). In the following decades, the studies of Erspamer and his colleagues as well as those of a number of other groups have shown that amphibian skin is indeed a rich source of a variety of peptides (for recent reviews, see Bevins & Zasloff 1990, Lazarus & Attila 1993). Most of these were discovered through their biological activities using a variety of isolated organ preparations. It was also noted that many of these skin peptides were similar or identical to peptide

hormones present in mammalian brain and other parts of the nervous system or in the gastrointestinal tract (Erspamer et al 1981). In addition, new peptide families were discovered for which mammalian counterparts have not yet been found. One example is a group of opioid peptides which contain a D-amino acid, generally D-alanine, in the second position (Erspamer 1992). Most of the dermorphins and deltorphins have a high affinity and selectivity for μ- or δ-opiate receptors, respectively.

Early on it was also noted that the skin secretions of *Bombina* sp. also contain haemolytic and antimicrobial peptides (Kiss & Michl 1962, Bachmayer et al 1967). This work led to the isolation of bombinin, a peptide that contained 24 amino acids and that exhibited haemolytic activity (Csordas & Michl 1970). Work on this type of skin peptide was not continued thereafter. On the other hand, in the following years, numerous studies dealt with the action of melittin, a peptide from bee venom that lyses animal cells and bacteria (Habermann 1972). This is the prototype of a whole family of positively charged, membrane-disrupting peptides that bind to phospholipid bilayers. The crystal structure of the melittin tetramer demonstrated that it can form an amphipathic helix with a kink in the central region (Terwilliger et al 1982) and in subsequent studies on similar peptides, the findings made with melittin have frequently served as references.

Peptides from the skin secretion of *Xenopus laevis*

Our own studies on amphibian skin peptides were initiated in 1980 with the aim of using the newly developed recombinant DNA techniques to investigate their biosynthesis. Since it was known at that time that many of the hormone-like peptides were present in skin in much higher concentrations than in mammalian brain or gastrointestinal tract, this seemed to be a more promising source for mRNAs from which to clone cDNAs encoding the respective precursors. In these investigations, we initially focused on the biosynthesis of caerulein (a relative of mammalian cholecystokinin) and thyrotropin-releasing hormone (TRH) in the skin of *Xenopus laevis* (Hoffmann et al 1983a, Richter et al 1984). Quite unexpectedly, we encountered a variety of new peptides which could at first be predicted only from the sequences of cDNAs. During the course of the sequence analysis of the caerulein precursors, for example, it was noted that the 'spacer' regions between caerulein copies potentially encoded a set of homologous basic peptides (Hoffmann et al 1983a, Richter et al 1985). A similar segment was found to be present in the precursor of xenopsin (Sures & Crippa 1984). In addition, several cDNAs encoding the precursor of yet another basic peptide were encountered by chance. This peptide was termed PGLa (*p*eptide with N-terminal *g*lycine and C-terminal *l*eucine *a*mide, see Table 1) (Hoffmann et al 1983b, Andreu et al 1985). A thorough analysis by Gibson, Williams and their colleagues (Gibson et al 1986, Giovannini et al 1987), as well as some of

TABLE 1 Antimicrobial peptides from the skin of different amphibian species

Species	Peptide	Sequence
Amphipathic peptides		
Xenopus laevis	Magainin/PGS	GIGKFLHSAGKFGKAFVGEIMKS
	PGLa	GMASKAGAIAGKIAKVALKAL.a
Rana brevipoda	Brevinin-2	GLLDSLKGFAATAGKGVLQSLLSTASCKLAKTC
Phyllomedusa sauvagei	Dermaseptin I	ALWKTMLKKLGTMALHAGKAALGAAADTISQGTQ.a
Bombina variegata	Bombinin	GIGGALLSAGKSALKGLAKGLAEHFAN.a
Other peptides		
Rana esculenta	Brevinin-IE	FLPLLAGLAANFLPKIFCKITRKC
		d
Bombina variegata	Bombinin-H5	IIGPVLGLVGSALGGLLKKI.a

'a' indicates that the C-terminus is amidated. The second residue of bombinin-H5 (marked d) is D-allo-isoleucine.

our own work (Andreu et al 1985, Richter et al 1985), demonstrated that all the basic peptides predicted from the sequences of cDNA clones were in fact present in the skin secretions of *X. laevis* It was noted by both groups that these peptides had the potential to form amphipathic helices and thus act as membrane-perturbing agents, as was already known for melittin and the cecropins (Hultmark et al 1982).

Further analysis of skin secretions, as well as cDNA cloning experiments, subsequently led to the discovery of additional peptides. In particular, these were the magainin/PGS peptides (see Table 1) (Giovannini et al 1987, Zasloff 1987). These are derived from precursors containing several copies of the two types of PGS/magainin entities (PGS stands for *p*eptide with N-terminal *g*lycine and C-terminal *s*erine). It was demonstrated by Zasloff (1987) that the magainins did indeed have antimicrobial activity. These observations and the extensive work on the biological activities of this group of peptides from the skin secretion of *X. laevis* are reviewed in Jacob & Zasloff (1994, this volume).

The studies on the peptides present in the skin secretion of a single species have revealed an unexpected complexity. In addition to a few hormone-like peptides (caerulein, xenopsin, TRH), at least a dozen different antimicrobial peptides have been found. In addition, peptides with unknown functions, such as levitide (Poulter et al 1988) and the xenoxins (Kolbe et al 1993), have also been detected.

Antimicrobial peptides from other frog species

The work on antimicrobial peptides from the skin of *Xenopus laevis* has in recent years been extended to a number of other amphibian species and this has led to the discovery of several new families of peptides. Some of these are basic peptides with the propensity to form α-helices while in other cases different structural motifs have been found which suggest alternative mechanisms of action. This work is briefly reviewed in the following sections.

Peptides from the skin of Ranidae

Studies with different species from the family of Ranidae have shown that their skin contains a large number of different antimicrobial peptides. The first of these, isolated from the skin of *Rana pipiens*, was shown to release histamine from mast cells (Horikawa et al 1985). More recently, homologous peptides with antimicrobial activity, termed brevinin-1 and brevinin-1E (see Table 1), were isolated from *Rana brevipoda* and *Rana esculenta*, respectively (Morikawa et al 1992, Simmaco et al 1993). These peptides possess a positive net charge and contain 24 amino acids, including two prolines. Two cysteines are present at positions 18 and 24 which form a disulphide loop (see Table 1). Interestingly, brevinin-1E is strongly haemolytic (Simmaco et al 1993).

This motif of two cysteines, one present at the C-terminus, separated by five amino acids, has also been found in a number of peptides present in the skin of these frogs. Besides the brevinins-1, these can be grouped into three subfamilies, namely brevinins-2 and esculentins-1 and -2. The N-terminal portions of these larger peptides, which contain up to 46 amino acids, can probably form amphipathic helices. It remains to be determined which parts of these molecules are essential for their biological activity.

In a thorough analysis, using both cDNA cloning and peptide isolation and sequencing, it was recently shown that the skin of a single species, *R. esculenta*, contains at least the following types of antimicrobial peptides: four brevinins-1, seven brevinins-2, three esculentins-1 and two esculentins-2 (Simmaco et al 1994). Just as in the case of *X. laevis*, a complex mixture of antimicrobial peptides is also present in the skin secretions of this *Rana* species.

Dermaseptins

The skin secretion of the Phyllomedusinae, a subfamily of hylid frogs in South and Middle America, has been shown to contain many different peptides. Of particular interest is the fact that a group of highly active opioid peptides which contain a D-amino acid has been isolated from this source (Erspamer 1992). Antimicrobial peptides were also detected in the skin of these species. The first was dermaseptin I (see Table 1), a 34 amino acid peptide isolated from *Phyllomedusa sauvagei* (Mor et al 1991). Evidence has been presented that residues (1–27) of dermaseptin I can form an amphipathic helix. Quite recently, additional dermaseptins have been characterized (Mor & Nicolas 1994a). These interesting peptides are very active against a variety of fungi and this property is retained in shorter fragments of dermaseptins. In particular, dermaseptin (1–18)-amide was found to be at least as active and less toxic than natural dermaseptin (Mor & Nicolas 1994b). From the skin of a related species, *Phyllomedusa bicolor*, a homologous peptide was isolated which enhances the binding of agonists to the A1 adenosine receptor (Daly et al 1992) and was therefore termed adenoregulin. It has since been demonstrated that this peptide also has antimicrobial, in particular fungicidal, activity (Mor & Nicolas 1994a).

Peptides from Bombina sp.

As was mentioned earlier, studies on the antimicrobial and haemolytic peptides in the skin secretions of *B. variegata* led to characterization of bombinin (Csordas & Michl 1970). In recent years this has been investigated in more detail using recombinant DNA techniques as well as peptide analysis (Gibson et al 1991, Simmaco et al 1991). In two related species, *B. variegata* and *Bombina orientalis*, several peptides related to bombinin have been characterized. These all contain 27 amino acids, with a constant C-terminal region and a variable N-terminal

segment. The sequence of one of these bombinins is shown in Table 1. They can all potentially form amphipathic helices. It seems likely that the original analysis of bombinin was carried out on a mixture of several peptides. Contrary to the earlier results, purified as well as synthetic bombinins are not haemolytic; however, these peptides show a high activity against different strains of staphylococci (Simmaco et al 1991).

From the sequence of cDNAs encoding the precursor of various bombinins, the existence of another peptide could be predicted (Simmaco et al 1991). It has since been shown that this and several related peptides with antibacterial activity occur in the skin secretion of *B. variegata* (Mignogna et al 1993). These have been named bombinins H as they are very hydrophobic and show some haemolytic activity. Of particular interest is the fact that some of these bombinins H contain a D-amino acid, D-allo-isoleucine, in the second position (see Table 1). These peptides cannot form an amphipathic helix and must act on membranes via a different mechanism.

Concluding remarks

Since the early 1960s, amphibian skin has been shown to contain a variety of peptides homologous or identical to mammalian hormones and/or neurotransmitters. In recent years, after the discovery of the magainins, the main interest has gradually shifted to antimicrobial peptides from this tissue. As is now known, the skin secretions of *X. laevis* contain a complex mixture of positively charged peptides with a propensity to form amphipathic helices. The activity of the individual peptides can be enhanced through synergistic effects.

Studies with other amphibian species indicate that *X. laevis* is by no means an exception. The skin secretions of *R. esculenta* contain numerous antimicrobial peptides belonging to four subgroups (Simmaco et al 1994), at least five different dermaseptins are present in the skin of *P. sauvagei* (Mor & Nicolas 1994a), and a complex mixture of bombinins and bombinins H has been found in *B. variegata* (Mignogna et al 1993). These are representatives of only four of some 20 families of amphibian species. It can thus be expected that a large variety of additional antimicrobial peptides exist in different frogs. Indeed, studies with two hylid species from Australia have led to the isolation of almost two dozen new peptides (Waugh et al 1993).

Most of these peptides can form amphipathic helices that encompass all or part of their sequence. This seems to be a frequent structural motif for antimicrobial peptides which otherwise show no discernible sequence homology. There is ample evidence that the positively charged peptides bind to phospholipid bilayers through ionic and hydrophobic interactions. In some instances, it has been demonstrated that synthetic peptides containing only D-amino acids are as active as the L-isomers (Wade et al 1990). However, other examples have

been found where the inhibition of growth and the killing of cells must proceed via different mechanisms. Brevinin-1 and bombinin-H are peptides which cannot form amphipathic helices and the structural motifs essential for their biological activity are currently not known. It would, for example, be interesting to test whether the all-D-isomers of these peptides are biologically active.

It is clear from these studies, which include just a few species, that in amphibians a variety of strategies have evolved to limit the proliferation of microorganisms on their skin. Whether homologues of these antimicrobial peptides are present in other species remains to be tested.

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DISCUSSION

Zasloff: My entry into the antimicrobial peptide field was prompted by my inability, as a physician, to understand how mucosal surfaces in vertebrates could withstand constant assault from microbes. It was only by circling around this issue that I appreciated that the frog, to some extent, presented a solution to that problem.

One thing that is extraordinary in the *Xenopus* family is that if you look at the family of antibiotic peptides they produce—amphipathic, α-helical, 21–27 amino acid residue peptides—other than them being amphipathic and cationic, they really show very little primary sequence homology. You would never be able to pick them out by a nucleic acid sequence search, for example. So they have been given the opportunity, for some reason or another, to drift rather surprisingly. In this setting, as Dr Kreil pointed out some time ago, the signal sequence is the most conserved portion of the preproteins. To some extent this has been the scenario in the frog work. We have never found the same antibiotic peptide sequence in two species—even *Xenopus borealis* has shown an extraordinary drift.

Another level of peptide–peptide interaction is the synergy that can be demonstrated very trivially to occur between PGLa and magainin (Juretic 1990, Williams et al 1990). What is amazing is that these very abundant peptides, which independently are exceedingly non-promiscuous (they don't lyse eukaryotic cells), when mixed become a potent haemolysin and a potent, promiscuous, melittin-like entity. We've mapped this at the peptide level and we now know it results from a peptide–peptide interaction, but this must be taking place in a membrane because we can't measure it physically in solution.

The system Dr Kreil has described is not only present on the skin. It is actually present throughout the wet skins of this animal, and the same structures—the granular glands—can be found throughout its gastrointestinal tract (Moore et al 1991). It is a defensive system that serves this animal not only on the surface but also on its internal epithelium.

Lehrer: The gland looks somewhat like a syringe designed to inject something outside, perhaps to coat the surface of the skin. If the same glands occur in the intestinal tract, does this system also work to defend the frog from organisms

that get into the lymphatic or the blood system? In other words, does it work inside the frog or just outside the frog?

Kreil: It is very unlikely that the peptides would get into the bloodstream. In fact, if some of these peptides (which may be present at high concentrations in the skin glands—several hundred micrograms per gram of skin) were to circulate in the blood, it would have severe consequences. From the anatomy of the skin glands—a large syncytium filled with elliptic granules with a duct to the surface of the skin—it is clear that they discharge in only one direction. The cells present in the stomach of *Xenopus laevis* also deliver the peptides to the outside, i.e. the lumen of the gastrointestinal tract.

Flajnik: The skin is very different during the tadpole stage; they don't have these glands (see e.g. Du Pasquier & Flajnik 1990). Do these glands all therefore appear during metamorphosis?

Zasloff: Although this differs slightly between species, these structures generally appear abruptly at metamorphosis and thus they are adult structures. Tadpoles of *Rana catesbeiana*, the common bullfrog, which has an extended tadpole life, express antibiotic peptides, but they are a product of the adult-type granular glands which generally arise during the extended metamorphosis they go through. We don't have the slightest idea how the frog defends itself so effectively from microbes during the tadpole phase. From my perspective, it's an extraordinarily fascinating area, because as you know, during the early phase of tadpole development, the tadpoles don't have much of a recognized immune system that could play a role in defending them. It's an intriguing problem.

Flajnik: This is very interesting, because tadpoles rarely get infected.

Zasloff: Tadpoles were the subjects of all of the early studies on regeneration; you can cut their tails off and they don't get infected.

Boman: It is strange that although there are 1–3 million insect species, representing a tremendous diversity of animals adapted to all sorts of ecological niches, they seem to have only a relatively small number of antimicrobial peptides, and the insect defensin seems to be very widely distributed. If we compare the insects with the frogs, it's very surprising that there is such a variety of frog antimicrobial peptides. It seems that *Xenopus* is the only frog that makes magainin. Why do frogs need such a range of defence peptides?

Flajnik: The amphibians (e.g. *Rana* and *Xenopus*) are evolutionarily very divergent. If they have so many different peptides, there is probably some redundancy in the system. It will be difficult to find the orthologous ones, both because the sequence divergence is rapid and possibly because some are lost through evolution, as there are so many of them.

Kreil: I look at this complex mixture of peptides and other components present in frog skin secretions as a kind of 'playground' for evolution. The only thing that counts is that the whole mixture has to fulfil certain functions, one being to act as a deterrent against predators (for some species at least, maybe not

for all of them), another one being inhibition of growth of microorganisms. But I have great difficulty in trying to explain why individual components are present in these secretions. Why is thyrotropin-releasing hormone (TRH) present in the skin secretions of some frogs? It may well be there just by accident, because some transcription factor acting in these specialized gland cells happens also to bind to the promoter of the gene for the TRH precursor. It probably makes no difference whether certain constituents are there or not—it is the biological activity of the whole mixture that is important. That's the only way for me to rationalize why some peptides are there, or why certain peptides are present in skin secretions of one species and not in those of a closely related one.

Hultmark: How much of this diversity is just because you're comparing different species and how much is within the species? Is there a whole range of different peptides in just one species?

Kreil: More than 30 different peptides have been identified in the skin secretions of *Xenopus laevis*. In another case, we sequenced seven cDNA clones from the skin of *Bombina variegata* encoding bombinin precursors (Simmaco et al 1991). Of these, six coded for different bombinins and no one knows how many exist. From the skin of a related species, *Bombina orientalis*, four additional bombinins were characterized (Gibson et al 1991), which were very similar but not identical to any of the peptides from the other species.

Hultmark: Are these all antibacterial?

Kreil: No, not all of them, but in those species where people have looked more closely (*Xenopus laevis, Bombina variegata, Rana esculenta*), the majority are antibacterial peptides.

Hultmark: That's comparable to what you find in insects.

Zasloff: No, the frog ones are all different, such that there's no sequence homology between any of them. So it would be as if you had 12 different classes of gene product expressed.

Hultmark: What you are seeing is probably a more rapid evolution of the frog peptides compared to the insect peptides.

Kreil: They may evolve at a high rate because there is little or no selection pressure for or against certain sequences. Anything goes, as long as the whole mixture works. That's the way I look at this bewildering multitude of frog skin peptides. An additional reason may be that through gene duplication, many genes encoding one type of precursor are present in the genome of a single species. The frequency of chance mutations could be higher in gene families.

Elsbach: Hans Boman, you mentioned that insects occupy a wide range of ecological niches; these also play an enormous role in the warm-blooded animals. For example, the evolution of bactenicins and some of the other bovine leukocyte peptides may well have to do with the fact that the cow and probably other ruminants have a different flora to deal with. This is something that we understand very little about yet: how different bacterial habitats influence the development of particular antimicrobial systems.

Flajnik: Especially for *Rana* and *Xenopus*; these have very different habitats.

Elsbach: We really haven't started to explore what particular bacterial or microbial challenges are due to in the course of evolution. Bob Lehrer has demonstrated that the defensins are not present in the leukocytes of mice, nor do they seem to have bactericidal permeability-inducing protein (BPI). Yet obviously the mice are very effective in dealing with a range of different microorganisms. There are aspects to the divergence of antimicrobial defences that are not adequately studied yet in terms of the microbial challenges that these various species have to face.

Lehrer: Defensins are not present in the leukocytes in mice (Eisenhauer & Lehrer 1992), but they are abundant in the leukocytes of rats (Eisenhauer et al 1990); they're also abundantly expressed in the small intestine of mice (Ouellette et al 1989, Eisenhauer et al 1992). The mice that we all work with are not creatures of Nature—they are highly inbred populations and no one has actually looked at the wild mouse yet. (By the way, I'm not so sure that a laboratory mouse would do that well in the wild.) Last summer I thought it would be fun to go back to a project that Tomas Ganz and I talk about occasionally and look at amphibian leukocytes. We got some bullfrogs and injected their dorsal lymph sacs with some living *Escherichia coli* and living *Staphylococcus aureus*. Nothing happened, so we started escalating the dose. Eventually, we were putting in billions of live organisms under the skin, presumably circumventing the skin antimicrobial defence system, and still nothing seemed to happen to the frogs. To my chagrin, I didn't see a white cell response, either, when I aspirated the fluid. I still don't understand why the frogs didn't get sick; they seemed to handle it. If I looked at the dorsal lymph fluids an hour or two after injection, I saw what I thought were some very sick looking *E. coli*. There was something miraculous going on here! Either this system does work internally or there's something else happening in the frog. Is there any evidence that these peptides may work as extracellular antibiotics *in vivo* or do they only work on surfaces?

Zasloff: The only real way to do these sorts of experiments is to produce mutant animals and show that they succumb to infection. Günther Kreil induces these animals to express antibiotic-laden secretions by putting 10 V across their backs or by just scratching them; if you just take the stuff off the animal's skin and put it on a bacterial lawn, you can pretty much eliminate every known organism. In the case of the skin secretion, it's pretty clear that for some period the surface is fully sterilized; how long that persists is another issue. Look at the tadpole: it is more interesting than the adult frog in many ways because it does not have the confounding antimicrobial peptide system of the adult. We have tried to induce this system by injecting them with *E. coli* and other bacteria. It is very difficult to kill these animals by injection; we could never induce an antimicrobial response. I conclude that the internal antimicrobial defence system of the tadpole must be labile, it may not be peptide based—or it is there and

we are just not smart enough to find it yet. We have tried to understand how a tadpole survives and we have failed embarrassingly.

Lehrer: In insects, there are high concentrations of antimicrobial peptides in the fluid-phase haemolymph. Do we know for sure that these are not present in the frog?

Zasloff: We have taken peritoneal fluid and blood from the frog and found that they have a number of antimicrobial substances in them. These are principally active against Gram-negative bacteria and are probably complement like. There's really no evidence for profound anti-Gram-positive bacterial activity, and yet if you introduce Gram-positive organisms into these animals, the animals do fine. It is really mysterious.

Flajnik: I've looked at 2D gels of the serum profiles of tadpoles versus adults and they change enormously in metamorphosis, so there may be something to this (unpublished results).

Ganz: The importance of using the right organism should be emphasized. I did the same experiments with the frog, where I injected bacteria intraperitoneally and looked for leukocyte responses. I didn't get any with standard laboratory strains and the frogs did fine. So in desperation I collected faeces from the frog and injected these; there was a beautiful leukocyte response. We cultured the bacterium that caused this, which turned out to be *Enterobacter cloacae*. This seems to be the organism that the frog responds to.

Boman: It's the same with the insect. I began using *Enterobacter cloacae* in 1970 in *Drosophila*. At the start I put in different antibiotic resistance markers, because I wanted to vaccinate the flies with live bacteria and then see how long different bacteria could persist in the vaccinated insect (Boman et al 1972). This is also the point of using the D21 and D31 strains of *E. coli*: they permit experimental infections without contamination from the natural flora, because that level of resistance is never present in the normal flora of any animals. We found that *E. coli* can exist at a steady-state level in *Drosophila* for almost the full lifespan of the fly. I think this may have survival value because these bacteria provoke the immune system at a suitable level. They are not too aggressive and not too weak. *Enterobacter cloacae* has the same property of being very suitable for stimulating the immune system in many insects without doing too much harm to the host.

Casteels: Amphibians can secrete whatever active peptides they want to on their skin, because it's not sensitive—it's not inside the animal itself. For instance, if amphibians had melittin, they could even secrete this on their skin. Because insects secrete their antimicrobial peptides into the haemolymph, the range of products they can release is very restricted. This could account for the large differences between the number of active peptides on the skin of the frog and in insect haemolymph.

Elsbach: I wonder if I may make a sacrilegious comment. We are assuming that all these 'antimicrobial peptides' are antimicrobial peptides biologically;

the possibility has to be kept in mind that the enormous diversity, especially in these amphibian peptides, may be a reflection of the fact that they are actually serving diverse functions that we don't yet know about. We measure what we know how to and like to measure. There are obvious limitations to our tendency to draw firm conclusions about the biological roles we assign to the agents we pull out of their natural environment.

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Gene-encoded antimicrobial peptides from plants

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Abstract. On the basis of an extensive screening of seeds from various plant species, we have isolated and characterized several different antimicrobial peptides. They were all typified by having a broad antifungal activity spectrum, a relatively low molecular weight (3–14 kDa), a high cysteine content and a high isoelectric point (pI > 10). With respect to their amino acid sequence, these peptides can be classified into six structural classes. Synergistic enhancement (up to 73-fold) of antimicrobial activity was demonstrated in some combinations of peptides belonging to different classes. cDNA clones corresponding to different antifungal peptides were isolated and used to transform tobacco plants. Extracts of these transgenic plants showed higher (up to 16-fold) antifungal activity than untransformed control plants. Such antimicrobial peptides may find applications in molecular breeding of plants with increased disease resistance.

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To protect themselves against pathogens, plants do not possess an immune system, such as that found in higher vertebrates. Their resistance is mainly based on a dynamic defence system composed of antimicrobial agents of different molecular weight classes. The first group covers low molecular weight compounds such as phytoalexins, i.e. secondary products of plant metabolism (Dixon et al 1983). Phytoalexins rapidly accumulate at the sites of infection to act as antibiotics against invading pathogens. Defence-related proteins of higher molecular mass form the second group of the plant's defence mechanism. They include structural proteins such as hydroxyproline-rich glycoproteins that incorporate into the cell wall to participate in confinement of pathogens (Showalter 1993). However, the best-known group of stress- or infection-induced plant proteins is formed by the diverse classes of pathogenesis-related (PR) proteins (recently reviewed by Stintzi et al 1993). Well-studied

examples of the PR proteins are the chitinases and β-1,3-glucanases, which are believed to combat fungal pathogens by hydrolysing their cell walls.

In this paper, we review our recent work on a third group of antibiotic plant compounds with an intermediate molecular mass. These represent cysteine-rich peptides and small proteins with masses ranging from 3 to 14 kDa.

Cysteine-rich plant antimicrobial peptides (AMPs): structural properties

With the aim of discovering new types of antifungal proteins, we screened seeds from about 600 plant species belonging to a wide range of plant families. On the basis of similarities in primary structure, the new antifungal proteins we discovered can be separated into six different classes.

Mirabilis jalapa antimicrobial peptides (Mj-AMPs)

A first class of plant AMPs occurs in seeds of *Mirabilis jalapa* L. (four o'clock plant) and related species from the Nyctaginaceae family. Two isoforms of 37 and 36 residues, designated *Mj*-AMP1 and *Mj*-AMP2, respectively, and differing from each other by only four amino acids, were isolated from seeds of *M. jalapa* (Table 1) (Cammue et al 1992). Both peptides are highly basic (pI > 10) and are stabilized by three disulphide bridges. Their amino acid sequence differs from all plant proteins hitherto known. The *M. jalapa* AMPs appear to be seed-specific proteins as they could not be detected by Northern blot analysis in non-seed tissues of *M. jalapa*, even under various stress conditions (M. F. C. De Bolle, unpublished results).

Amaranthus caudatus antimicrobial peptides (Ac-AMPs)

A second type of plant AMP was discovered in seeds of *Amaranthus caudatus* (amaranth) (Broekaert et al 1992). As for the *Mj*-AMPs, two near-identical isoforms, termed *Ac*-AMP1 and *Ac*-AMP2, could be distinguished. Both amaranth peptides are also very basic (pI > 10), small (3025 and 3181 Da, respectively) and contain three disulphide bridges (Table 1). As for the *Mirabilis* AMPs, the disulphide bridges are formed by the six cysteine residues present, of which the third and fourth residues are at adjacent positions and flanked by a serine. Besides these structural similarities between AMPs from *M. jalapa* and *A. caudatus*, no further sequence homology could be found. However, striking primary structure homologies exist with the cysteine/glycine-rich domain of chitin-binding plant proteins such as chitinases, chitin-binding lectins and hevein (recently reviewed by Raikhel et al 1993). As with the latter proteins, the *Ac*-AMPs can be reversibly bound on chitin substrates (Broekaert et al 1992). Like the *Mj*-AMPs, *Ac*-AMPs appear to be seed-specific proteins (De Bolle et al 1993).

Raphanus sativus antifungal peptides (Rs-AFPs)

Analysis of the imbibition medium of germinating radish (*Raphanus sativus*) seeds revealed the presence of two highly homologous antifungal proteins (*Rs*-AFP1 and *Rs*-AFP2) of about 5 kDa, each containing four disulphide bridges (Terras et al 1992a). These peptides show striking amino acid sequence homology with other plant proteins thought to be involved in plant defence mechanisms. These include inhibitors of insect α-amylases present in sorghum, γ-thionins from wheat and barley endosperm, and two proteins derived from pea genes that are specifically induced by fungal attack. Antifungal proteins almost identical to the *Rs*-AFPs were isolated from seeds of four other Brassicaceae tested (Terras et al 1993a). Proteins homologous to the *Rs*-AFPs were also observed to accumulate in radish leaves in response to infection (F. R. G. Terras, unpublished results).

Raphanus sativus non-specific lipid transfer protein (Rs-nsLTP)

A 9 kDa protein with antifungal activity was partially co-purified during the isolation of the *Rs*-AFPs from radish seeds. Characterization of this protein revealed extensive N-terminal sequence homology with non-specific lipid transfer proteins (nsLTPs) from other plant sources (Table 1) (Terras et al 1992b). Besides this sequence homology, the radish nsLTP-like protein shares other characteristics with lipid transfer proteins, such as its molecular structure (it is a dimer of 2×9 kDa) and its high isoelectric point (> 10.5). The *R. sativus* nsLTP (*Rs*-nsLTP) was the first lipid transfer protein from plant seeds reported to exert antifungal activity. Almost simultaneously, leaf nsLTPs from maize and barley were demonstrated by others to have antifungal properties (Molina et al 1993).

Raphanus sativus 2S albumins (Rs-2S)

Another type of antifungal protein isolated from radish seeds was identified as one of the well-known seed storage proteins called 2S albumins (Terras et al 1992a). This identification was based on the following characteristics: a molecular mass of about 14 kDa, the existence of multiple isoforms, a molecular structure consisting of a small (4 kDa) and a large (10 kDa) subunit, and an extensive homology in primary structure to reported cDNA-derived sequences of radish 2S albumins (Table 1). As for the *Rs*-AFPs, 2S albumins present in seeds of other species of Brassicaceae were demonstrated to exert antifungal activity (Terras et al 1993b).

Thionins

Because of some common features (small size, basic nature), all the above-mentioned classes of AMPs could be purified using a standard sequence of

chromatography steps. Using the same basic protocol, we were able to isolate from barley (*Hordeum vulgare*) another class of potent AMPs (F. R. G. Terras, unpublished results); these turned out to be the well-known thionins, of which the antimicrobial properties are well established (Redman & Fisher 1969, Fernandez de Caleya et al 1972, Bohlmann et al 1988). Thionins are a group of cysteine-rich peptides with a compact (5 kDa) amphipathic structure stabilized by three or four disulphide bridges. They are generally located intracellularly in the endosperm of monocotyledonous plants such as wheat (purothionins), barley (hordothionins), rye, maize and oat. Both intracellular and extracellular forms have also been detected in leaves of the same plants, where their expression is induced by several stress factors and upon challenge with pathogenic fungi (Bohlmann et al 1988, Reimann-Philipp et al 1989). Thionins were also detected in leaves of a few dicotyledonous species (e.g. *Pyrularia pubera* and *Viscum album*) (Vernon et al 1985, Samuelsson et al 1968). To avoid confusion, we should mention that some recently discovered proteins from barley and wheat endosperm, termed γ-thionins, were originally classified as thionins (Mendez et al 1990, Colilla et al 1990). However, more detailed analysis of their primary structure indicated that they belong to a different protein family that includes *Rs*-AFPs (Terras et al 1992a).

Cysteine-rich plant antimicrobial peptides: antimicrobial activities

Antifungal activity

Extended analysis of the antifungal activity of the AMPs described above revealed that they are fungistatic towards a broad spectrum of phytopathogenic fungi. From these data, summarized in Table 2, it can be concluded firstly that the *Mj*-AMP2, *Ac*-AMP2, *Rs*-AFP2 and wheat thionin exhibit very high antifungal activity; this is reflected by the very low concentrations (0.5–8 μg/ml) necessary for 50% inhibition (IC_{50} values) of the fungi tested. Both the 2S albumins and nsLTPs from radish exhibit relatively moderate antifungal activity with IC_{50} values from 15 up to 500 μg/ml. To approximate physiological ionic strength conditions, we performed similar antifungal assays in growth medium supplemented with 1 mM $CaCl_2$ and 50 mM KCl (Table 2). In this medium, antifungal activity was drastically reduced or even abolished for most of the AMPs tested, with the exception of *Rs*-AFP2 and β-purothionins, which were less salt sensitive. Further experiments demonstrated that this reduction in antifungal activity was most pronounced in the presence of divalent cations (results not shown). Morphological analysis of the antifungal activity revealed important differences between some of the AMP classes. Most of the AMPs severely delay the growth of the fungal hyphae without changing considerably the normal mycelial morphology (Fig. 1B,C). The *Rs*-AFPs, in contrast, exhibit a totally different type of fungal growth inhibition, characterized by hyperbranching

TABLE 2   Overview of the antibiotic activity of antimicrobial peptides belonging to different classes

Cell type	Peptide					
	Mj-AMP2	Ac-AMP2	Rs-AFP2	Rs-nsLTP	Rs-2S	β-purothionin
Fungi LIS	+ +	+ +	+ +	+	+	+ +
MIS	−	−	+	−	−	+ +
Yeast	+ +	+ +	−	ND	−	+ +
Bacteria						
Gram +	+ +	+ +	−	ND	+	+ +
Gram −	−	−	−	ND	+	+ +
Plant	−	−	−	ND	−	−
Insect	−	−	−	ND	−	ND
Human	−	−	−	−	−	+ +

Activity levels are expressed as: + +, high activity ($IC_{50} \leqslant 10\,\mu g/ml$); +, moderate activity ($10\,\mu g/ml < IC_{50} < 100\,\mu g/ml$); −, no or low activity ($IC_{50} \geqslant 100\,\mu g/ml$). IC_{50} determined as the minimum concentration of the peptide necessary to cause 50% inhibition of cellular growth (for fungi, yeast and bacteria) or 50% loss of cell viability (for plant, insect and human cells). LIS, low ionic strength medium (Cammue et al 1992); MIS, medium ionic strength medium = LIS + 1 mM $CaCl_2$ + 50 mM KCl; ND, not determined.

and swelling of the hyphae (Fig. 1D). Furthermore, all the AMPs tested seem to act fungistatically, with the exception of the thionins, which cause a pronounced decrease of fungal viability. This lethal effect was not found for the 2S albumins, although both thionins and 2S albumins were shown to have a similar effect on fungi, namely permeabilization of the fungal plasmalemma around hyphal tips (Fig. 1E,F) (Terras et al 1993b).

To assess possible synergistic effects between AMPs of different classes, we tested dual combinations of different types of AMP on a range of phyto-pathogenic fungi. So far, we have only observed a clear synergism between thionins and 2S albumin-like proteins. Depending on the test fungus and the ionic strength of the growth medium, IC_{50} values of thionins (from either wheat or barley) could be lowered 2–73-fold by adding subinhibitory concentrations of 2S albumins of various Brassicaceae (Terras et al 1993b). Comparable synergism was observed by combining thionins with trypsin inhibitors from barley and wheat grains. These inhibitors are known to show homology in primary structure with the radish 2S albumins. Another synergistic combination has recently been reported for wheat thionin and an nsLTP from barley leaves (Molina et al 1993).

Other antibiotic properties

To address the question of whether the inhibitory effect of the AMPs—purified on the basis of their antifungal activity—is restricted to fungi, we investigated their effect on other cell types. Table 2 shows that the AMPs from
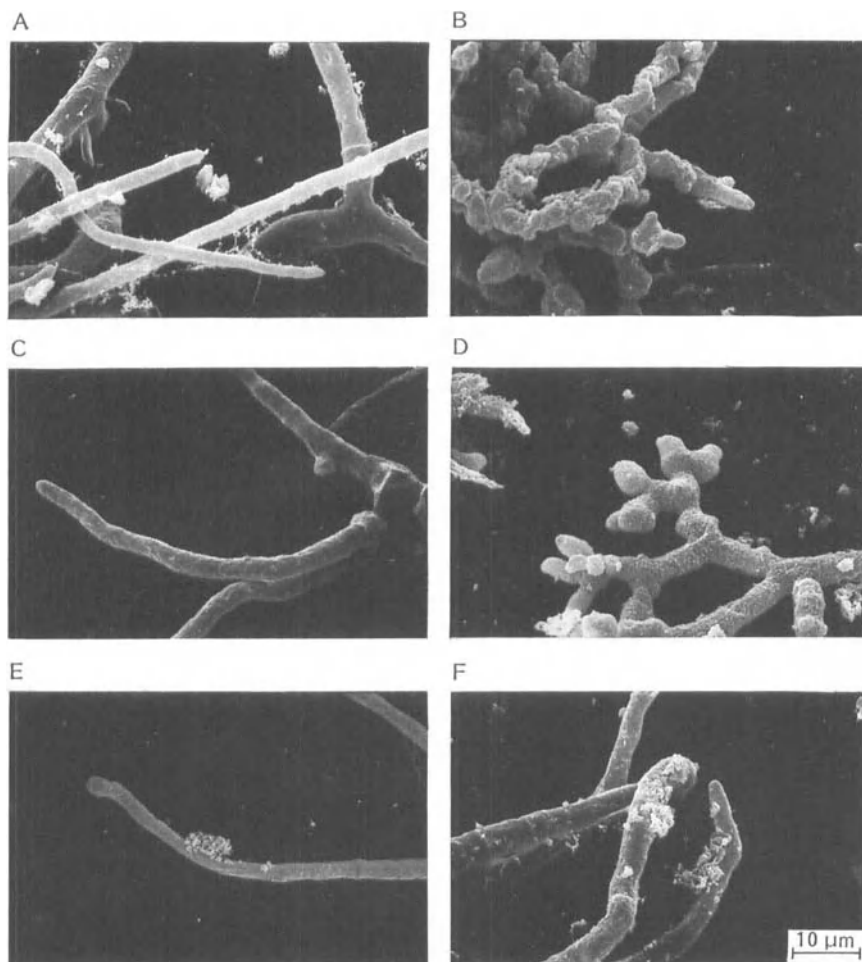
FIG. 1.   Scanning electron micrographs of hyphae of *Botrytis cinerea* grown for 36 h in the presence of: A, water (control); B, *Mj*-AMP2; C, *Ac*-AMP2; D, *Rs*-AFP2; E, *Rs*-2S (isoform 4); F, β-purothionin. The final concentration of the antimicrobial peptides was 50 μg/ml. Bar = 10 μm. Scanning electron microscopy was performed as described by Terras et al (1993b).

Mirabilis jalapa and *Amaranthus caudatus* exhibit rather similar antimicrobial spectra, which is restricted to fungi, yeast and Gram-positive bacteria. The *Rs*-AFPs, on the contrary, do not affect any of the organisms tested except for filamentous fungi (at concentrations below 100 μg/ml). They are therefore termed antifungal proteins (AFPs) rather than AMPs. Antibacterial activity assays have not yet been performed with the radish seed nsLTPs. However, a recent report on

leaf nsLTPs from maize and barley indicates they have strong inhibitory activity on some Gram-negative phytopathogenic bacteria (Molina et al 1993). Besides their antifungal activity, 2S albumins also seem to exhibit a previously unreported (moderate) antibacterial effect on both Gram-positive and Gram-negative bacteria (Terras et al 1992a). Thionins, which showed a similar permeabilizing effect on fungi to the Rs-2S, have a devastating effect on all cell types tested, except plant cells. Our results confirm the earlier reported general cytotoxicity of thionins (reviewed by Bohlmann 1994). We also looked to see if thionins and 2S albumins had a similar synergistic effect to that mentioned above when they were applied together to yeast, bacteria and cultured human cells. From these experiments, we concluded that thionins and 2S albumins exert synergistic effect only on cell types that are inhibited by either of both types of protein separately (Terras et al 1993b).

Transformation with antimicrobial peptide-encoding genes and evaluation of transgenic plants

Complementary DNA clones encoding the Mj-AMP2, Ac-AMP2 and Rs-AFP2 have been isolated and characterized (M. F. C. De Bolle, unpublished results 1993, De Bolle et al 1993, F. R. G. Terras, unpublished results 1993). Their coding regions seem to be restricted to a putative signal sequence (of 81, 75 and 87 base pairs, respectively) and a domain corresponding to the mature protein. In addition, the coding region of the Ac-AMP2 cDNA is extended by 93 base pairs encoding a C-terminal propeptide with a putative N-glycosylation site (De Bolle et al 1993). Whether this propeptide plays a role in vacuolar targeting, as reported for some homologous chitin-binding proteins (Bednarek & Raikhel 1992), remains to be determined. The isolated cDNAs were inserted into plant expression vectors (under the control of CaMV35S promoters) and transferred to tobacco. Expression of the introduced genes in the primary transformants was confirmed by Western blot analysis for Mj-AMP2 and Rs-AFP2. High-expression lines were self pollinated and the progeny analysed. The introduced AMP-encoding genes seem to be stably inherited through (at least) two generations. Extracts from these transgenic plants showed up to 16-fold higher in vitro activity against Fusarium culmorum when compared with untransformed control plants (M. F. C. De Bolle, F. R. G. Terras & R. W. Osborn, unpublished results 1993). This indicates that the expressed AMPs are correctly processed and folded, because they retain their antifungal activity. Moreover, their antifungal activity was characterized by the typical 'hyperbranching effect' when extracts of transgenic tobacco expressing the Rs-AFP2 were added to the test fungus. Homozygous lines expressing Mj-AMP2 and Rs-AFP2 at about 0.1% of soluble protein are currently being evaluated for disease resistance.

Conclusions

In this paper we have reviewed our recent work on antimicrobial plant peptides. Primarily on the basis of their amino acid sequences, these AMPs can be separated into six different classes. With respect to their more general structural properties, they appear to belong to a superfamily of small, highly basic, cysteine-rich proteins exhibiting antibiotic activities. This superfamily can even be extended to include proteins with similar structures from outside the plant kingdom, including defensins from mammals (Lehrer et al 1991) and insects (Lambert et al 1989), bovine bactenecins (Romeo et al 1988), tachyplesin from the horseshoe crab (Katsu et al 1993) and an antifungal protein secreted by the fungus *Aspergillus giganteus* (Nakaya et al 1990). For most of these antimicrobial peptides, a role in host defence has been demonstrated. Although some of our data on the localization, inducibility and specific antibiotic activity of the plant AMPs suggest a similar protective role, their biological function(s) *in planta* need(s) to be investigated further.

On the other hand, the plant AMPs we have described exhibit several attractive properties in the light of their possible use as transferable resistance traits for genetic engineering of crop plants. Firstly, they show a relatively high inhibitory activity against a broad spectrum of phytopathogenic fungi. This suggests that when these AMPs are expressed in transgenic crops, they could be active against a wide range of fungal pathogens causing soil-borne, vascular, foliar and post-harvest diseases. Secondly, with the exception of thionins, the antibiotic activity of the plant AMPs is specific for fungi, yeast and bacteria—they are not toxic to human cells. This property is important if they are to be expressed in edible crops. Moreover, their fungistatic rather than fungicidal activity allows a minimal growth of the fungus, which *in planta* can trigger other endogenous defence mechanisms. Thirdly, with respect to their inhibitory mode of action, at least three different types of AMPs have been distinguished. Combining two (or more) AMPs with a different mode of action in the genetic manipulation of a crop plant would considerably slow down the emergence of resistant pathogens. Finally, although the synergism we have observed between different AMPs might not be biologically relevant, combinations of synergistically acting AMPs could drastically enhance their antimicrobial potency *in planta*.

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DISCUSSION

Casteels: I have a question about the microtitre plate-based assay you used. We also carry out all our antibacterial tests on microtitre plates. You read your plates on a scanner; do you think that the optical density readings of the medium give accurate values, because fungi tend to grow as clusters, which could cause strong aberrations in your readings?

Cammue: We published a paper on this (Broekaert et al 1990): the technique is based on a straight-line correlation between absorbance and the fungal dry weight. That's why we based our experiments on this assay. But it is true what you are saying, especially in cases where you observe effects like hyperbranching caused by the *Rs*-AFP2. We always confirm our results by microscopy.

Ganz: Was there anything in your selection scheme that biased the selection towards cysteine-rich antimicrobial peptides, or is just that the various antibiotics naturally tend to be cysteine rich in plants?

Cammue: We started by screening for proteins with antifungal activity; this was the sole criterion. It may be a consequence of our screening or isolation procedure that we always came up with proteins that were small, basic and had quite a high cysteine content.

Ganz: I'm thinking of one particular aspect: when you disrupt plant cells, proteolytic enzymes are released to which cysteine-rich peptides may be more resistant than are other peptides. Was that a factor in the extraction procedure?

Cammue: Originally, we just screened plant extracts saturated with ammonium sulphate, in order to enrich the extract in protein components. This extract was tested for antifungal activity.

Lehrer: Was it tested in the presence of the ammonium sulphate, i.e. in a high-salt solution?

Cammue: No, it was dialysed first.

Lehrer: Some of our earliest work with antimicrobial peptides was done with rabbit lung macrophages; we sought to identify components with activity against fungi. We ended up with the cysteine-rich defensins.

Cammue: There are, of course, other antifungal proteins present in plants, like the chitinases and glucanases that I mentioned earlier. Currently, with our screening and isolation procedures, we have purified more than 20 different proteins, which we have always been able to place in one of the six classes I outlined.

Lehrer: Do you have a feeling of how these peptides protect plants? Do they prevent invasion?

Cammue: At present, we don't know the exact mode of action of our proteins on fungi. We are currently investigating their toxicity towards fungi in more detail.

Lehrer: What about their localization in the plants you isolated them from?

Cammue: All these peptides were originally isolated from seeds.

Lehrer: What about mature plants?

Cammue: For the first two classes, the *Mj*-AMPs and *Ac*-AMPs, we analysed the mature plants and we were not able to detect any of these proteins. However, we were able to induce *Rs*-AFP-like proteins (the third class of antifungal proteins that I mentioned), which we call plant defensins, in leaves of radish plants after fungal attack. So far we have been unable to purify these leaf proteins. Induction of defence proteins by pathogen attack has been demonstrated by other research groups for leaf thionins (see review by Bohlmann 1994).

Lehrer: What you are describing is approaching an immune response in plants. Have you tried experiments to see whether this system has any memory?

Cammue: No, we haven't. At least our results prove that the defence system in plants is not just a static system—it is also a dynamic system.

With respect to the antifungal proteins present in seeds, it is worth mentioning some studies that we have done on the release of antifungal proteins from germinating radish seeds. On average, a single radish seed contains about 5 μg of *Rs*-AFP. When radish seeds were slightly incised and put in a germination medium, we showed that 1.5 μg of *Rs*-AFP per seed is released into the imbibition medium after 4 h incubation. This is about 30% of the total the seed contains.

On the other hand, when the same calculations were done for the total seed protein, we found that only 0.6% of the total soluble seed protein could be detected after 4 h in the medium. This suggests that the *Rs*-AFPs are preferentially released and might have a specific function during early seed germination. Because of their antifungal activity, we propose that this function is to protect the germling against fungal attack.

Zasloff: Plants also make an array of low molecular weight organic compounds that are present in very large amounts in many species, and I guess in some cases are induced. Do they co-exist with these classes of antimicrobial substances?

Cammue: Yes, I think so. They include the phytoalexins, which occur in many plant species and are in most cases induced by microbial attack. The dry seed, however, is a resting organ, so you cannot induce anything in it. Instead, any antimicrobial defence has to be by means of something that is present at the time of fungal attack.

We observed that these antifungal proteins accumulate mainly during the later stages of seed maturation, so that they are present at their highest concentrations at the time of germination. Germination includes the breaking of the seed coat, which acts as a tough physical barrier against microbial pathogens. A quick release of antifungal protein at this time might be necessary to protect the weak germling.

The situation in vegetative tissues is different: there, the production of some antifungal proteins can be induced upon attack by pathogens, as I mentioned for the induced *Rs*-AFP-like proteins in radish leaves.

Sahl: I was interested to hear that when you increase the ionic strength of your test medium the activity of these peptides is much reduced. We observed a similar effect with the lantibiotics. One reason for this is that the rate of efflux of cellular solutes is decreased when the ionic strength is increased. A second reason might be that in order for the peptides to have their effect they first have to bind to the cell wall of the Gram-positive bacteria, which is a highly anionic polymer because of the teichoic acids. With isolated cell walls, we could show that the peptides actually bind and that these cell walls act as a cation exchanger from which you can remove the bound peptide with increasing salt concentrations. This is also a relevant point for the activity of these peptides. What is the ionic strength *in vivo* where your peptides are acting? Do you know what the ionic strength is in the plant?

Cammue: The salt sensitivity of our proteins is most pronounced in the presence of divalent cations. Sensitivity varies greatly with the type of test fungus. From this, it is likely that there is an interaction of the fungus with the cations, rather than a direct protein–cation interaction. In that sense, the salts could antagonize the binding of the protein to the fungus (as you remarked for the lantibiotics on bacteria) and could protect the fungus against the activity of the antifungal proteins.

One can't, of course, say anything about the ionic strength of the dry seed tissue in which the peptides occur. When the seed is germinating, these peptides are (partially) released into the soil surrounding the seed. It's very difficult to give an idea of the ionic strength in that particular environment.

With respect to vegetative plant tissues, there are some reports of the cellular concentration of free Ca^{2+}, Mg^{2+} and K^+, indicating that relatively high ionic strengths occur in most compartments. But it is also difficult to predict what will happen when a fungus enters these tissues, breaks open the cells and creates a mixture of components that are normally separated in different cellular compartments. In other words, it is difficult to predict the ionic conditions under which the antifungal proteins will react with the penetrating fungus.

At first, when we saw that activity of the *Mj*-AMP proteins was very sensitive to the presence of Ca^{2+} and K^+ (but especially divalent cations), we were worried about the effectiveness of its antifungal activity in transgenic plants. That's also why we were happy to discover other peptides, such as the *Rs*-AFP2, which were much less sensitive to divalent cations. From the first analysis of transgenic plants, we found that the transgenic plants expressing the *Rs*-AFP2 exhibit increased resistance to fungal attack. *Mj*-AMP2-expressing plants are currently being evaluated.

Ganz: I think it is very beautiful that you were able to complete the circle and show that these peptides, when exogenously introduced, can protect the plant. We've all been trying to do that with animal antimicrobial peptides, and we haven't yet been able to do it.

Lehrer: Although I agree that this work is very beautiful, I don't think the circle is yet completed. You need to show that you can remove the peptide from the parent plant, have the plant become susceptible to fungal disease, then put the peptide back in and restore resistance.

Cammue: We are considering doing this, for example, by antisense suppression of these genes in the native plant.

Kreil: Starting with these model experiments in tobacco, what would be your dream experiment? Is there an important crop plant in some region which could be protected by these antifungal peptides, thereby changing dramatically the quantity that can be harvested and stored?

Cammue: There are many candidate crops for transformation with these antifungal genes. Fungal diseases are devastating in many crops throughout the world. I have to add, though, that our proteins are not the only candidates for use as tools for resistance in crops: several groups are working with other types of antifungal protein, for example, the previously mentioned chitinases/glucanases and thionins (reviewed in Punja & Zhang 1993, Bohlmann 1994). Transgenic plants producing thionins show increased resistance to fungal attack.

From a commercial point of view, besides demonstrating the scientific potential of these transgenic plants, it important for us also to be able to convince public opinion before we can sell plants expressing foreign genes. If the foreign

gene is coming from another plant, which ideally is also edible, one will have much more chance of convincing people that it is safe.

Zasloff: There are several patents, especially in the European patent literature, demonstrating the transfection of cecropins and magainins into food plants.

Cammue: A lot will depend on how consumers are informed about these transgenic plants. As an illustration of this, I will briefly mention the following anecdote. One or two years ago I participated in an international banana meeting at which most of the attendees were classical banana breeders. Because of a few presentations made by groups working on genetic transformation of banana, the following question was raised at the end of the meeting: 'who would accept eating bananas containing proteins from another organism?' No one raised a hand except the molecular biologists. I asked the question more specifically: 'who would accept eating transgenic bananas if they contain genes from another (edible) plant?', and nearly all raised their hands.

In some cases, even bad terminology can have a negative impact on public opinion. This was the case for the Bt proteins, insecticidal proteins isolated from the bacterium *Bacillus thuringiensis.* Now, people try to avoid calling them 'endotoxins', as they were originally termed, just because of the word 'toxin'.

Bevins: Have you considered making a transgenic plant where these antimicrobial peptide genes are driven by inducible promoters? This would allow you to induce these peptides just when they are needed to fight infection and you could avoid having high concentrations of them in, say, the fruit.

Cammue: The first thing that we wanted to prove was the concept that our antifungal proteins could be expressed in other crops and give a certain resistance. Therefore we used only one promoter to drive the expression of the different antimicrobial peptide genes. This was the widely used cauliflower mosaic virus 35S promoter, which gives constitutive expression. On the basis of the results that we now have, we can start thinking about increasing or fine-tuning this expression. In this context, inducible promoters are considered. They would use less of the plant's energy than does constitutive expression of the proteins.

We have also considered using tissue-specific promoters to drive these genes. In this case, you could avoid having high concentrations of antifungal proteins in the fruits, as you mentioned. However, this might not be desirable: because of the broad spectrum of antifungal activity of these proteins, they are potentially useful in protection against post-harvest rots, which are often caused by fungi.

Zasloff: What are your feelings about overlap in defence systems? There's a large and an old literature on phytoalexins. There are clearly interesting inducible alkaloids. Do you see them as complementing this system in some plants? Or do you believe that there are species where antibiotic peptides are not expressed and the plant is solely protected by secondary metabolites? Are there systems where both are operating?

Cammue: Plants, like other organisms, defend themselves against pathogens using combinations of different defence mechanisms. These include different types of physical barrier as well as the production of a variety of antibiotic compounds. Similarly, if you're aiming at creating transgenic crops that would express a more durable resistance, you should not focus on just one type of antimicrobial system. You have to try several combinations of different antimicrobial components. In that sense, I think that it is important in our work that we possess proteins with at least three different modes of action. We will probably use combinations of these different types of antifungal protein in the final transgenic crops. This would decrease considerably the rate of emergence of resistant pathogens. In addition, I think you also have to take advantage of the endogenous defence mechanisms that are already present in the target plant. This is one of the advantages of the first five classes of our proteins, which act fungistatically. In contrast to the thionins, they allow a little outgrowth of the fungus. This might be enough to trigger the endogenous defence mechanisms, which can complement the action of the antifungal proteins. At first sight you might think that fungicidal proteins would be more interesting than those that are fungistatic, but I think the opposite might be true.

Lehrer: In your various classes of antifungal proteins, are all of the cysteines present as disulphides?

Cammue: Yes.

Lehrer: If you reduce and alkylate the disulphides in the peptides, do they retain activity or lose it?

Cammue: They lose their activity. I think the 3D structure of the protein, stabilized by the disulphide bridges, is important. On the other hand, because of this compact structure, these proteins are also very stable; you can heat them for 10 min at 100 °C without any loss of antifungal activity. Coming back to the question Tomas Ganz asked earlier, this stability might be a reason why these small cysteine-rich petides were predominantly found as active substances in early screenings for antifungal proteins in both animal and plant systems.

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Drosophila as a model system for antibacterial peptides

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Abstract. As a defence against bacteria, infected insects synthesize cecropins and a large number of other bactericidal proteins and peptides. To understand this response and its possible relationship with similar systems in mammals, we need to characterize the induced components and how they act, as well as how this antibacterial response is initiated. To study the molecular basis for this response we cloned the genes for cecropins and other bactericidal peptides from *Drosophila*, 14 genes in total. The cecropin genes were selected as convenient markers for the immune response because they are strongly induced by different microbial substances. In contrast the lysozyme gene family is constitutively expressed in the digestive tract. We have developed an inducible blood cell line from *Drosophila* for studying the immune response *in vitro*. Using this system we are now investigating the function of membrane proteins and signal pathways in the transcriptional activation of immune genes in *Drosophila*.

As a defence against bacterial infection, insects have been found to produce a suprisingly diverse array of antibacterial proteins and peptides that accumulate in the blood (for reviews, see Faye & Hultmark 1993, Hultmark 1993). With the exception of insect lysozyme (related to c-type lysozymes in vertebrates), these inducible effector molecules represent novel types of factors which were first discovered in insects. They include broad-spectrum antibiotic peptides such as the cecropins (Hultmark et al 1980) that kill and lyse a majority of the bacteria that have been tested. The sapecins (Matsuyama & Natori 1988), or insect defensins (Lambert et al 1989), are also potent bactericidal peptides, mainly active against Gram-positive bacteria. Attacin (Hultmark et al 1983), sarcotoxin II (Ando et al 1987), diptericin (Dimarcq et al 1988) and coleoptericin (Bulet et al 1991) are more restricted in their antibacterial spectra and the isolated proteins affect a limited number of Gram-negative bacteria. Finally, several proline-rich antibacterial peptides, such as apidaecin (Casteels et al 1989), abaecin (Casteels et al 1990) and drosocin (Bulet et al 1993) have been described; the list of bactericidal insect peptides continues to grow.

With respect to this impressive defence system, we have to answer three basic questions: which peptides are produced, how do they work and how does an infection activate the system? On the way we may also find clues as to the evolutionary origins of this system; more specifically, whether it is related to our own defence mechanisms.

To investigate these problems, we have chosen to work with the fruit fly, *Drosophila melanogaster*. Problems that stem from the small size of *Drosophila* are more than compensated for by the powerful genetic and molecular tools that are available for the study of this insect. These techniques are of special help in the study of the activation of the antibacterial response, but they may also aid the study of the peptides themselves. Here I review what is known about the antibacterial proteins and peptides in *Drosophila*, and I will also touch on the question how they are activated. For other recent reviews on the antibacterial response in *Drosophila*, see Hultmark (1993, 1994) and Hoffmann et al (1993).

Cecropin

Cecropins were originally discovered as the major bactericidal factors in immunized pupae of the cecropia moth, *Hyalophora cecropia* (Hultmark et al 1980). Homologous substances have been found in several other insects, including the flesh fly, *Sarcophaga peregrina* (Okada & Natori 1983). The cecropins are highly amphipathic peptides of 35–39 amino acid residues. They interact with lipid membranes (Nakajima et al 1987, Steiner et al 1988), creating voltage-dependent ion channels of variable size (Christensen et al 1988). In bacteria, they cause the permeability barrier of the cell membrane to break down (Okada & Natori 1985) and it must be assumed that they also penetrate the outer membrane of Gram-negative bacteria. At concentrations in the micromolar range, these peptides are able to kill and lyse a majority of the bacteria that have been tested, both Gram-positive and Gram-negative (Hultmark et al 1982). Eukaryotic cells are generally resistant to the cecropin concentrations reached in haemolymph (Steiner et al 1981) which can be up to 50–100 μM (Samakovlis et al 1990, Gudmundsson et al 1991).

We used a cDNA clone for a *Sarcophaga* cecropin, sarcotoxin IA (Matsumoto et al 1986), as a probe for cloning the cecropin genes in *Drosophila*. Four cecropin genes were found (Kylsten et al 1990, Tryselius et al 1992), tightly linked in a cluster at 99E on the third chromosome. They code for only three different cecropins, since two of the genes, *CecA1* and *CecA2*, encode the same peptide, *Drosophila* cecropin A.

The different *Drosophila* cecropins may have slightly different functions, since they differ in how they are expressed. All the cecropin genes are induced when bacteria are injected into the haemocoel, but in larvae and adults, the *CecA* genes are expressed at much higher levels than *CecB* and *CecC* (Samakovlis et al 1990, Tryselius et al 1992). In these developmental stages, the fat body is

the major site of cecropin synthesis, but induced expression of the cecropin genes was also seen in haemocytes (Samakovlis et al 1990). In the pupae, the situation is somewhat different. At this stage, significant expression of the cecropin genes is often detected even in untreated animals. However, it appears that this constitutive expression also depends on the presence of bacteria, since it is almost abolished in axenically reared animals (Samakovlis et al 1990). Whereas the *CecA* genes are considerably less active in pupae than in larvae or adults, *CecB* and *CecC* are preferentially induced at this stage (Samakovlis et al 1990, Tryselius et al 1992). The tissue distribution is also different in the pupae. *CecC* is mainly expressed in degenerating larval tissues such as hindgut and salivary glands (Tryselius et al 1992). Scattered foci of expression are also seen in the pupa, possibly representing haemocytes.

The induction of the cecropin genes is relatively rapid in *Drosophila*. Significant levels of cecropin mRNA accumulate within an hour after bacteria have been injected, reaching a peak a few hours later (Kylsten et al 1990).

Whereas lepidopteran cecropins differ considerably in their sequences, the dipteran cecropins are highly conserved. The major cecropins in *Drosophila* and *Sarcophaga* are identical; other dipteran cecropins differ by a few residues at most. The two species are not believed to be closely related and the conservation is not reflected in the DNA sequence, as illustrated by a comparison of phylogenetic trees constructed on the basis of protein and DNA sequences (Fig. 1). The dipteran cecropins form a tight cluster in the amino acid sequence tree. The different rates of evolution indicate that the cecropins may play somewhat different roles in Diptera and Lepidoptera.

Andropin

Closely linked to the *Drosophila* cecropin gene cluster is the *Anp* gene, which codes for another bactericidal peptide, andropin (Samakovlis et al 1991). There is little recognizable sequence similarity between andropin and the cecropins, but the peptides are predicted to form similar secondary structures, with two amphipathic helices, and it is possible that they share a common origin.

In contrast to the cecropins, andropin expression is confined to the ejaculatory duct of the adult male fly. The *Anp* gene is constitutively expressed and there is no further increase in expression after an injection of bacteria, although the gene is more strongly expressed after mating. It is likely that the bactericidal activity of andropin helps to keep the seminal fluid free from bacterial growth. This may be especially important when sperm is stored by the female for extended periods after mating.

Considering that the *Anp* gene is separated from the *CecA1* gene by a distance of less than 600 base pairs, it is remarkable that the two genes show entirely different patterns of expression.

Amino acid sequence

Lepido-ptera

A-like — Hyalophora A

B-like — CMIV — Hyalophora B, Antheraea B, A2 } Bombyx, B2

Bombyx CMPh

D-like — Hyalophora D, Antheraea D, B2, B5P } Manduca, B4, B3

Diptera — Sarcophaga I A — A, B, C } Drosophila, I C, I B, I D } Sarcophaga
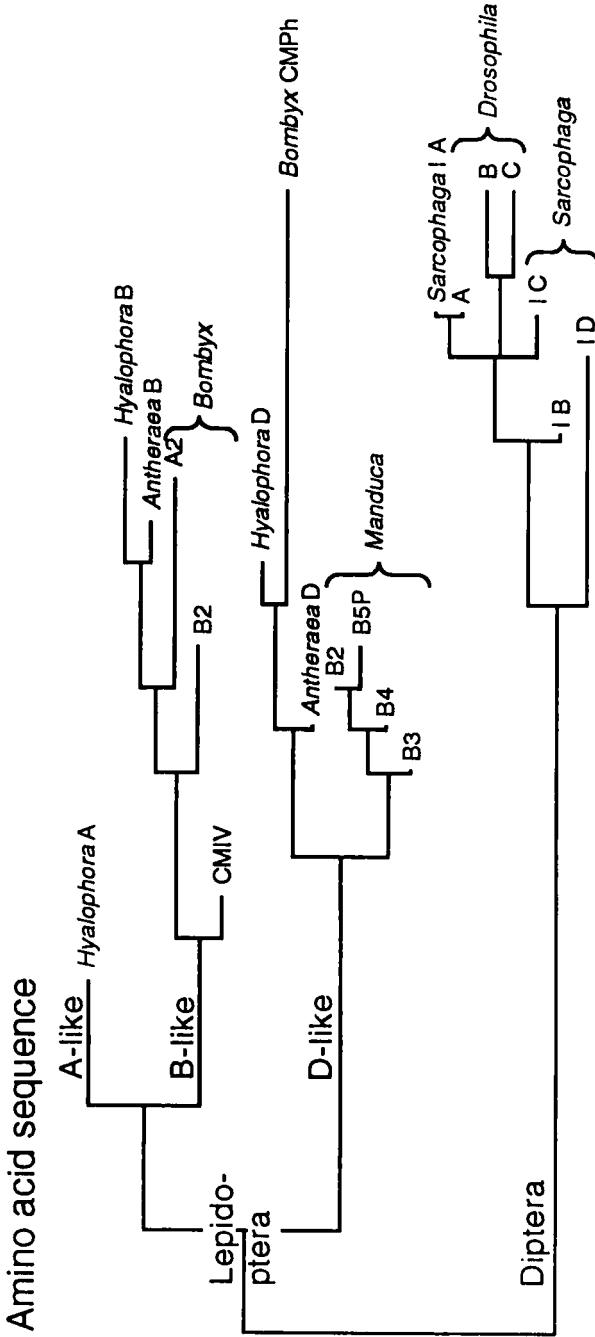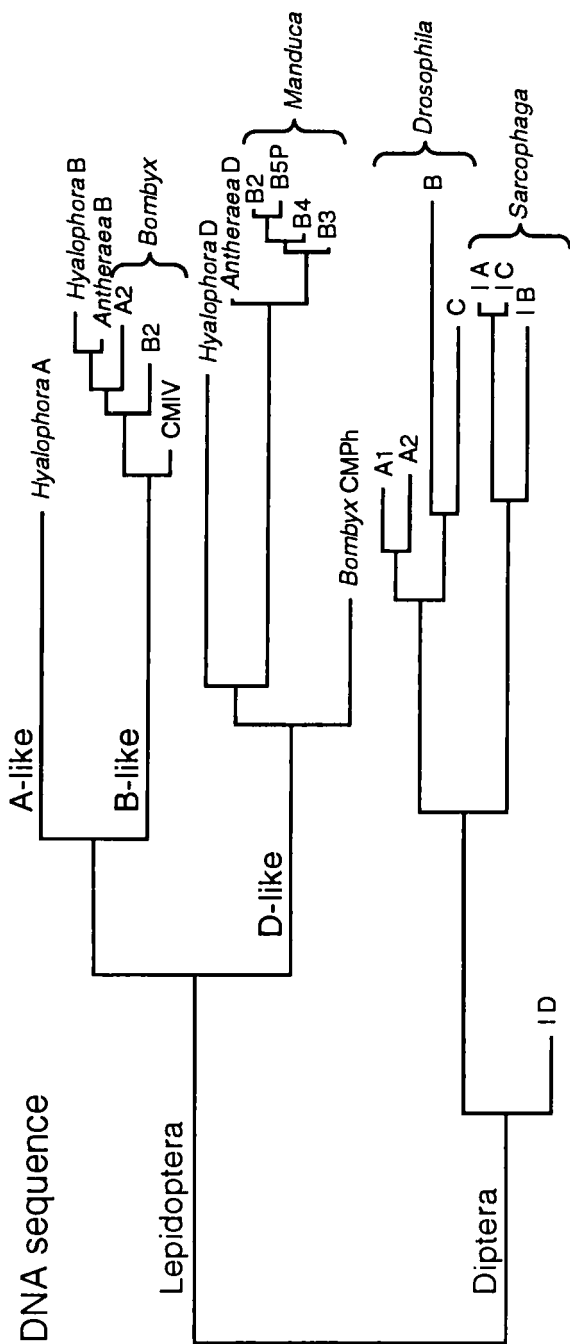
FIG. 1. Phylogenetic tree of cecropins. The lepidopteran cecropins fall in three deeply rooted classes: A-, B- and D-like, whereas the dipteran cecropins are all very similar. The most parsimonious trees are shown for both DNA and protein sequences. When DNA sequences were not available, they were obtained by back-translation from the amino acid sequence. The trees are arbitrarily rooted between the dipteran and lepidopteran sequences. Branch lengths are proportional to the minimal number of replacements. Trees were constructed with the PAUP program. Sequence alignments and references can be found in Faye & Hultmark (1993).

Diptericin and other attacin-like factors, drosocin

The diptericins are 9 kDa bactericidal proteins that were initially isolated by Dimarcq et al (1988) from immunized flesh flies, *Phormia terranovae*. In a collaboration with us, Wicker et al (1990) used a *Phormia* diptericin probe to isolate homologous cDNA clones from *Drosophila*. *Drosophila* diptericin has subsequently been investigated extensively in J. A. Hoffmann's lab. It is encoded by a single gene (Reichhart et al 1992) at 56A on the second chromosome and it can be induced in the fat body at all developmental stages.

Surprisingly, the diptericins are distantly related to the much larger antibacterial proteins, attacin and sarcotoxin II (Wicker et al 1990) and perhaps also to coleoptericin (Fig. 2). Diptericin contains a single copy of a weakly
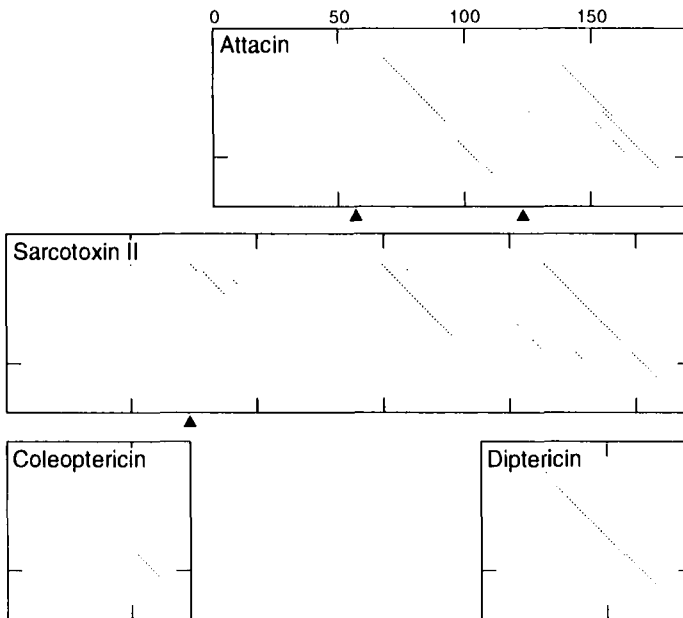
FIG. 2.   Presence of similar G domains in attacin, sarcotoxin II, diptericin and possibly in coleoptericin. The amino acid sequences for *Drosophila* diptericin (Wicker et al 1990), *Zophobas* coleoptericin (Bulet et al 1991), *Hyalophora* acidic attacin (Kockum et al 1984) and *Sarcophaga* sarcotoxin IIA (Ando & Natori 1988) (horizontal axis) were searched for similarity to a G protein consensus sequence (vertical axis) and the result is shown in a diagonal plot. Each dot corresponds to a stretch of 25 amino acids with > 50% similarity. The positions of introns in the genes are indicated by arrowheads. In the attacin gene they correspond exactly with the domain borders.

conserved 'G domain' that is present at least twice in attacin and sarcotoxin. The relationship between sarcotoxin II (from *Sarcophaga*) and attacin (from *Hyalophora*) is further supported by our recent discovery of the gene for a homologous protein from *Drosophila* (B. Åsling, unpublished results). This protein is most closely related to sarcotoxin II (43% sequence identity in the sequenced part) but retains several of the residues found in the attacin sequence (38% identity).

The members of the attacin superfamily, including diptericin, have similar effects on bacteria. Their activity is restricted to growing cells of certain Gram-negative bacteria, causing them to grow in long chains (Hultmark et al 1983, Ishikawa et al 1992). The mechanism of action has been investigated for attacin, which specifically blocks the synthesis of the major outer membrane proteins in *Escherichia coli*, leading to the breakdown of the integrity of the outer membrane (Carlsson et al 1991).

In addition to cecropins and attacin-like proteins, several other bactericidal factors are induced in the fly, as reflected in the complex pattern of antibacterial bands that can be detected after electrophoresis of the haemolymph (Samakovlis et al 1990). The characterization of such factors is under way in Hoffmann's lab (Hoffmann et al 1993). One of these factors, drosocin (Bulet et al 1993), has been analysed in detail and was found to be a 19-residue proline-rich peptide with an *O*-linked glycosylation. The glycosylation was found to be necessary for the biological activity of the peptide.

Lysozyme

Lysozyme is an enzyme that catalyses the breakdown of the peptidoglycans in bacterial cell walls. This enzyme is one of the major proteins induced after infection in insects like *Hyalophora* and *Manduca* (Faye & Hultmark 1993). It therefore came as a surprise when we isolated two lysozyme genes from *Drosophila* and found that they were repressed, not induced, when we injected flies with bacteria (Kylsten et al 1992). These genes are members of a larger multigene family in *Drosophila* of which we have so far cloned seven members (Daffre et al 1994). The *Drosophila* lysozyme genes are all expressed in the digestive tract; the different genes show a complex pattern of expression in different parts of the digestive tract and at different times of development (Table 1). This is in striking contrast to the inducible lysozyme in moths and to the majority of the antibacterial molecules in *Drosophila* that are mainly expressed in the fat body and haemocytes.

The aberrant behaviour of the *Drosophila* lysozyme genes is probably related to their recruitment for the digestion of bacteria in the decaying fruits that are the natural food source of this species. The sequences of the *Drosophila* lysozymes show that they constitute a highly specialized set of enzymes that must have undergone very rapid evolution (Fig. 3). In this respect, they present

TABLE 1   Properties of the *Drosophila* lysozyme genes and their products, compared with the single lysozyme in *Hyalophora*

Gene	Developmental stage	Tissue	pI[a]
LysB	Larva, adult	Anterior midgut	5.1
LysC	Larva, adult	Anterior midgut	5.1
LysD	Larva, adult	Anterior midgut	5.1
LysE	Larva, adult	Anterior midgut	5.1
LysS	Larva	Gastric caecae	4.4
LysX	Wandering larva, white prepupa	Midgut	ND[b]
LysP	Adult	Salivary gland	8.6
Hyalophora lysozyme	Larva, pupa[c]	Fat body, haemocytes	8.4

[a]Isoelectric point, calculated from the sequence.
[b]The full sequence has not been determined for *LysX*. The pI for the sequenced part (4.4) is very close to that of the corresponding parts of *LysB–E* (4.3).
[c]Embryos and adults have not been investigated.

an interesting and more extreme parallel to the lysozymes in the cow and other ruminants, which have also evolved rapidly as an adaptation for a digestive function (Irwin et al 1992). The inhibition of lysozyme expression could probably be seen as a part of a 'malaise response' in the diseased insect, in which digestive processes are turned off.

Induction of the antibacterial response

The inducible antibacterial genes serve as convenient markers for the immune response in *Drosophila*, and we have selected the cecropin genes as a model system in which to study this response. To develop a tissue culture system where the response could be more easily studied, we investigated a number of *Drosophila* cell lines. In two of them, the cecropin genes were induced when microbial components, such as bacterial lipopolysaccharide (LPS), were added to the tissue culture medium (Samakovlis et al 1990, 1992). The strongest response was seen in Gateff's mbn-2 cells (Samakovlis et al 1992), originally isolated from haemocytes of the blood cell tumour mutant *l(2)mbn* (Gateff et al 1980). These cells show several haemocyte-like characteristics, including the capacity to phagocytose; they even attack bacteria that accidentally infect the culture (Gateff et al 1980).

Among the substances that induce cecropin gene expression in mbn-2 cells, LPS, laminarin (a β-1,3-glucan) and flagellin (the major structural component of bacterial flagella) are active at concentrations as low as 0.1 ng/ml
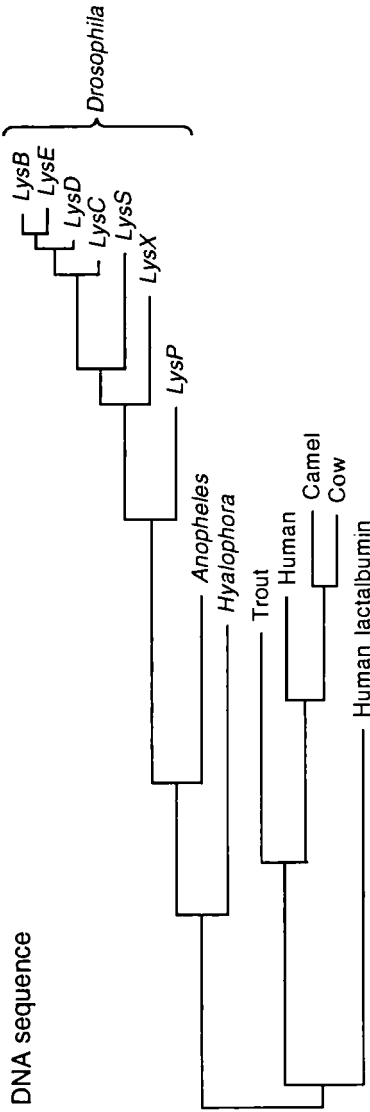
DNA sequence

FIG. 3. Phylogenetic tree of lysozymes. The *Drosophila* lysozyme sequences are compared to those of two other insects, a mosquito (*Anopheles gambiae*) and a moth (*Hyalophora cecropia*), and to a selection of vertebrate lysozymes and lactalbumin. The most parsimonious tree is shown for the DNA sequences. It is arbitrarily rooted between the insect and vertebrate sequences. Branch lengths are proportional to the minimal number of replacements. Trees were constructed with the PAUP program. Sequence references can be found in Daffre et al (1994). The *Anopheles* sequence is from Jong-Youn Lee (personal communication).

(Samakovlis et al 1992). Obviously, the mbn-2 cells must carry molecular receptors that mediate the response to these microbial substances; these cells should be a useful system for the study of such receptors.

Upstream of each of the cecropin genes we found a conserved stretch of DNA (Tryselius et al 1992). This sequence includes a GGGRAYYYYY motif (where R = A or G; Y = C or T), similar to the xB-like motif first noted by Sun et al (1991) in several insect immune response genes. Extra copies of this motif are also present upstream of the *CecA2* and *CecB* genes. In mammals, the xB motif has been characterized as a binding site for the transcription factor NF-xB and other members of the Rel family of DNA-binding proteins (Liou & Baltimore 1993). The functional importance of the xB-like element in the *CecA1* promoter was verified experimentally with different promoter constructs fused to a β-galactosidase reporter gene and tested after transfection into the mbn-2 cells (Engström et al 1993). Deletion of the xB-like element was found to destroy the promoter and a trimer of this element is sufficient to drive induced expression from a minimal promoter in mbn-2 cells. Similar results have been obtained for two xB-like motifs in the *Drosophila* diptericin gene (Kappler et al 1993).

Sun & Faye (1992) purified a 65 kDa nuclear factor, which they called CIF (cecropia immunoresponsive factor), from the fat body of pupae of the cecropia moth, *Hyalophora cecropia*, which binds specifically to the xB-like site. Active CIF was found in nuclear and cytoplasmic extracts from the fat body of *Hyalophora*, but only after the animals had been treated with known inducers of the immune genes. A similar factor, DIF, was also found in *Drosophila* extracts (Engström et al 1993). Recently, Ip et al (1993) cloned a *Drosophila* gene for a new member of the Rel family of transcription factors. This gene, *Dif* (for *Dorsal-related immunity factor*) is prefentially expressed in the fat body. The *Dif* gene product binds specifically to the xB site in *CecA1*, and it cross-reacts immunologically to the DIF complex. It appears likely that the *Dif* gene is directly involved in the transcriptional activation of the cecropin genes, in much the same way that NF-xB and other Rel proteins mediate the induction of different genes in the mammalian immune response (Liou & Baltimore 1993). Another Rel protein, the product of the developmental gene *dorsal*, has been implicated in the induction of the *Drosophila* diptericin gene (Reichhart et al 1993). The relative importance of the two Rel proteins in insect immunity will have to be further investigated.

Conclusion

In spite of obvious differences, the immune systems of insects and vertebrates are likely to be of common origin. Like the insects, vertebrates are now also known to rely on bactericidal proteins and peptides for their antibacterial defence, even if the homology between these substances is in most cases difficult

to judge. Furthermore, the involvement of similar transcription factors in the immune reactions of insects and vertebrates is strong evidence for a true relationship and the similarity between the serine protease cascades involved in the activation of insect phenoloxidase and mammalian complement is hardly a coincidence.

The initial mechanisms for the recognition of microorganisms in the insect and the signals that lead to the activation of cecropin and other immune genes are not understood. The mbn-2 cell line now allows us to study these phenomena at the molecular level. The use of *Drosophila* also enables us to dissect the immune system genetically and by screening for mutations in this response it should be possible to identify the major factors involved.

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DISCUSSION

Elsbach: You mentioned that the attacins inhibit synthesis of outer membrane proteins in *E. coli*. Carlsson et al (1991) showed that this general effect on outer membrane protein synthesis is not mediated through the *ompB* locus. What about LPS synthesis: is that affected by the attacins?

Hultmark: I don't know.

Elsbach: It is quite possible, because the diffuse effect on all outer membrane proteins suggests a more general toxicity.

Boman: In 1969 we isolated and mapped a mutant of *E. coli*, called D22, which was extremely sensitive to many classical antibiotics. It has since also been found to be hypersensitive to attacins, lysozyme and PR-39. The mutated gene, *envA*, was cloned and its function has very recently been discovered: it is involved in the synthesis of lipid A. These results are not published yet, but the information has appeared in a minireview by Raetz (1993).

Elsbach: There is evidence to indicate that the regulation of the synthesis of the outer membrane proteins, as part of the physiology of *E. coli*, is very discretely regulated in response to environmental changes, including through the *ompB* locus.

Boman: Loretta Leive (Leive et al 1968) showed that you can remove the Ca^{2+} with EDTA and make the outer membrane permeable.

Elsbach: Outer membrane proteins, such as OmpF and OmpC, are functional pores; their effects are very distinct from the effect LPS has on overall permeability properties of the outer membrane. These are totally separate regulatory systems.

Ganz: Are the products of these induced genes primarily secreted by the fat body, or primarily induced in haemocytes? Do you know the relative effects on the fat body and on the haemocytes?

Hultmark: Only about 10% of the haemocytes are inducible in our experiments. The haemocytes are small and there aren't many of them. By far the majority of the cecropin comes from the fat body. The question is: why would the insects bother to make a little cecropin in the haemocytes? This might be because what is produced by the haemocytes is important locally, when they're accumulating around foreign objects and so on.

Boman: The situation in the cecropia moth is the same as Dan Hultmark has described for *Drosophila*. The number of haemocytes is far too low to account for the massive synthesis of immune protein made by the fat body.

Natori: You showed three inducers—β-1,3-glucan, LPS and flagellin. All these are very different compounds, especially the last one, which is a pure protein. Do you think that all these compounds use the same receptor to activate the *Drosophila* immune protein genes?

Hultmark: I would be surprised if it were the same receptor, but we have no evidence one way or the other.

Flajnik: Is one of the helices in the cecropin structure more conserved than the other when you do the phylogenetic trees?

Hultmark: Yes, the most strongly conserved motif is a repetitive pattern of lysines at the N-terminal end.

Boman: It is amazing what is coming out from the *Drosophila* work. In fact, the regulatory mechanisms in immunity are much more conserved than the effector molecules. The closest similarity so far seen is between NF-xB and the cecropia counterpart, CIF (cecropia immunoresponsive factor). Antibodies to NF-xB cross react with CIF, which was isolated by Sun & Faye (1992) a year before the cloning of the *Drosophila* immune factor DIF (*dorsal*-related immune factor) (Ip et al 1993). If we now have similarity between a gene for differentiation and one for immunity, an interesting question arises: which came first, differentiation or immunity? What is the significance of having an oncogene-like product such as Dif with a Rel domain? One can guess that the early primitive eukaryotic organisms—about a thousand million years ago— had to compete with microorganisms. The need for immunity may have come before the need for differentiation, because a slow-growing, complex single-celled eukaryotic microorganism may still have had problems dealing with fast-growing bacteria competing for food and space. There was probably a very early need for a defence mechanism to counteract the significant differences in growth rates.

Hultmark: The connection between the Rel proteins in mammals, including NF-xB, and Dif and perhaps other factors in the insects, is the best evidence for a true relationship between the mammalian and insect immune responses. When it comes to effector molecules, it's much more difficult to see any relationships, perhaps because they have evolved so much faster.

The connection with embryology is extremely surprising: why would you have perhaps the very same system involved in immunity and in establishing dorsoventral polarity in the embryo? It is quite likely, as Ip et al (1993) have argued, that the involvement of these proteins in embryogenesis is a later phenomenon. I have suggested that when the insects became terrestrial, it became important to coordinate the dorsoventral polarity of the embryo with the dorsoventral polarity of the eggshell (Hultmark 1994).

Bevins: We isolated a tracheal antimicrobial peptide from the cow airway. The gene that encodes this peptide is expressed in epithelial cells. When we cloned and sequenced the gene, we found an NF-xB recognition site 180 nucleotides upstream from the transcription start site. We've since gone on to grow airway epithelial cells in primary culture, and we find the gene is dramatically up-regulated by the addition of LPS (Diamond & Bevins 1994).

Zasloff: Are deletion mutants of the cecropin locus available yet?

Hultmark: No, and we are not trying to produce them at the moment. This is a region which has a number of minute loci which are haploinsufficient. This means that it may be difficult to obtain deficiencies in this region.

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Function of antimicrobial proteins in insects

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Abstract. We have isolated and characterized various antimicrobial proteins from the haemolymph of *Sarcophaga peregrina* (flesh fly) larvae. Of these the sarcotoxin I family is a group of proteins mainly active against Gram-negative bacteria whereas sapecin is active mainly against Gram-positive bacteria. In addition to its function in defence, sapecin also plays a role in insect development. Recently, we identified a hendecapeptide of the sapecin homologue sapecin B that has the same antibacterial activity as the original sapecin B. Both sarcotoxin I and sapecin are inducible proteins synthesized *de novo* by the fat body and/or haemocytes and secreted into the haemolymph when the insect is in the acute phase response to bacterial infection. Antifungal protein (AFP) is constitutively present in the haemolymph and is active against certain fungi but not bacteria. These various antimicrobial proteins interact with microbial membranes. Sarcotoxin I interferes with membrane functions such as ATP synthesis and amino acid transport. The fungicidal activity of AFP is enhanced synergistically by sarcotoxin I, although sarcotoxin I alone has no appreciable antifungal activity. It is clear that the flesh fly has the ability to mount a potent defence response against microbial parasites by mobilizing several antimicrobial proteins.

1994 Antimicrobial peptides. Wiley, Chichester (Ciba Foundation Symposium 186) p 123–134

The antimicrobial defence reactions of insects have been extensively studied and it is now clear that insects synthesize a battery of potent antibacterial proteins (Hultmark et al 1980, Steiner et al 1981, Okada & Natori 1983, Dimarcq et al 1988). These antibacterial proteins are not normally present, but are promptly synthesized by the fat body and/or haemocytes and secreted into haemolymph when insects are in an acute phase. Most of these proteins are small cationic molecules exhibiting a broad spectrum of activity against Gram-positive and/or Gram-negative bacteria. We have been studying antimicrobial proteins of the flesh fly, *Sarcophaga peregrina*. So far, we have purified and characterized six groups of proteins. These are sarcotoxin I, II and III, sapecin, diptericin and antifungal protein (AFP) (Okada & Natori 1983, Ando et al 1987, Baba et al 1987, Matsuyama & Natori 1988a, Ishikawa et al 1992, Iijima et al 1993).

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Mode of action of antimicrobial proteins

Sarcotoxin I

The sarcotoxin I family is a group of cecropin-type antibacterial proteins each consisting of 39 amino acid residues (Okada & Natori 1985a). At least five sarcotoxin I congeners are present in this insect. Of these, we chose to use sarcotoxin IA to study their mode of action. Sarcotoxin IA has potent bactericidal activity against Gram-negative bacteria. The N-terminal half of this molecule is rich in positively charged amino acids and is hydrophilic, whereas the C-terminal half is hydrophobic. Thus, we expect this molecule to be amphiphilic and to interact readily with bacterial membranes.

We found that sarcotoxin IA cancels electrochemical membrane potential, resulting in cessation of ATP synthesis and amino acid transport in *Escherichia coli* (Okada & Natori 1984, 1985b). It is likely that the C-terminal half of this molecule penetrates into the bacterial membrane and its N-terminal half interacts with acidic phospholipids in the membrane, causing perturbation of membrane and loss of its function. We found that an *uncA* mutant of *E. coli* that has a defect in oxidative phosphorylation is much less sensitive to sarcotoxin IA than wild-type *E. coli* (Fig. 1). This may be due to the fact that the *uncA* mutant does not require membrane potential for the synthesis of ATP, as ATP is supplied by substrate-level phosphorylation. These results suggest that the bactericidal activity of sarcotoxin IA is mainly due to its ability to disrupt membrane potential and cause cessation of ATP synthesis.
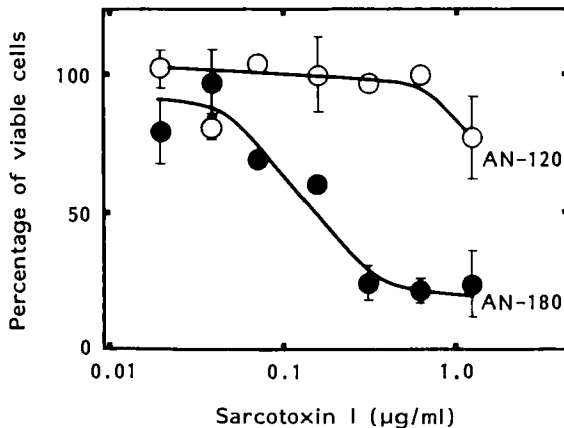
FIG. 1.   Effect of sarcotoxin IA on viability of the *uncA* mutant of *Escherichia coli*. *E. coli* strains K-12 AN 120 (*uncA* mutant) (○) and AN 180 (wild-type) (●) were incubated with various concentrations of sarcotoxin IA for 10 min at 37 °C. The mixture was then diluted and plated.

From the analysis of the genes coding for proteins of the sarcotoxin I family, it became evident that these genes align tandemly forming a gene cluster in a relatively short fragment of *Sarcophaga* DNA (Kanai & Natori 1989). A similar gene cluster was identified in *Drosophila* (Kylsten et al 1990). Why are so many proteins with such similar structures induced simultaneously? It is probably because they have different antibacterial spectra and thus the different proteins are needed for the insect to be able to respond to a wide range of bacteria. In support of this hypothesis, *E. coli* was found to be less sensitive to sarcotoxin IC than to sarcotoxin IA and IB (Nakajima et al 1987).

Sapecin

Sapecin is another antibacterial protein of *Sarcophaga*; it is more effective against Gram-positive bacteria (Matsuyama & Natori 1988a,b). This protein consists of 40 amino acids including six Cys residues that form three intramolecular disulphide bridges (Kuzuhara et al 1990). As with sarcotoxin I, the primary target of sapecin is likely to be the bacterial membrane (Matsuyama & Natori 1990). However, antibacterial activity of sapecin against *Staphylococcus aureus* was weak below 20 °C and the number of viable bacteria did not decrease appreciably on treatment with sapecin at 0 °C. Nevertheless, the antibacterial activitiy of sapecin became obvious above 20 °C and the viability of treated bacteria was almost completely nil at 37 °C. These facts suggest that the primary target of sapecin is indeed the bacterial membrane, whose physicochemical nature is known to change with temperature. *S. aureus* is more sensitive to sapecin than *E. coli*. Therefore, we prepared two types of liposomes with similar phospholipid compositions to those of the membranes of *S. aureus* and *E. coli*, respectively, each containing trapped glucose, and examined the effect of sapecin on them by measuring the release of glucose. An increase in sapecin concentration resulted in the release of glucose from liposomes resembling the phospholipid composition of *S. aureus* membrane, formed from phosphatidylglycerol and cardiolipin in a molar ratio of 3:1. Liposomes prepared from phosphatidylethanolamine, phosphatidylglycerol and cardiolipin in a molar ratio of 7:2:1, resembling the phospholipid composition of *E. coli* membrane were not affected (Fig. 2). We found that sapecin has high affinity for cardiolipin, which is a major acidic phospholipid of *S. aureus* membranes, but its affinity for other acidic phospholipids was low.

We assume that the interaction between sapecin and cardiolipin is a prerequisite for the bactericidal activity of sapecin because although *E. coli* is less sensitive than *S. aureus* to sapecin, it is sensitive to sapecin at sufficiently high concentrations. Therefore, we examined the effect of sapecin on an *E. coli* mutant with a defect in cardiolipin synthetase. The cardiolipin content of this mutant was less than 10% of that of wild-type *E. coli*. This mutant was clearly more resistant to sapecin than the wild-type, suggesting that the content of
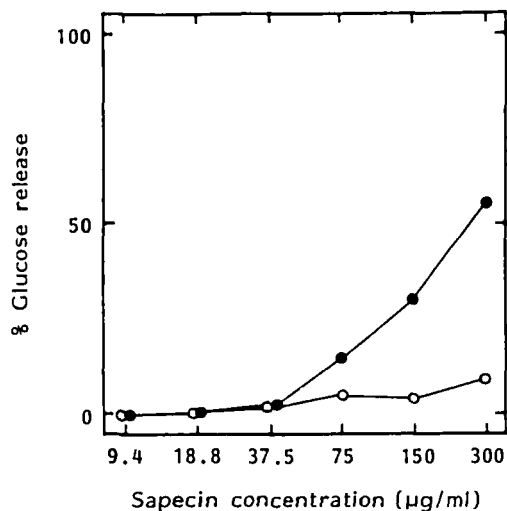
FIG. 2. Release of glucose from liposomes in the presence of sapecin. Two types of liposomes with entrapped glucose were prepared from phosphatidylethanolamine, phosphatidylglycerol and cardiolipin with phospholipid compositions resembling those of the membranes of *Staphylococcus aureus* (●) and *Escherichia coli* (○). Increasing concentrations of sapecin were added to these liposomes. They were incubated for 30 min at 22 °C, after which the amount of glucose released from the liposomes was measured.

cardiolipin is a crucial factor for bacterial sensitivity to sapecin. However, the membrane cardiolipin content alone cannot fully explain the preferential sensitivity to sapecin of Gram-positive bacteria. We found that the lipopolysaccharide (LPS) of Gram-negative bacteria is an effective barrier to sapecin. A rough mutant of *E. coli*, which lacks the polysaccharide chains of LPS molecules, was more sensitive to sapecin than wild-type. Moreover, *E. coli* became more sensitive to sapecin when treated with EDTA (conditions under which it is known to lose more than 60% of its LPS). Thus, LPS seems to be a barrier to sapecin and to make Gram-negative bacteria less sensitive than Gram-positive bacteria to this protein.

Antifungal protein (AFP)

In addition to bacteria, fungi and viruses are also well-known infectious agents of insects. As nothing is known about insect proteins with antifungal activity, we looked for haemolymph proteins of *Sarcophaga* that could repress the growth of the fungus *Candida albicans*. Haemolymph from third instar larvae was found to have significant fungicidal activity; from this we purified AFP. Analysis of its cDNA sequence revealed that AFP is a 67-residue basic protein rich in
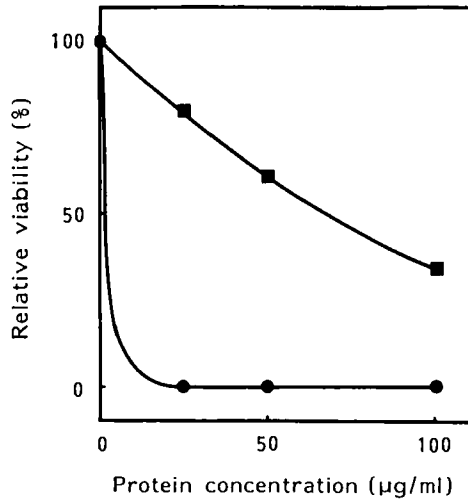
FIG. 3.   Antifungal effects of antifungal protein (AFP) in saline and distilled water. *Candida albicans* was cultured in distilled water (●) or saline (■) in the presence of increasing concentrations of AFP for 2 h at 37 °C. Aliquots of the mixture were then plated to examine viability.

His and Gly residues (Iijima et al 1993). The ionic environment seems to influence the fungicidal ability of AFP. As shown in Fig. 3, treatment of *C. albicans* in saline with AFP for 2 h at 37 °C resulted in a gradual decrease in the viable cell number with increase in AFP concentration. But AFP had a much more potent effect—even at lower concentrations—in distilled water. Under the same conditions no appreciable loss of fungal viability was detected in the absence of AFP. The protein alone had no effect on the viability of either Gram-positive or Gram-negative bacteria.

An obvious difference between AFP and the antibacterial proteins is that AFP is a constitutively expressed protein of haemolymph, whereas antibacterial proteins are inducible. Thus *Sarcophaga* seems to have both types of defence proteins. It is noteworthy that the fungicidal activity of AFP was increased significantly in the presence of a low concentration of sarcotoxin IA, as shown in Fig. 4. This experiment was performed in Sabouraud medium. With the increase in the concentration of sarcotoxin IA, which on its own has no appreciable fungicidal effect on *C. albicans*, the fungicidal ability of AFP was greatly enhanced. Thus, there is a synergism between AFP and sarcotoxin IA. These results suggest that this insect has the ability to construct an efficient defence network against various microorgansims by means of a combination of both constitutive and inducible defence proteins. We found similar synergism between sarcotoxin IA and sarcotoxin IIA, which are both inducible antibacterial proteins.
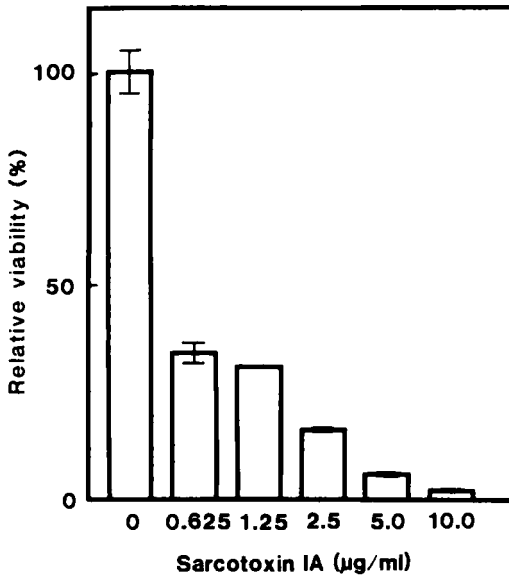
FIG. 4. Synergism between antifungal protein (AFP) and sarcotoxin IA. *Candida albicans* was incubated with 100 μg/ml of AFP and increasing amounts of sarcotoxin IA for 2 h at 37 °C. Part of the mixture was then plated to examine viability. Numbers of colonies relative to those not treated with AFP are shown.

The mode of action of AFP is not known, but we found that about 8×10^5 molecules of AFP bind per cell of *C. albicans*. This binding results in progressive leakage of a cellular component with an absorbance at 260 nm, suggesting that AFP has some effect on membranes. However, AFP is a very hydrophilic protein and it is difficult to deduce its interaction with fungal membranes from its primary structure.

Novel features of insect antimicrobial protein

Possible involvement of NF-κB motifs in the activation of antimicrobial protein genes

It is known that the defence protein genes of *Sarcophaga*, including those for antibacterial proteins, are activated in response to larval body injury or bacterial infection. The 5' upstream regions of these genes were found to have common motifs showing similarity to the mammalian NF-κB-binding consensus sequence. We purified a 59 kDa protein with affinity to these NF-κB-binding motifs (Kobayashi et al 1993). This protein is probably a common transcription factor for these genes, since NF-κB-binding motifs were shown to be essential for

their transcription. Similar results were obtained with other insects (Sun et al 1991a,b, Reichhart et al 1992). How, then, does the stimulus of body injury or bacterial infection signal to the fat body to activate these genes? On the basis of the results of ligation experiments, we suggest that a humoral factor released from the anterior part of the larva stimulates the fat body to activate the defence protein genes (Shiraishi & Natori 1988, 1989).

Dual roles of an antimicrobial protein

We have accumulated evidence supporting the hypothesis that some antibacterial proteins of *Sarcophaga* play a dual role in both defence and development (Natori 1990). Northern blotting experiments showed that the sapecin gene and sarcotoxin IA gene are expressed transiently in the embryonic stage and in the early pupal stage without any outside stimulus (Nanbu et al 1988, Matsuyama & Natori 1988b). These results suggest that these proteins participate in the development of this insect. At present, the function of sarcotoxin IA during development is unknown. But sapecin seems to act as a growth factor for embryonic cells (Komano et al 1991). We found that the DNA synthesis of NIH-Sape-4 cells, an embryonic cell line of *Sarcophaga*, was clearly enhanced when cultured in the presence of sapecin. Cell number was also found to increase significantly under these conditions. Insects seemingly have the flexibility to use their antibacterial proteins in multiple ways.

Fragmentation of sapecin B

Recently, we isolated two homologues of sapecin, termed sapecin B and C (Yamada & Natori 1993). Sapecin B had significant structural similarity to charybdotoxin, a K^+ channel blocker synthesized by scorpion venom glands. We divided sapecin B into four regions according to the structural model of charybdotoxin and synthesized four amidated peptides corresponding to these regions. We found that only the 7R–17K fragment (RSLCLLHCRLK-NH$_2$, amino acid residues 7–17 of sapecin B), which forms an α-helix, repressed the growth of *S. aureus*. The antibacterial activity of this peptide was almost comparable to that of sapecin B, its concentrations for 50% growth inhibition (ID$_{50}$) being $0 \cdot 7$–$1 \cdot 5\,\mu M$ (Yamada & Natori 1994). The three other peptides had essentially no antibacterial activity at concentrations less than $10\,\mu M$ (Fig. 5). The 7R–17K fragment repressed the growth of *E. coli* as well as that of *S. aureus*. Furthermore, this fragment also suppressed the growth of *C. albicans*, although the ID$_{50}$ value of this fungicidal activity was one order of magnitude higher than that of its antibacterial activity.

Sapecin B is preferentially active against Gram-positive bacteria, but this hendecapeptide derived from sapecin B showed a much wider antimicrobial spectrum. There are two possibilities: one is that the hendecapeptide is the active
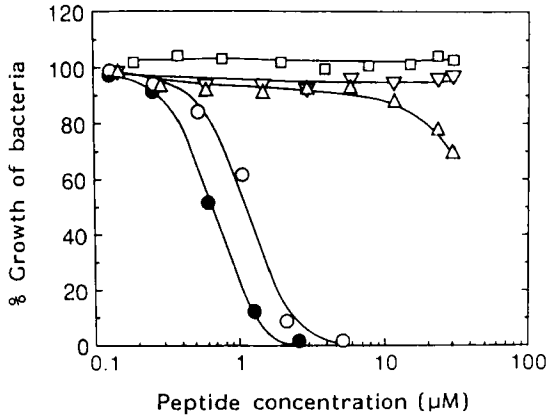
FIG. 5.   Antibacterial activities of synthetic peptides from sapecin B. The antibacterial activities of amidated fragments of sapecin B were measured using *Staphylococcus aureus* as a target bacterium. Increasing amounts of test samples were added to the medium and relative bacterial growth was determined by measuring the A_{650} after incubation for 3 h at 37 °C. ●, sapecin B; ⊓, 1L–6D fragment; ○, 7R–17K fragment; △, 18G–28K fragment; ▽, 29V–34Q fragment.

site of sapecin B, and so sapecin B is potentially active against various microorganisms, but its other regions interfere with its interaction with Gram-negative bacteria and fungi, resulting in it being active against only Gram-positive bacteria. The other possibility is that the antibacterial activity of sapecin B and the hendecapeptide are coincidental and independent. At present, it is difficult to know which possibility is correct. However, this hendecapeptide may be useful as a lead for designing better antimicrobial peptides.

Discussion

Insects are fascinating in their ability to induce antimicrobial proteins, creating chemotherapeutic compounds in their haemolymph in response to outside stimuli. This inducible system may have the advantage of decreasing the frequency of the appearance of bacteria resistant to these proteins. Because the proteins are synthesized transiently and disappear rapidly, the chances of bacteria interacting with them are small. If these proteins were present constitutively in the haemolymph, the likelihood of bacteria evolving resistance would probably increase. On the other hand, AFP is a constitutive protein. The synergism between AFP and inducible antibacterial proteins such as sarcotoxin IA may help to prevent the emergence of resistant fungi.

  We suggested that sapecin is a growth factor for embryonic cells as well as a defence protein of this insect (Komano et al 1991). Previously, we demonstrated clearly that *Sarcophaga* lectin, another defence protein of *Sarcophaga*, is

essential for the development of imaginal discs (Kawaguchi et al 1991). Many defence proteins seem to have a dual role in both defence and development. The developmental functions of these antibacterial proteins deserve much attention and should not be underestimated.

Using sapecin B, we showed that it is possible to identify a core peptide carrying antibacterial activity. This strategy is not necessarily applicable to other antibacterial proteins, because there is no general rule for fragmentation of an active antibacterial protein. We succeeded in obtaining the 7R–17K fragment of sapecin B. Once relatively small core peptides of various antibacterial proteins are identified, they may provide clues for the development of novel antimicrobial compounds.

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DISCUSSION

Andreu: Professor Natori, have you tried making a simplified version of your antifungal protein, with a shorter number of structural repeats?

Natori: No, we haven't done that experiment.

Lehrer: There are histidine-rich salivary proteins that have been described in humans (Xu et al 1989). Is there any similarity between these and the antifungal protein you described?

Natori: We carried out a computer search using the sequence of the antifungal protein, but we didn't find any proteins with significant sequence similarity. The antifungal protein is similar to the human salivary antifungal protein in that both have a high histidine content, but we found no sequence similarity between them.

Lehrer: Is your protein expressed either in salivary glands or in the intestine?

Natori: We don't know anything about its expression in these tissues because we haven't done Northern blots with them. All I can say is that it is synthesized by the fat body and secreted into the haemolymph.

Lehrer: Have you looked at the effect of pH on antifungal activity?

Natori: We haven't measured the pH dependency precisely, but antifungal protein is active at neutral pH.

Lehrer: The salivary antifungal proteins of humans are much more active at low pH.

Boman: Shunji Natori, I have followed and admired the work you have done over the years. But it is sometimes difficult to compare the results you have obtained with those on other antimicrobial peptides because of the differences between the assays you have used and those chosen by other groups for measuring antimicrobial activity. I think some of your fragments may be much more active than you think, but it is quite difficult to tell from your present results.

In addition, we seem to be calling similar peptides by different names (for instance, sarcotoxin IA is really a cecropin). Perhaps it would make things easier if we were to agree on the nomenclature of these peptides.

Hultmark: What is the mechanism of action of sapecin/insect defensin? Do you think that it is generally membrane active or that it has a specific action on K^+ channels?

Natori: I think the primary site of action of sapecin is the phospholipid in the bacterial membrane, especially acidic phospholipids such as cardiolipin. So far as the K^+ channel is concerned, this is a completely different story. We found that sapecin B inhibited the Ca^{2+}-activated K^+ channel of rat Purkinje cells. Sapecin B probably regulates the activity of the K^+ channel in the development of this insect, but this is another role of sapecin B and it may not be directly related to its bactericidal activity.

Gazit: Professor Natori, you mentioned the similarity in the primary structure between sapecin B and charybdotoxin, the K^+ channel blocker. Is there also similarity in the 3D structures?

Natori: Yes, the molecules are very similar.

Zasloff: Conceptually, one of the most helpful simplifications for me has been the belief that the peptides we are working with recognize their targets through their more or less specific recognition of certain lipids. These lipids are placed in such a fashion as to be accessible in those sensitive targets. What's amazing about sapecin is its severe restriction to cardiolipin; most of the peptides that we as a group work with see anionic phospholipids more promiscuously. Do you have any structural basis to explain the sapecin–cardiolipin interaction?

Natori: One of my colleagues is now analysing the interaction between sapecin and cardiolipin using NMR. It is possible that there is a pocket in the sapecin molecule that fits the structure of cardiolipin.

Boman: I think the mechanism of action is a very important issue. The action of cecropin is stoichiometric, so a very important question for the interpretation of your results is the ratio between the number of liposomes and the peptide concentration you have. One of the main difficulties in pinning down the mechanism of action of the cecropins is the speed with which they lyse bacteria. This is why we decided to return to studying their action on mitochondria, because mitochondria do not lyse and using them you may get a better idea of the dose–response and the order of events.

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General discussion II

Antimicrobial peptides from bees

Casteels: There are four different types of antibacterial peptides that are induced upon infection in bees. (1) The apidaecins, which are small proline-rich peptides of 18 residues (Casteels et al 1989). (2) Abaecin, which is also proline rich and is made up of 34 residues (Casteels et al 1990). (3) Hymenoptaecin, which is bigger and is active on both the inner and outer membranes of Gram-negative bacteria (Casteels et al 1993). (4) Bee defensin, which is slightly larger than the other insect defensins and which appears similar to royalisin, an insect defensin-like peptide purified from royal jelly (Fujiwara et al 1990). The bee lymph defensin has one minor modification compared with royalisin—an Arg/Tyr substitution at the end—and it is probably the only insect defensin that is amidated at the C-terminus. The C-terminal part can be projected on an α-helical wheel and shows an amphipathic character, a typical feature of membrane-disrupting peptides with α-helical conformation. The purpose of amidation here is probably to stabilize the C-terminal helix.

Apidaecins are made of tandemly repeated peptide precursors, which is quite unique (Casteels-Josson et al 1993). The prepro form consists of a repeat of cassettes. Each cassette is composed of the mature peptide, preceded by dipeptides which are then cleaved by the dipeptidyl-peptidase (dipeptidyl aminopeptidase). In fact, this system is very similar to the maturation of yeast α mating factor (which involves KEX-2, KEX-1 and dipeptidyl-peptidase).

The apidaecin precursors are still active against bacteria, but the activity goes down quickly the more dipeptides are present on the N-terminus. The N-terminus doesn't seem to be very important for activity, since the removal of a couple of residues from this end of the mature peptide has little effect. However, removal of the C-terminal leucine abolishes the entire activity of the peptide.

Paul Tempst synthesized a conserved peptide, to check whether a specific interaction with the bacterial target is necessary for antibacterial activity, or whether the interaction is non-specific. He conserved all the prolines, the histidine and the C-terminal leucine. This peptide had only 1% of the original activity, which suggested that there was something specific going on.

There were two alternative methods we could have used to investigate this. One would have been to start synthesizing different peptides, but screening all possible synthetic analogues of an 18 residue peptide, without any clue about structure/function, is impossible. So we decided to look at what was present in Nature and try to purify apidaecin-like peptides from other insects to see what the constraints are. We therefore purified apidaecins from a range of

hymenoptera. From the primary structures it was immediately clear that the C-terminal part was pretty well conserved (Casteels et al 1989). The only small difference is the Ile–Leu substitution in the honeybee, which doesn't affect the activity at all (Casteels at al 1989). Other motifs are also conserved. Proline in position 9 is completely conserved, except for in one honeybee peptide. However, this peptide was never found in lymph: we just know the sequence from cDNA clones Katty Josson picked up, and in the pre sequence of this peptide there's also a mutation that affects its maturation (Casteels-Josson et al 1993). Therefore it is likely that this cDNA sequence never makes it up to the peptide level. Another part which is also conserved in all known apidaecins is the Arg–Pro sequence, although in the small parasitic wasp this is changed to Lys–Pro, which is a very similar motif: a basic residue followed by Pro. All these peptides were chemically synthesized and their antimicrobial activity was compared (Casteels et al 1994).

The activities of the various apidaecin-related peptides were compared to the activity of cecropin P1 (Table 1 [*Casteels*]): I think this was a good choice, because it's a mammalian representative of antibacterial peptides. The results showed that an *E. coli* mutant Apid R that was resistant to the honeybee (Hb) peptides still proved to be susceptible to the apidaecins from the hornet (Ho), yellow jacket (Yj) and the parasitic wasp (Cd). This indicates that the resistance to bee apidaecins is probably not a result of, for example, a barrier mutation, because these peptides are very similar in size, hydrophobicity and isoelectric point. We're still working on this.

Also of interest are the almost mirror-image results obtained with two bacteria, *Yersinia enterocolitica* and *Campylobacter jejuni*. The first class of apidaecins (Hb, Bb, Ck) was not active against *Campylobacter* but it was against *Yersinia*, whereas the peptides from the small parasitic wasp were very active against *Campylobacter* but not against *Yersinia*. This proves that these peptides have a rather specific interaction, and that the differences in activity are probably related to the bacterial target.

I would also like to draw your attention to the fact that the all-D-apidaecin (Hb-D) is inactive. For the L-form, if you optimize your assay and use dilute medium, you need less than 0.1 μg/ml to inhibit the growth of *E. coli*. But one can add the D-form at more than 500 μg/ml in a similar assay and there's still no inhibition, so it's not active at all. This indicates that a stereospecific interaction with a bacterial target is needed.

There is significant sequence similarity between apidaecin from the hornet and drosocin from *Drosophila* (Bulet et al 1993). The major difference is that drosocin is glycosylated, and needs the glycosylation for optimal activity. Since last year there have been other proline-rich peptides reported, for instance PR-39, which was purified from the pig by Hans Boman's lab (Agerberth et al 1991). I think that in the near future more proline-rich peptides will be discovered, because they are a separate class of antibacterial peptides and they probably

TABLE 1 (*Casteels*) Antibacterial activity of apidaecins from a range of hymenoptera

Bacterium	Peptide																	
	Hb Ib	Hb III	Hb-D	Bb-A	Bb+A	Ck P	Ck A	Ho-	Ho+	Yj+S	Yj-S	CD 1+	Cd 1-	Cd 2+	Cd 2-	Cd 3+	Cd 3-	CP1
Escherichia coli	••••	-	-	••••	••••	•••	•••	•••	••••	•••	•••	••	•••	•••	••	••	••	-
Escherichia coli Apid R	-	-	-	-	••••	•••	-	•••	•••	••	••	••	••	••	••	••	••	-
Escherichia coli K514	•••	-	-	•••	••	••	••	-	•••	•••	•••	•••	••	••	•	•	•	•
Yersinia enterocolitica	•••	••	-	•••	••••	••••	•••	••	••••	••	••	••	-	-	-	•	••••	••••
Campylobacter jejuni	-	-	-	-	-	-	-	-	-	-	-	-	•••••	•••••	•••	•••••	••••	•••
Morganella morganii	-	-	-	-	-	-	-	••	••	-	••	••	••	••	••	••	••	-
Rhizobium meliloti	••••	•••••	-	••••	••••	•••	•••	•••	•••	ND	•••	•••	•••	•••	•••	•••	•••	•

Apidaecins isolated from honeybee (Hb), bumble bee (Bb), circadia killer wasp (Ck), hornet (Ho), yellow jacket (Yj) and *Coccypomimus distaris* (Cd). The activities were determined with an agar diffusion assay and are expressed with dots: the more dots, the higher the activity (larger inhibition zone). (-) Denotes no inhibition was found; CP1, cecropin P1 (Agerberth et al 1991); ND, not done.

have a special mode of action. They have no activity on bacterial membranes even at extremely high concentrations and it is likely that there's a chiral interaction involved in their activity.

Sahl: Are *Campylobacter jejuni* or *Yersinia enterocolitica* pathogens for bees?

Casteels: No. These are clinical pathogens. The activity spectrum of apidaecins is quite interesting and points to possible clinical applications. They are very active against Enterobacteriaceae, such as *Salmonella* and *Shigella* (Casteels et al 1989), and they're very active against plant-associated bacteria such as *Rhizobium*, some *Pseudomonas* species, *Agrobacterium* and *Erwinia*, which are likely to be encountered by bees.

Sahl: Should we care about pathogens which are pathogenic for humans? This occurred to me when we were talking about injecting pathogens into frogs—it's pretty clear that *Staphylococcus aureus* is a pathogen for humans, but perhaps it's not pathogenic for a frog at all: this might be the reason why they're quite happy when they are injected with them.

Casteels: What we have here are two different approaches. One is to induce the immune response *in vivo*, the other is to look for potential applications *in vitro*. The results which I showed are a selection of *in vitro* tests where we focused on clinically relevant bacteria. *In vivo*, in an insect, you can inject whatever you like—even inert substances such as Chinese ink—and you will get the same kind of humoral response. The levels of induction are going to be different, but it is a non-specific response: all the peptides are going to be induced.

Hultmark: I think it's highly relevant to look at non-pathogens. People who work with vertebrate immunology have been too concerned about pathogens; potentially any heterotrophic bacterium would be a pathogen unless there were defences against it. So I think it's highly relevant to show that antimicrobial peptides kill non-pathogens—that's probably why they are not pathogens. The real 'pathogens' are still pathogens in spite of these defences because of long co-evolutionary series of events—a kind of 'arms race'.

Boman: I think the proline-rich peptides are probably of great significance in Nature, and unfortunately they are not very well covered in this symposium. It is clear that our proline and arginine-rich peptide PR-39 does not lyse bacteria (Boman et al 1993). Unfortunately, it is difficult to synthesize PR-39. Peter Casteels, it is very nice that you have been able to synthesize both enantiomers of apidaecin, and I think that it is an important result that the D-enantiomer is totally inactive. Here we have an example where a peptide does not lyse membranes and where there's clear evidence of stereospecificity, probably in the interaction of the peptide with a receptor. This has not previously been demonstrated for any of the antibacterial peptides.

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Antibacterial peptides in insect vectors of tropical parasitic disease

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Abstract. The induction and characterization of immune peptides in two groups of medically important insects, the mosquitoes and blackflies, is currently an important research area. Mosquitoes transmit a variety of viral and parasitic diseases including yellow fever, dengue, malaria and lymphatic filariasis. Simuliid blackflies are vectors of river blindness. The diseases are together responsible for death and morbidity in millions of people each year. The relationship between inducible peptides and bacterial and parasitic infections in these insects is proving to be a complex one. The identification of an insect defensin (4 kDa) in *Aedes aegypti*, the yellow fever mosquito, has proved to be the first peptide characterized in a vector of human disease. This inducible molecule appears in the haemolymph in response to bacterial and to a lesser extent filarial infection. The characterization of inducible blackfly peptides has revealed potent inducible anti-Gram-positive as well as anti-Gram-negative activity. In addition, non-self recognition molecules such as phenoloxidase may play a part in differentiating one species of eukaryotic pathogen from another of the same genus. The interactions between the peptides and these other proteins are likely to be important in the establishment of a successful immune response against a parasitic pathogen, particularly as we now know these peptides to have anti-eukaryotic activity (against a range of parasite species). As well as being of fundamental interest in our understanding of host–parasite relationships, the indication that antibacterial peptides are toxic to parasitic organisms has implications for their possible use in the disease vector control strategies of the future. It may also mean that a revision in our understanding of their mode of action, loose as it is, has to take place.

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A number of blood-feeding or haematophagous insects are known vectors of parasitic and viral diseases of humans and animals. The largest order of such insects, the Diptera, includes the mosquitoes which transmit the protozoan parasites responsible for malaria, metazoan filarial nematodes which cause lymphatic filariasis, and many viruses, such as those giving rise to dengue

haemorrhagic fever and yellow fever. Also included within the Diptera are the Simuliidae, species of which transmit ocular onchocerciasis (river blindness), caused by another species of filarial nematode. The tsetse flies (Glossiniidae) are vectors of another disease, trypanosomiasis (sleeping sickness). In addition to the Diptera, several other groups of blood-sucking insects carry a variety of important infections. Studies on the mosquitoes and simuliid blackflies will be described in this paper.

   A particularly important characteristic of these diseases is that in order to complete its life cycle, the parasite undergoes an essential developmental stage within the insect. Transmission does not normally occur passively from individual to individual. Often the parasite also needs to undergo a series of migrations within the insect, occupying, as a result, different microhabitats. These may include the midgut lumen, where the blood meal is digested. If the parasite leaves the gut, it often does so via the midgut epithelium. Malaria parasites spend several days developing within a growing cyst on the gut epithelium before the cyst ruptures, liberating parasites into the haemocoel. These find their way into the salivary glands, resulting in transmission at a subsequent blood meal. Filarial nematodes migrate through the midgut epithelium straight after the blood meal and into the haemocoel before further migration through and penetration of a flight muscle cell, where they develop for a number of days. They later migrate out of the muscle cell, back into the haemocoel, and on into the mouthparts of the insect. Here they await a further blood meal by the insect, when infection of the human host will occur. Figure 1 shows the development of one such species of filarial nematode, *Onchocerca volvulus* (the causative organism of river blindness) in an African vector, *Simulium damnosum* (Blacklock 1926). Development of some of the filarial nematodes in mosquitoes is very similar. The important point to note is that the haemocoel is an important route on one or more occasions in the migration of filarial nematodes and malaria parasites during their life in the insect. At these times, they are exposed to an array of defence molecules which may be innately present or induced on infection.

   Several humoral haemolymph components of the immune response in these insects have been identified as being induced or activated in response to infection with parasites (Ham 1992, Christensen & Severson 1993). These include phenoloxidases and carbohydrate-binding lectins, a number of serine and cysteine proteases, and antibacterial peptides. It is these antibacterial molecules that we focus on here and which are now receiving considerable attention in our laboratory. We will refer to two specific areas of study: first, work on an insect defensin in the mosquito *Aedes aegypti*, a laboratory vector of the filarial nematode *Brugia pahangi* and the avian malaria *Plasmodium gallinaceum*; second, studies on antibacterial activity in *Simulium* sp., vectors of human and bovine *Onchocerca* species. Antibacterial activity with similarities to known immune peptides has been reported in other vector groups, in
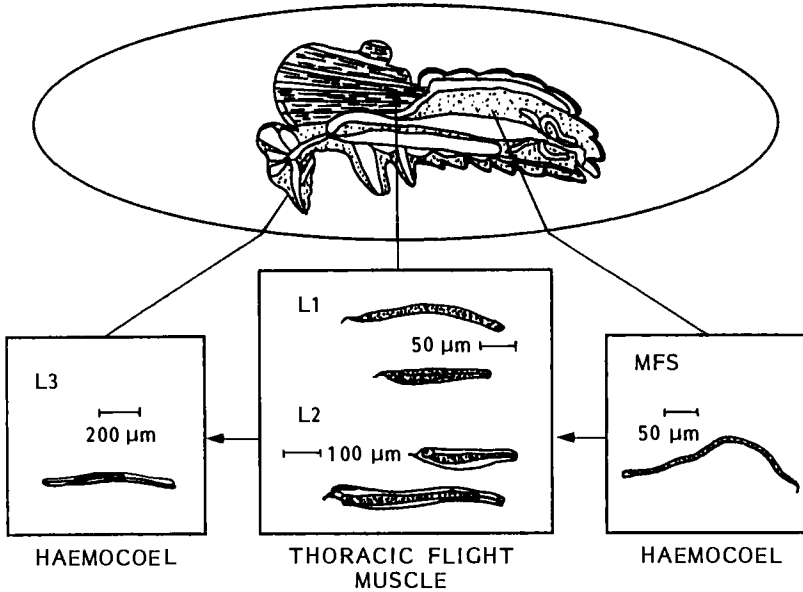
FIG. 1. The developmental stages of the filarial nematode *Onchocerca* spp. in the blood-feeding blackfly vector, *Simulium*, showing different microhabitats occupied during the life cycle. (MFS, juvenile microfilariae ingested by insect; L1, L2 and L3, sequential larval stages of development.)

particular in bugs (de Azambuja et al 1986) and *Glossina*/tsetse flies (Kaaya et al 1987).

Aedes aegypti defensin

Townson & Chaithong (1991) and Chalk et al (1994) observed the induction of a peptide of approximately 4 kDa in *Ae. aegypti*, which was inducible following bacterial infection via intrathoracic inoculation (*Escherichia coli*, D31 strain, Boman & Monner 1975). This was visualized on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) using a tricine system (Schagger & von Jagow 1987). Activity was assayed using an *E. coli* inhibition of growth zone bioassay system in agarose, modified from that of Hoffmann et al (1981). This bioassay was more sensitive to synthetic cecropin (Hultmark et al 1982) activity than previous assays and incorporated lysozyme into the agarose. Subsequent work (Chalk et al 1994) showed that lysozyme and cecropin acted synergistically against *E. coli*. Significant antibacterial activity, though far less than with *E. coli*, was also induced by infection with the filarial nematode *Brugia pahangi*. A number of protein bands appear in the haemolymph in response to bacterial infection, several of which were below 10 kDa. Ultrafiltration and

high-performance liquid chromatography (HPLC) fractionation revealed anti-bacterial activity in the 4 kDa peak. N-terminal amino acid sequencing revealed 85% homology to sapecin (Matsuyama & Natori 1988) and other insect defensin-like molecules.

Subsequently, we have examined the induction of this peptide in two strains of *Ae. aegypti*, selected from the same stock, for high (re[fm]) and low (rep RR) susceptibility to infection with *Brugia pahangi*. Preliminary work suggests that only two molecules appear in the haemolymph in response to filarial infection, the 4 kDa peptide and a 66 kDa protein that, following substrate analysis, we believe to be phenoloxidase. Earlier work with synthetic cecropin showed that at what may be physiological concentrations (50 μM) the peptide was toxic to *Brugia pahanagi in vitro* and reduced its development in the insect *in vivo*. It is possible that the insect defensin in *Ae. aegypti* may also show some
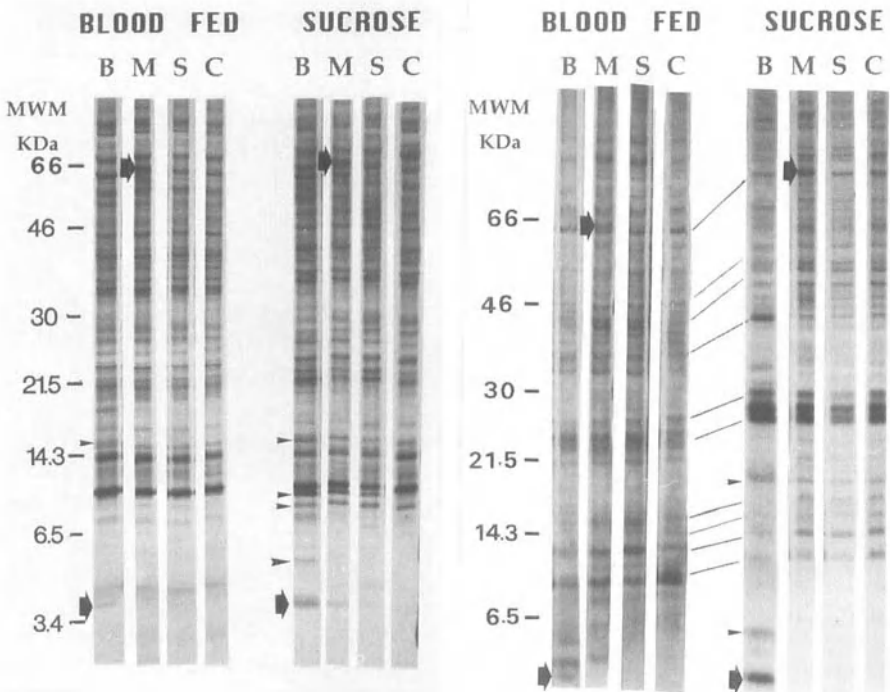
FIG. 2. SDS–PAGE (tricine gradient gels) of pooled haemolymph samples from adult female *Aedes aegypti* mosquitoes of strains either susceptible (a) or refractory (b) to filarial infection. Each group was fed either blood or sucrose prior to receiving different infection treatments as follows: B, intrathoracic inoculation of *E. coli* (D31) bacteria; M, inoculation of 30 *Brugia pahangi* microfilariae per fly; S, sham inoculation of medium alone; C, control (uninjected). Large arrows at ≈ 4 kDa and ≈ 66 kDa denote micro-filarially induced molecules. Minor arrows denote some of the additional bacterially induced molecules.

toxicity, either on its own, or in conjunction with an opsonin such as phenoloxidase, or in synergy with lysozyme (which may be uninduced but constitutive in the haemolymph of *Ae. aegypti*).

There seem to be differences in the appearance of the 4 kDa peptide in the two strains of *Ae. aegypti* (see Fig. 2a,b). Here, pooled haemolymph samples from the filariae refractory (rep RR) and the susceptible (re^{fm}) mosquitoes have been subjected to electrophoresis on SDS tricine gradient gels and stained with silver reagent. Four groups of insects are presented (see legend). The three points to note with respect to the 4 kDa peptide are that the bacteria induce an apparently stronger response than filariae, the filariae response is apparently stronger in the re^{fm} strain than in the rep RR (see Fig. 2a versus 2b) and a blood meal given to the mosquitoes prior to the infection seems to reduce the quantity of peptide free in the haemolymph. The presence of free peptide in the haemolymph appears to be linked to filariae susceptibility and to the nutritional status of the insect. If the peptide is active against the eukaryotic parasites, one might expect it to be localized onto the worm surface via some sequestering mechanism, where it might be more potent. The discovery of a reduced level in the re^{fm} strain could be explained by such an hypothesis. However, studies on transcription and protein synthesis need to be carried out to clarify the situation.

Antibacterial activity in *Simulium*

Filarial nematodes belonging to the genus *Onchocerca* undergo similar development to that of the filariae that develop in and are transmitted by mosquitoes. The period of development from the juvenile 'microfilariae' ingested by the insect to the third stage 'infective larva' that infects a person at a subsequent blood meal, is shorter (minimum 6–7 days) than for the mosquito-borne filariae (minimum 9–10 days). However, as Fig. 1 shows, the micro-habitats within the insect are midgut, haemocoel and flight muscle, as for *Brugia*.

Humoral immunity in Simulium—background

Our interest in immunity in blackflies was initiated when we found that innate variation in susceptibility to *Onchocerca* occurred and that this correlated with haemolymph-mediated reductions in microfilarial motility *in vitro* (Ham & Garms 1988). Furthermore, haemolymph from infected blackflies conferred a filariae refractory status on naive susceptible insects when passively transferred from one donor to recipient (Ham 1986). Such 'infected' haemolymph was also more effective in killing microfilariae *in vitro* compared with 'uninfected' control haemolymph. The induction of a number of peptides was observed in *Simulium* following injury and infection trauma and preliminary results with antibody to a *Sarcophaga* cecropin-like molecule, sarcotoxin IA (supplied by Professor

S. Natori) indicated binding to a doublet in traumatized *Simulium* haemolymph of around 4–6 kDa (A. J. Baxter, personal communication). Southern blot analysis of DNA samples from different *Simulium* species has revealed homologies with consensus oligonucleotides for cecropin and a *Drosophila* cDNA probe (supplied by Dr D. Hultmark).

Synthetic cecropin at $50 \mu M$ was found to reduce significantly the motility of *Onchocerca* microfilariae *in vitro* (Ham 1992) and to reduce the development rate of the parasites by around 80% *in vivo*, following inoculation into infected, filariae-susceptible blackflies. It appears, therefore, that cecropin is toxic to filariae at concentrations at which it is present in other insects. The gene for cecropin, or a similar peptide, seems to occur in *Simulium* and encodes molecules antigenically similar to cecropin (our unpublished results).

Current studies

Our current studies are focused on: (1) identifying the functional antibacterial molecules induced in *Simulium* using bioassays together with serological assays and protein/peptide purification techniques; and (2) isolating the genes for cecropins and other immune peptides present in *Simulium*.

Current functional studies using SDS–PAGE followed by silver staining, and native acid gel electrophoresis (Gabriel 1971) together with bacterial overlays of either *E. coli* or *Micrococcus luteus* (Hultmark et al 1982), have revealed a 4–5 kDa peptide that is inducible by *E. coli* in *Simulium equinum* a temperate surrogate vector of *Onchocerca* (see Fig. 3). Subsequent overlays with *M. luteus* have shown a constitutive molecule with activity, as well as a molecule induced by bacterial infection (see Fig. 4). When similar studies were carried out with *Simulium ornatum*, a vector of bovine onchocerciasis in the UK, three bands appeared on the *M. luteus* overlays in haemolymph from blackflies infected with filariae (B. Smithies, unpublished result). Therefore Gram-positive antibacterial activity is inducible by eukaryotic filarial parasites, as well as bacteria. When the same samples are examined for activity against *E. coli* following native gel electrophoresis, this appears to be observed constitutively. However, in whole haemolymph, there seems to be an inducible anti-Gram-negative activity. It is possible, therefore, that different components in the haemolymph act in synergy with one another to give functional activity.

Although whole haemolymph has antiparasitic-filariae activity, it is not yet known whether the purified peptides do; as mentioned above, synthetic cecropin does. We believe that cecropin is likely to be present in the insects, but our objective is to purify the peptides, as we have done for *Ae. aegypti*, in order to carry out the definitive experiments. There is circumstantial evidence of cecropin-like activity, in that antibodies to cecropin and cecropin-like molecules ameliorate the cidal effect of immune blackfly haemolymph *in vitro*. Polyclonal rabbit antisera raised against sarcotoxin IA partially block killing of *Onchocerca*
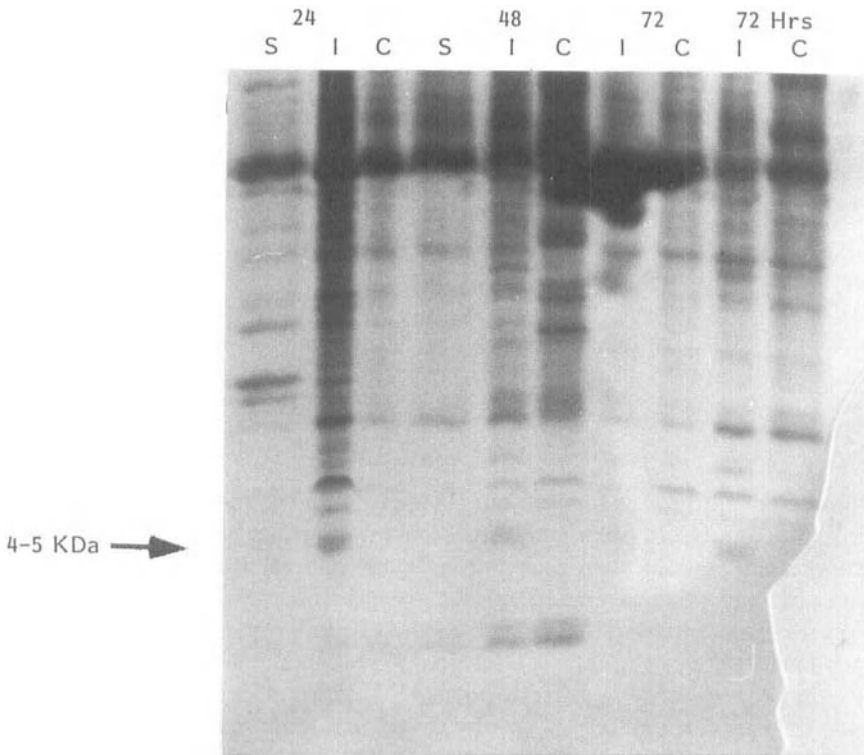
FIG. 3. SDS–PAGE (tricine gradient gels) of haemolymph samples from pooled adult female *Simulium equinum*, 24, 48 or 72 hours after: intrathoracic inoculation with D31 strain of *E. coli* (I), or sham intrathoracic inoculation of sterile tissue culture medium (S) and from untreated control insects (C). Arrow denotes position of 4–5 kDa peptide.

microfilariae (see Fig. 5a) and a monoclonal antibody that recognizes a doublet at between 4 and 7 kDa in immune *Simulium* haemolymph, as well as synthetic cecropin, but not defensin, also partially protects microfilariae from killing by immune haemolymph (see Fig. 5b). In contrast, anti-sarcotoxin IA antibody has no protective activity against microfilariae in immune *Ae. aegypti* haemolymph (Ham 1991). In view of the fact that the inducible 4 kDa peptide in *Aedes* was an insect defensin, this is perhaps not surprising.

Of further interest is the fact that a polyclonal rabbit antibody raised against synthetic *Hyalophora* cecropin and which recognizes this molecule in Western blots, also recognizes an inducible 4–5 kDa peptide in *S. ornatum* haemolymph following immunization with lipopolysaccharide. However, there appears to be little recognition of any peptide in haemolymph from insects immunized with *E. coli*. This may be either because little induction occurs in this group of
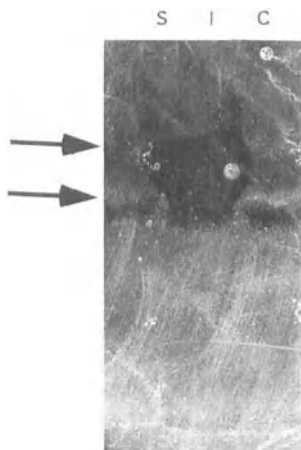
FIG. 4. Native gel electrophoresis of pooled haemolymph samples from adult female *Simulium equinum* following infection as in Fig. 3. After electrophoresis the acrylamide was briefly washed and laid onto an agarose gel seeded with *Micrococcus luteus*. Arrows denote clear zones where haemolymph contains antibacterial activity. Upper band appears to be enhanced in infected group. Lower band appears to be constitutive.

simuliids, or because humoral peptide (cecropin) is sequestered onto the bacterial surface, thus removing it from circulation.

Protease activity

It is important to note that *Simulium* haemolymph contains a relatively high level of humoral protease activity following trauma and/or infection (see Fig. 6). This consists of several inducible molecules and includes serine proteases which are known to be involved in initiation of the phenoloxidase pathway. It is possible that one or more of these proteases will break down excess peptides rapidly as observed with proteases from American foulbrood scales (Jarosz & Glinski 1990), explaining why visualization of free peptide can be relatively inconsistent in *Simulium* species.

The role of other immune molecules in Simulium

In addition to the peptides and proteases, other molecules are known to be induced or activated following infection. Increased titres of haemagglutinins occur in *Onchocerca*-infected *Simulium* (Smail & Ham 1989). In addition Hagen et al (1994) have demonstrated the activation and molecular weight of phenoloxidase in similarly infected *S. damnosum* from West Africa. This molecule of M_r 66 000 appears at various concentrations, depending on the

(a)

(b)
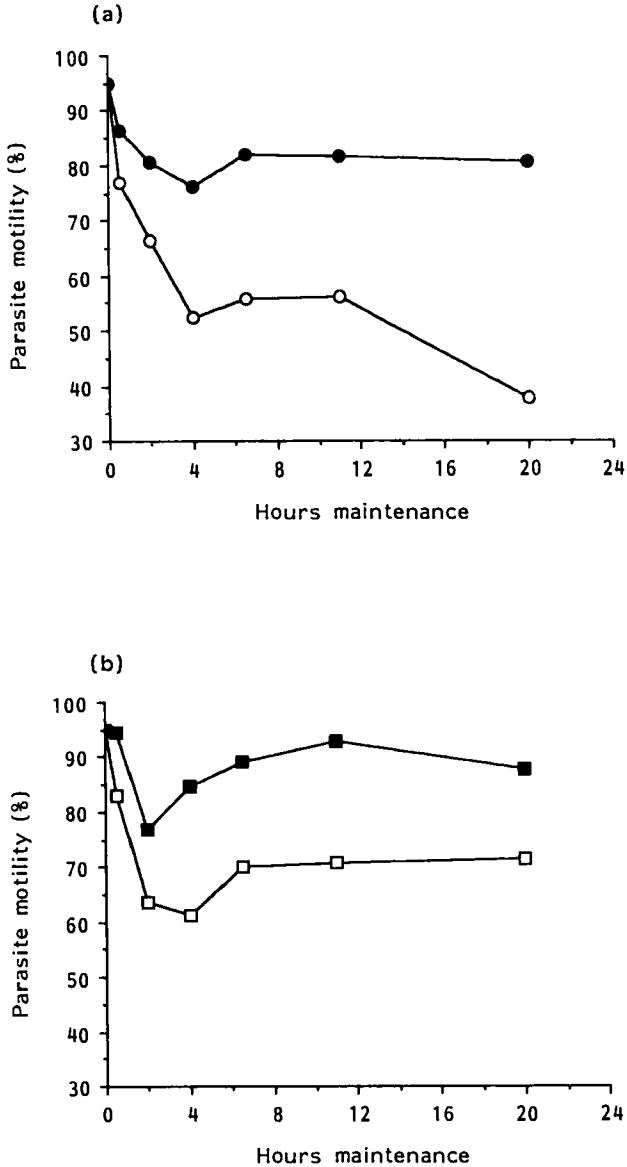
FIG. 5. Graphs showing motility of *Onchocerca lienalis* microfilariae during maintenance in immune haemolymph from *Simulium ornatum*. The haemolymph was pretreated by (a) preincubation in polyclonal rabbit antibody to sarcotoxin I peptide (●) or normal rabbit serum (○), or (b) by preincubation in monoclonal antibody 3/3A7F9 (■) or control ascites fluid (□). Each point represents the mean proportion of microfilariae showing motility in five replicates.
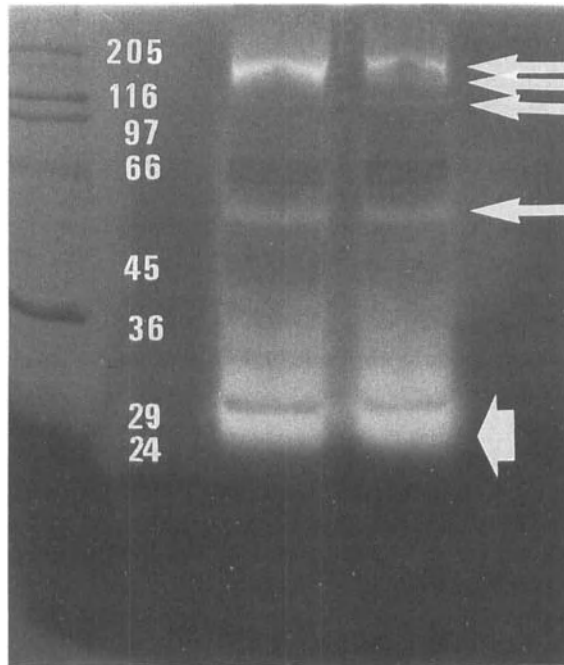
FIG. 6. Zymogram of pooled immune haemolymph from adult female *Simulium ornatum* following SDS–PAGE in casein acrylamide, detergent washing to remove SDS, overnight incubation at 37 °C, pH 7.21 and protein staining. Clear zones denote protease activity. Major molecules denoted by arrows. Numbers on left denote molecular weight markers.

species of *Onchocerca* infecting the blackfly. The working hypothesis behind this is that the enzyme is sequestered onto the parasite surface depending on the species of worm and acts as a recognition molecule for subsequent deposition of cidal molecules, perhaps via haemocytes.

It is possible that recognition molecules, such as phenoloxidase or carbo-hydrate-binding lectins, act as opsonins for the sequestration of the more potent immune peptides. This is feasible, because such peptides, which were previously known to act against bacteria, are now known to act against eukaryotes, particularly parasites transmitted by insects (Jaynes et al 1988, Gwadz et al 1989, Ham 1992, Chalk et al 1994, Ingram & Molyneux 1988). It is for these reasons that the studies in our laboratory are now focusing on these peptides but also incorporate their interaction with the other immune functional molecules known in insects and observed in *Simulium* and mosquitoes.

Of course, when dealing with vectors of medically significant diseases such as those mentioned here, one application of the study of these immune peptides

is immediately apparent—that of disease control. Current transgenic technology, which has been so important in the study of *Drosophila* genetics, is not yet feasible for mosquitoes. However, we hope that the use of immune genes will soon play a role in novel integrated control strategies for major tropical diseases such as malaria (WHO 1991).

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DISCUSSION

Lehrer: You mentioned lines of *Aedes* that are relatively resistant and those that are sensitive. Do you have any insight as to why some are resistant?

Ham: The experiments in which I showed a series of tracks (blood-fed versus non-blood fed and with different infections), we have done in two strains: the filariae refractory and susceptible strains of mosquito. The interesting thing is that this 4 kDa peptide is inducible by the parasite in one strain but it is apparently not inducible in the other. When I say inducible, that is probably a misleading term; it doesn't appear in the haemolymph. At the molecular level we don't know.

Lehrer: Is it induced in the resistant strain?

Ham: That is the problem, because it appears in the susceptible but not in the resistant strain. But I have some theories about that. Both strains were selected from the same original stock, and the only selection criterion was susceptibility to the filarial nematode. This is manifested in many forms; it's also manifested in the prophenoloxidase pathway as well, not just in this peptide. My hypothesis is that if these peptides are playing a defence role against the parasite, they have to be localized on the parasite surface—they have to be targeted there. I don't think it's going to be any good the parasites swimming

around in a mixture of these peptides, because the peptides would then have to be present at a much higher concentration than would be necessary for an effect against bacteria. Such a level might be toxic to the fly itself. Certainly, when we see the peptide in response to a parasite, the levels are quite low compared to what happens when you put bacteria in. So if the peptides are going to be active, my hypothesis is that they have to be targeted onto the surface of the parasite. This targeting may be done via some ligand or opsonin. Phenoloxidase may act as an opsonin. We wonder whether phenoloxidase or some other lectin is binding to the parasite surface, and is acting as a bridging molecule for the haemocytes to come in and specifically target these peptides onto foreign organisms.

Lehrer: In mammalian systems, the targeting system is often a leukocyte; in your system it would be the haemocyte. Have you also looked at expression of the 4 kDa peptide in the haemocytes?

Ham: No, we haven't done that.

Lehrer: It sounds like this is a wonderful model: if you have two strains that differ in their expression, perhaps you would see the opposite pattern in haemocytes.

Ham: Jaynes et al (1988) and Gwadz et al (1989) published papers on activity of defensins against *Plasmodium* and trypanosomes. Cecropin was put into mosquitoes which had malarial infections: there was an effect on the malarial parasite, but there was also an effect on the fly; they were putting so much in, it was killing the mosquitoes as well. My feeling was that they were actually looking at the wrong stage of the parasite, because the malarial parasite lives in a cyst on the gut wall of the mosquito for a large portion of its life cycle— it's not actually in the haemocoel. They were challenging these mosquitoes with synthetic cecropin by inoculation when these parasites were in the cyst. I think that they should have taken just a few more days and waited for the cyst to rupture and then the emerging parasites, which pass through the haemocoel to get to the salivary glands, would have been very much more susceptible to the cecropin.

Zasloff: I was one of the authors on that paper!

Ham: I spoke to Louis Miller about this and he said he would try the experiment described above again, but I don't know if it has been done yet.

Zasloff: But the schizont stage was also susceptible, as well, I think.

Boman: For those who may not have first-hand experience of insect work, I would stress that Peter Ham's work is really admirable because of the technical difficulties involved. To manipulate this system as he has done is extremely difficult—this is very nice research.

Ham: The mosquitoes and blackflies we used are really not a lot smaller than *Drosophila*. I think that the big difference is that the blackflies, in particular, are much more difficult to maintain, and we don't have a mutant system available.

The mosquitoes can be colonized quite readily. The blackflies really are a problem, so we have to get a lot of our material out in the tropics. We use the British species as a model to work out the optimum conditions for the experiments. Then we do the work in Africa, freeze everything down and bring it back in liquid nitrogen, and hope it's going to work.

Zasloff: One intriguing problem in this particular model is the mechanism of action of defensins or linear amphipathic peptides on the nematode worms. These are pretty complicated animals. A long time ago we began to play with planaria. At exceedingly low (micromolar) concentrations, the common planarian exhibits an extraordinary response when exposed to, for instance, magainin. The creature stops moving and begins to exude a mucus from all of its glands. When we looked at this, I began to wonder whether or not we were acting on channels of some sort. As you know, most of the common antihelminthics in one way or another block the chloride channels and similar gates. Would you describe the inhibition of movement as the principal effect of these peptides on filarial nematodes?

Ham: Yes, and inhibition of development, too. They aren't killed *in vitro*. We have tried to look at this by electron microscopy which should tell us more. The parasites are strongly negatively charged; I don't know if that means there might be some affinity there. They are covered by a cuticle somewhat like the insect cuticle, with a high level of glucosamine. But this is not impenetrable because they feed through it, they have excretory pores and they have a rudimentary gut which develops as the parasite develops. My other speculation is that the peptide interferes *in vivo* with the bacterial fauna and flora of the insect which the parasite depends on, but this doesn't explain the *in vitro* experiments where you just get this effect.

Boman: This is very important, because many insects have symbionts. In a few cases the symbionts can be grown *in vitro*, but in most cases they cannot. The evidence for them is largely morphological (you can see bacteria-like structures); also, in a few cases, treating insects with tetracycline has eliminated the symbionts seen morphologically.

Zasloff: Beyond that, I'm pretty sure that some of the molecules that we've played with hit neuromuscular or electrically active targets in the worm—at least in some of the model worms we've played with. We've not explored this in any depth, but the effects are almost instantaneous: you can stop a planarian, for example, almost immediately. This brings to mind the K^+ channel-blocking activity of sapecin. One wonders whether that activity is perhaps being expressed.

Hultmark: It's very surprising that you have this effect at concentrations as low as $50\,\mu M$, because, with cecropins, whenever effects have been seen on eukaryotic cells, it has usually been at higher concentrations. We did a few experiments on *Leishmania*, where we started to see cecropins having an effect at $100\,\mu M$, but strong effects were only seen at a few hundred micromolar concentrations; this is way above physiological concentrations, whereas $50\,\mu M$

really is a level that can be obtained in the insect. Can you exclude the possibility that there are symbiotic bacteria in the worm, not in the insect, that are killed, and that they somehow affect the worm? Maybe this also explains the effects on planarians, because they feed on bacteria.

Zasloff: But the planarians just stop dead—you add magainin and they sort of flatten out.

Hultmark: Assuming that your tests are done in culture in the presence of bacteria, what happens at micromolar cecropin or magainin concentrations is that the bacteria will be lysed. Could the effect on the nematodes be a reaction to released LPS?

Ham: For the later stages of the worm, where you have a fully developed gut, there is a known microfauna and flora, but for the microfilaria at this stage I suspect it's not the case. But even if it were, I would still find it difficult to understand how that would actually kill a parasite.

Zasloff: But Ivermectin, which is the most effective drug for the treatment of *Onchocerca*, works by inhibiting a GABA receptor. Clearly, that type of activity is sufficient to produce a clinical cure: *Onchocerca* is almost being wiped out in those communities in Africa where Ivermectin is being effectively used.

Ham: But Ivermectin kills parasites *in vivo* relatively slowly, and that's the reason it's so successful. You don't get this 'Mazzoti' reaction, where you have a massive death of parasites resulting in intense itching, which is what happened with the old DEC. What we're seeing with the antimicrobial peptides is usually a difference between the control and the infected groups within 6 h. With an immune haemolymph, you actually see the difference within half an hour to an hour; these two lines are diverging straight away. In fact, microfilarial motility in immune haemolymph will often increase; you may get a spike first and then you know they're on their way out. It's an established fact that, in toxicity trials for drugs, they may become hyperactive before they die. This seems to be what you see in the insect, as well.

The big difference here is that we are talking about a fundamental defence system that the insect is relying on, whereas the antimicrobial peptides on the surface of the frog are in addition to their internal defences.

Ganz: The biochemistry and genetics of *Caenorhabditis elegans*, another nematode worm, have been well worked out. Is this a similar enough system to the filariae for you to try and explore these issues?

Ham: Colleagues of mine are working in that system purely because it's so easy to manipulate.

Lehrer: Two comments—you will tell from this that I'm a haematologist. One of the things that concerns me as I listen to the insect story is that it is assumed that everything happens in fluid phase in the haemolymph. There is another compartment, the haemocytes, which seems to have some of the same peptides. When people have looked at interactions between mammalian leukocytes and worms, the leukocytes were found to adhere tightly to the surface

of the worm. This tight apposition could simulate a phagocytic vacuole and allow leukocytes to deliver very high local concentrations of molecules the worms could find noxious. Things can happen to peptides at high concentrations which you would not necessarily predict from their effects at the low concentrations you've worked at. I think your field may not be paying enough attention to the haemocytes and what they could be doing.

I also wanted to say that it's not enough to talk about concentration. We're dealing with stoichiometric reactions, so the relative numbers of effector molecules and targets has something to do with it too.

Elsbach: Dan Hultmark mentioned earlier today that only about 10% of the haemocytes seem to be induced for cecropin production. Is there any evidence of heterogeneity among the haemocytes? Is there a specialization? In other words, are the haemocytes like the mammalian leukocytes where there are many different types with different functions?

Hultmark: Classification of insect blood cells is so far based entirely on morphological criteria. Several different haemocyte types have been described from different insects, but it's difficult to put this together into one picture, partly because there are strongly specialized cells present in some insects that don't look like the haemocytes present in other insects. Functionally, very little is understood about this specialization.

Elsbach: Can you culture these cells?

Hultmark: We work with the mbn-2 cell line, isolated by Gateff et al (1980). It shows some of the morphologies of normal haemocytes, but it also shows a lot of pathological morphologies.

What's needed are functional and molecular criteria to define the insect haemocytes.

Lehrer: The tools are there; one could use *in situ* hybridization. What I would like to see done in your worm is an *in situ* hybridization with a probe for sapecin, to see whether the haemocytes express it, and whether its expression is any different in your resistant and sensitive lines.

Boman: My feeling is that when insects get infected, low levels of bacteria are taken care of by the phagocytes. But insects can very easily run into such massive infections that the number of phagocytes (which varies greatly in different insects) can't deal with them. Whenever this level is reached, the antimicrobial peptides are induced. They may even be induced by fat body cells to which haemocyte cells with phagocytosed bacteria are attached, but this hasn't been clearly investigated.

Elsbach: One interesting possibility is that when antimicrobial peptides are induced, proliferation of haemocytes is also induced. Is it at all possible that when you see only 10% of haemocytes lighting up that this reflects a young differentiating population, so if you look at different time points you get a different kinetic picture?

Ham: Cell proliferation is a function of the immune system.

Elsbach: Yes, but your inducing agent is also mobilizing more of the haemocytes. It could be a subpopulation that you see lighting up, and if you look at different time points you may see more of the new cells.

Ham: We know that with our filarial infections, within 48 h the haemocyte population has tripled.

Hultmark: But not necessarily by proliferation. Many such studies have been done; in some studies numbers go down and in others they go up (e.g. Geng & Dunn 1989). These changes are much too fast to be a result of proliferation. They are probably a consequence of the release of a sessile population.

Ham: We've done experiments with mitotic inhibitors, such as colchicine, in the haemolymph. In this case there is no change in the haemocyte numbers after infection. We actually feed the insect with colchicine several days before we infect it and haemocyte numbers do not go up, whereas in the infected flies without colchicine the haemocytes triple in number. This is pretty good evidence that the increase in haemocyte numbers is a result of proliferation.

Ganz: Does colchicine also affect haemotaxis of the leukocytes?

Ham: We don't know at this stage.

Boman: You mentioned that the mature filariae have a digestive system with bacteria in the gut, but that the microfilariae don't. Where does this gut flora come from and how does it enter?

Ham: I don't know the answer to that.

Boman: If you take nematodes which live on insects, such as Neoaplectana, they have intestinal bacteria. There are only about 50 bacteria in the gut of each nematode, which isn't very many. There is a slow release of these bacteria from the digestive system. Many years ago, when Peter Götz took the trouble of preparing axenic Neoaplectana, they turned out to be virtually non-toxic to the insect. However, the bacteria, which were quite difficult to cultivate, turned out to be extremely toxic. What happened in this case was that the nematode was the invasive mechanism. The bacteria could never enter the insect, but the nematode served as the injection needle. The nematodes themselves could not kill the insect, but once the bacteria were released inside the insect, they could grow and kill it. Only when this occurred could the nematodes go through the reproductive cycle and release a new generation of nematodes (Götz et al 1981).

Ham: One could speculate that the microfilariae pick up bacteria within the gut of the mosquito.

Flajnik: Do you think there's a direct induction of the haemocytes or do you think there are signalling molecules such as cytokines involved as well?

Hultmark: We would all like to believe that there are cytokines involved, especially in the activation of the fat body, because there is no evidence for a direct interaction between bacteria and the fat body. However, results from Kanost et al (1988) and Taniai et al (1992) indicate that the signals released from the haemocytes may in fact be fragments of bacteria, like LPS and peptidoglycan fragments; both of these are good inducers of the fat body. So you

don't have to invoke cytokines, but I still believe there are such signalling molecules present.

Flajnik: So the phagocytes release fragments of the bacteria?

Hultmark: That's right. Taniai et al (1992) found that only in the presence of haemocytes could washed bacteria induce the fat body in tissue culture. Also, the cell-free supernatant from these haemocytes induced the fat body. But they could demonstrate the presence of LPS in this fraction at a concentration high enough to explain this effect. This makes it difficult to demonstrate the presence of cytokines. On the other hand, sterile wounds (at least in some systems) can also induce cecropin synthesis, admittedly at a lower level and for a shorter time. In that case I guess you have to assume that there are endogenous signals.

Ganz: Not necessarily, because you may be breaking mitochondria.

Hultmark: Yes, the signals could be released cell contents.

Elsbach: Of course, it's very difficult to distinguish between what the bacterial product does directly and what a bacterial product does to release cytokines that secondarily stimulate the overall host defence. Certainly, 'toxic' effects are entirely mediated by the effect of LPS on host responses—it induces toxic cytokines. In fact, whole bacteria, under conditions where they seem to remain intact, are capable of signalling their presence to host cells, causing release of cytokines or mediating priming of host cells by an LPS-like phenomenon. There are conditions where a single live bacterium primes more than 200 polymorphonuclear leukocytes. This raises the question: can small numbers of bacteria trigger responses in a few host cells that then mobilize the rest of the population? We wonder if the host recognizes whole bacteria without having to rely on release of bacterial products, such as LPS, for mounting a response. We are trying to address this question.

Boman: I would like to introduce the role of phenoloxidase and other serine proteases into our discussion. This system has been worked out very well in two organisms: by Söderhäll in crayfish and by Ashida in *Bombyx mori*. A lot is known about the cascade reaction that is needed to activate phenoloxidases, and many groups have tried to find a function for this system. It's quite clear that in many insects you have melanization of encapsulated bacteria. However, the function of this is not fully clear. Except for Söderhäll's demonstration that fungi are killed by phenoloxidase, there is no evidence that the system kills bacteria or other parasites.

Ham: In *Simulium*, which is the blackfly, there is never any melanization of foreign invading material, which you do get regularly in mosquitoes. But we know that phenoloxidase is activated in the haemolymph. We find that if you put different species of the parasite in the same insect, for different species of parasite which are compatible or not, you get differential appearance of phenoloxidase in haemolymph. What we believe might be happening is that the parasite is sequestering phenoloxidase onto its surface (Hagen & Ham 1994); that's one alternative. But it is not giving rise to melanization. This is one of

the reasons we believe it may be acting as a bridging molecule, as part of a non-self recognition molecule system, targeting possibly haemocytes. But we do see a clear activation of phenoloxidase.

Boman: If you use phenolthiourea, which is a powerful inhibitor of phenoloxidase, do you see any difference in the biology?

Ham: We haven't done that; I think it would be a very good thing to do.

Lehrer: Along these lines, there is clear evidence in mammalian systems that, in addition to antimicrobial peptides, the host uses a variety of oxidative systems to damage microorganisms and even worms (Klebanoff 1992). Some of the systems are peroxidase mediated, some of them depend on products of NADPH oxidase, others on inducible nitric oxide synthase. It's entirely conceivable that phenoloxidase is acting in a similar manner by producing free radicals. Just as you have shown synergy between lysozyme and your *Aedes aegypti* peptide, there may well be similar synergy taking place with phenoloxidase.

Ham: I think synergy is the key word, because we fractionated haemolymph from blackflies, and we've incubated different fractions with our parasites. The fraction which contains the 66 kDa phenoloxidase molecule is the one in which they thrive the best.

Lehrer: Have you mixed such fractions together, to look for synergistic effects?

Ham: No, there's a lot more we have to do. It may be a side issue, in a way, but with the mosquitoes we have two bands which appear to be inducible around 64–66 kDa and we believe that at least one of them is phenoloxidase. The top one is apparently induced by the parasite but the lower one is apparently induced by the bacteria and not the other way round. So it does look like there might be some differentiation of induction.

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Structure–function relationships of tachyplesins and their analogues

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Abstract. Haemocytes of the horseshoe crab (*Limulus*) contain a new family of arthropodous peptide antibiotics, termed the tachyplesin family. These cationic peptides are composed of 17–18 amino acid residues with a C-terminal arginine α-amide. Tachyplesin I takes on a fairly rigid conformation constrained by two disulphide bridges and adopts a conformation consisting of an antiparallel β-sheet connected by a β-turn. Isopeptides of tachyplesin I with amino acid replacements, tachyplesins II and III, and polyphemusins I and II have also been found in the haemocytes of the South-East Asian species and *Limulus polyphemus*. These peptides are present in abundance in the small granules of the haemocytes and inhibit strongly the growth of not only Gram-negative and Gram-positive bacteria but also fungi such as *Candida albicans*. Tachyplesin exists in the prepro form consisting of 77 residues; this precursor is probably processed by intracellular proteases and an amidation enzyme before incorporation into the small granules of the haemocytes. We examined the mode of action of tachyplesin I on biomembranes, comparing it with that of gramicidin S. Tachyplesin caused an efflux of K$^+$ from *Staphylococcus aureus* and *Escherichia coli* cells similar to that caused by gramicidin S. Another antimicrobial substance, anti-LPS factor, has been isolated from haemocytes.

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Invertebrates have characteristic host defence systems different to mammalian immune systems (Brehelin & Zachary 1986). In the horseshoe crab (*Limulus*), this defence system is carried in the haemolymph which contains a cell type called amoebocyte or haemocyte. The haemocytes are extremely sensitive to lipo-polysaccharide (LPS), a bacterial endotoxin which is a major component of the cell surface of Gram-negative bacteria (Armstrong 1991). Electron microscopy has shown that two types of granule, large and small (L and S), are present in the haemocyte (Toh et al 1991). When the haemocytes make

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TABLE 1 Antimicrobial peptides and lipopolysaccharide (LPS)-binding proteins found in horseshoe crabs (*Tachypleus tridentatus* and *Limulus polyphemus*)

Components	M_r	Total amino acid residues	Function	Intracellular localization	References
Tacyplesin precursor	9335	77	ND	Golgi (?)	Shigenaga et al (1990)
Tachyplesin I	2263	17	Antimicrobial	S-granule	Nakamura et al (1988)
Tachyplesin II	2263	17	Antimicrobial	S-granule	Miyata et al (1989)
Tachyplesin III	2245	17	Antimicrobial	S-granule	Muta et al (1990)
Tachyplesin I intermediate	2450	19	ND		Muta et al (1990)
Polyphemusin I	2410	18	Antimicrobial	ND	Miyata et al (1989)
Polyphemusin II	2402	18	Antimicrobial	ND	Miyata et al (1989)
Anti-LPS factor (*T. tridentatus*)	11 600	102	Antimicrobial & LPS-binding	L-granule	Tanaka et al (1982)
Anti-LPS factor (*L. polyphemus*)	11 786	101	Antimicrobial & LPS-binding	ND	Muta et al (1987)
L-6	27 000	221	LPS-binding	L-granule	(unpublished results)

ND, not done; L-granule, large granule; S-granule, small granule.

contact with Gram-negative bacteria or LPS, they begin to degranulate and the resulting granular components initiate haemolymph coagulation (Levin & Bang 1964, Ornberg & Reese 1981, Armstrong & Rickles 1982, Toh et al 1991). This response is thought to be important for the host defence in engulfing invading microbes, in addition to preventing the leakage of haemolymph. In our ongoing studies on the molecular mechanism of haemolymph coagulation in *Limulus*, we have established a serine protease cascade system (Nakamura et al 1986), much like the mammalian plasma clotting cascade (Iwanaga et al 1992, Iwanaga 1993a,b). During the purification of several clotting factors we found two types of potent antimicrobial substance in haemocytes that inhibit the LPS-mediated activation of this coagulation cascade, anti-LPS factor and the peptide tachyplesins (Table 1). In particular, the concentration of tachyplesins in haemocytes is extremely high—approximately 10 mg are found in the haemolymph of an individual horseshoe crab, suggesting that the peptides contribute to the host defence of *Limulus*. Here, we shall concentrate on the structure–function relationships of these antimicrobial substances.

The structure of tachyplesin and its analogues

Tachyplesin I is a cationic peptide that was first isolated from acid extracts of the Japanese horseshoe crab (*Tachypleus tridentatus*) haemocytes (Nakamura et al 1988). Later, two peptide analogues of tachyplesin I, named tachyplesins II and III, were isolated from haemocytes of two species of South-East Asian horseshoe crabs, *Tachypleus gigas* and *Carcinoscorpius rotundicauda* (Miyata et al 1989, Muta et al 1990). Two tachyplesin analogues, named polyphemusins I and II, were also found in the haemocyte debris of *Limulus polyphemus* (Miyata et al 1989). Furthermore, a tachyplesin peptide derivative with a C-terminal extension of Gly–Lys has been found in the haemocytes (Muta et al 1990). Tachyplesin I is highly stable at low pH or high temperature; this stability is due to the rigid structure imposed by two disulphide linkages (Nakamura et al 1988). The primary structures of the tachyplesin family are shown in Fig. 1. They are composed of 17 or 18 residues with arginine α-amide at the C-terminal end. Tachyplesin has a characteristic structure, with three tandem repeats of a tetrapeptide sequence, namely hydrophobic amino acid–Cys–hydrophobic amino acid–Arg, indicating its amphipathic nature.

   Figure 2 shows the conformational structure of tachyplesin I (Kawano et al 1990, 1991). The secondary structure is substantiated by interpretations of the nuclear Overhauser effect, coupling constant, amide exchange rate and temperature dependence of the amide chemical shift. Tachyplesin I takes on a fairly rigid conformation constrained by two disulphide bridges and adopts a conformation consisting of an antiparallel β-sheet (residues 3–8 and 11–16) connected by a β-turn (residues 8–11). In this planar conformation, five bulky hydrophobic side groups are located on one side of the plane and six cationic

Tachyplesin I   **Tachyplesin II**   **Tachyplesin III**

Tachyplesin   **Polyphemusin I**   **Polyphemusin II**
precursor
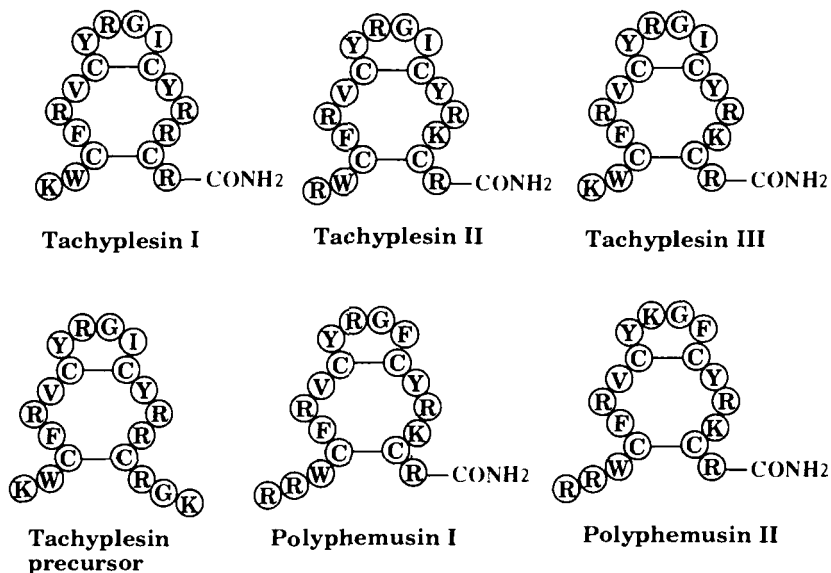
FIG. 1.   Primary structures of six kinds of tachyplesin and tachyplesin analogues isolated from four species of horseshoe crab. Tachyplesin III was isolated from *Tachypleus gigas* (Muta et al 1990), tachyplesin I from *T. tridentatus* (Nakamura et al 1988), *T. gigas* and *Carcinoscorpius rotundicauda*, and tachyplesin II from *T. tridentatus*. Polyphemusins I and II were isolated from *Limulus polyphemus* (Miyata et al 1989). Reproduced with permission from Iwanaga et al (1992).
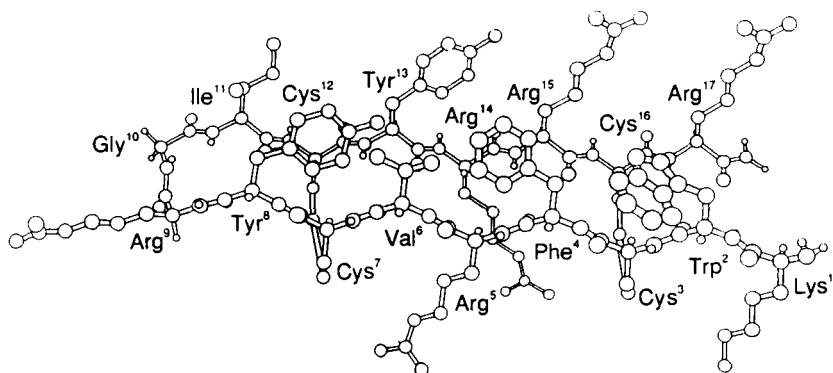
FIG. 2.   Structure of tachyplesin I in aqueous solution, presumed on the basis of NMR experiments (Kawano et al 1990, Park et al 1992).

side groups are distributed at the 'tail' of the molecule (residues 1–5 and 14–17). This amphipathic structure may have a crucial role in the interaction of tachyplesin with LPS or bacterial membranes (Yonezawa et al 1992). In fact, the energy-transfer experiment indicates that some conformational change of tachyplesin I occurs such that the Tyr and Trp residues are positioned more closely to each other in acidic liposomes than in buffer solution, suggesting that an amphipathic lipid bilayer causes its structure to change from an energetically stable β-structure to a less ordered, probably more amphipathic structure (Park et al 1992).

Tachyplesin precursors and their intracellular localization

Recently, we succeeded in cloning the cDNAs encoding the tachyplesin precursors and showed their distribution in various tissues of the horseshoe crab (Shigenaga et al 1990). The precursors of the tachyplesins consist of 77 amino acids with 23 residues in a pre segment, and there are two types of mRNAs corresponding to the isopeptides of tachyplesins I and II (Fig. 3). Both precursors contain a putative signal peptide, a processing peptide sequence and a C-terminal amidation signal, Gly–Lys–Arg, connected to the mature tachyplesin peptide. Moreover, an unusual acidic amino acid cluster with Asp–Glu–Asp–Glu–Asp–Asp–Asp–Glu–Glu–COOH is present in the C-terminal portions of both

FIG. 3.   Amino acid sequence of tachyplesin I precursor predicted from its cDNA sequence. The residues circled in bold constitute a mature tachyplesin. The charged residues are indicated by + and − (Shigenaga et al 1990). Reproduced with permission from Iwanaga et al (1992).

precursors. This acidic region might interact with a cationic part of the tachyplesin peptide to stabilize a conformational structure of the precursor for proteolytic degradation. The amino acid sequence of the precursor molecule suggests that the mature tachyplesin is generated through several processing steps. First, the N-terminal signal sequence of the tachyplesin precursor is processed in a stepwise fashion by a signal peptidase followed by a dipeptidyl-peptidase. This is suggested by the fact that the Glu–Ala–Glu–Ala sequence preceding the mature tachyplesin peptide fits the substrate specificity of a dipeptidyl-peptidase. Second, the Arg–Asn bond at positions 20 and 21 is cleaved by a processing enzyme that recognizes the dibasic sequence of Lys–Arg at positions 19 and 20. Third, release of Lys–19 and Arg–20 is effected by a carboxypeptidase B-like enzyme, and finally an oxidative amidation is catalysed by an enzyme recognizing the C-terminal Gly residue.

Northern blot analyses of total RNA prepared from various tissues indicate that the tachyplesin precursor is expressed exclusively in the haemocytes, in accordance with the high levels of tachyplesin peptides recovered from these. The mRNAs from heart and brain tissues also contain a transcript identical in size (0.7 kb) to that found in the haemocytes, but the signals are relatively weak. The amount of the tachyplesin precursor mRNA in other tissues, including those of the hepatopancreas, stomach, intestine, muscle and coxal gland, is negligible. Although little is known about the haemopoietic tissue from which circulating haemocytes are derived in the horseshoe crab, our observations suggest that cardiac tissues may be the site of their formation. Immunohistochemistry has shown that tachyplesin is localized in the S-granules in the haemocytes (Fig. 4).

We recently designed a method for separating L- and S-granules from the post-nuclear supernatant of *T. tridentatus* haemocytes mechanically disrupted in the presence of phenylmethanesulphonyl fluoride and propranolol (Shigenaga et al 1993). Through this procedure, the L- and S-granules are clearly separated at around 2 M sucrose density. Comparisons of granular components indicate that proteins and peptides in L- and S-granules are quantitatively unique. Protein profiles obtained from different batches of each of the granules are always similar, showing the coexistence of four major components (coagulogen, factor C, proclotting enzyme and anti-LPS factor) in the L-granules and a major tachyplesin I peptide in the S-granules. In particular, coagulogen and tachyplesin are predominant in L- and S-granules, respectively. The existence of mature tachyplesin I free from the precursor polypeptide in S-granules strongly suggests that it is generated not in the granule but in the Golgi complex through conventional processing mechanisms. We have no explanation as to why this tachyplesin family, which has a strong amphipathic nature and is therefore autotoxic, is stored in the granule. A mechanism may exist to prevent lysis of the granular membrane by tachyplesin.

FIG. 4.   Localization of tachyplesin in horseshoe crab haemocytes. Haemocytes were fixed, stained with anti-tachyplesin IgG and gold-particle-conjugated second antibody; sections were viewed under the transmission electron microscope. Numerous gold particles are present in the more electron-dense S-granules (Toh et al 1991, Shigenaga et al 1990). The less dense L-granules contain anti-LPS factor, in addition to several clotting factors essential for haemolymph coagulation (Iwanaga et al 1992).

Biological function of tachyplesin

In Table 2, the minimal inhibitory concentrations of tachyplesins I and II and polyphemusins I and II for various bacterial strains are summarized (Miyata et al 1989). The chemically synthesized peptides are also included for comparison. Native tachyplesins and polyphemusins display potent antimicrobial activity towards several microbial strains. Tachyplesin II shows almost the same potency and inhibitory spectrum as tachyplesin I against Gram-negative (*Salmonella* and *Escherichia* strains) and Gram-positive (such as *Staphylococcus*) bacteria. In general, polyphemusins I and II show the same as or somewhat lower potency than the tachyplesins. The growth of fungi, such as *Candida albicans* M9 and *Cryptococcus neoformans* IMF 40040, is also strongly inhibited by tachyplesin peptides (Niwa et al 1990, Morimoto et al 1991, Murakami et al 1991). On the other hand, synthetic tachyplesins I and II and polyphemusin I show the same or a rather stronger inhibitory effect on the growth of microorganisms, as

TABLE 2 Antimicrobial activity of native and synthetic tachyplesins and polyphemusins

Strain	Minimum inhibitory concentration ($\mu g/ml$)						
	Tachyplesin I		Tachyplesin II		Polyphemusin I		Polyphemusin II
	Native	Synthetic	Native	Synthetic	Native	Synthetic	Native
Gram-negative bacteria							
Salmonella typhimurium LT2	3.1	1.6–3.1	3.1	1.6–3.1	3.1	3.1–6.3	6.3
Salmonella typhimurium 1102	0.8–1.6	0.8–1.6	1.6	1.6–3.1	3.1	3.1	3.1
Escherichia coli K12	1.6–3.1	1.6–3.1	3.1	1.6–3.1	6.3	6.3	12.5
Salmonella minnesota 1114W	3.1	6.3	3.1	6.3	6.3	12.5	12.5
Salmonella minnesota R595	1.6	1.6	1.6	1.6	3.1	3.1	3.1
Pseudomonas aeruginosa	12.5	ND	ND	ND	ND	ND	ND
Gram-positive bacteria							
Staphylococcus aureus 209P	3.1	3.1	1.6–3.1	6.3	6.3	6.3	6.3
Staphylococcus aureus ATCC 25923	6.3	12.5	6.3	12.5	6.3	6.3	12.5
Bacillus subtilis	3.13	ND	ND	ND	ND	ND	ND
Fungus							
Candida albicans M9	3.1	3.1	3.1	3.1	6.3	6.3	6.3
Cryptococcus neoformans IMF40040	1.56	ND	ND	ND	ND	ND	ND

ND, not done.

compared with the native preparations (Akaji et al 1989). These antimicrobial potencies of native and synthetic peptides are comparable to that of anti-LPS factor described later.

To elucidate the mode of action of tachyplesin I on biomembranes, we examined its effects on the morphological and permeability changes of bacterial cells and human erythrocytes, comparing them with those of gramicidin S (Katsu et al 1993). Tachyplesin I increases the K^+ permeability of *Staphylococcus aureus* and *Escherichia coli* cells, concomitantly reducing cell viability. At higher concentrations, this peptide also enhances the permeability of human erythrocytes. Tachyplesin decreases the phase-transition temperature of an artificial membrane composed of dipalmitoylphosphatidylglycerol and, further, broadens it extensively, while it does not affect that of a dipalmitoly-phosphatidylcholine membrane. The latter result relates closely to the fact that this peptide acts weakly on erythrocyte membranes, in which acidic lipids are a minor component. Tachyplesin alters the normal discoid shape of human erythrocytes to a crenated form, suggesting that the peptide accumulates predominantly in the outer half of the membrane bilayer and destabilizes the membrane structure, thus causing the change in permeability. Therefore, the mode of action of tachyplesin I appears to be essentially similar to that of gramicidin S, a peptide forming an amphiphilic structure analogous to tachyplesin.

Structure and function of anti-LPS factor

Limulus anti-LPS factor (ALF) is a small, basic protein which binds and neutralizes LPS and has a strong antibacterial effect on the growth of Gram-negative R-type bacteria (Morita et al 1985). In haemocytes, this protein is contained in the L-granules together with several clotting factors (Toh et al 1991). The primary structure of ALF isolated from the Japanese horseshoe crab was first determined in 1986 (Aketagawa et al 1986); that of the American crab was established in the next year (Muta et al 1987). Both ALFs are single-chain polypeptides composed of 101 or 102 amino acid residues with a relative molecular mass of 12 000 (Table 1). No carbohydrate is attached. The primary structures are shown in Fig. 5. Whereas the Japanese ALF has its N-terminus masked with pyroglutamic acid, the American ALF has its N-terminus masked with an aspartyl residue. The Japanese ALF has two sites of heterogeneity; it has either valine or isoleucine at position 36 and glutamine or glutamic acid at its C-terminus. On the other hand, American ALF contains either asparagine or lysine at position 13. Both molecules contain two cysteine residues at positions 31 and 52 linked with an intramolecular disulphide bridge. The sequence identity between the two molecules is 83%. This value is rather higher than that obtained from comparison of coagulogen molecules from the same two species (69%), indicating that the entire molecule is closely associated with its biological function (Miyata et al 1984). The disulphide loop region is highly basic; in particular,

```
                            20                          40
(a)  D G I W T Q L I F T L V WN NL A T L W Q S G D F Q F L D HE C H Y R I K P T F R RL K W K Y K G K F
                          K
     -                                    -         -  +- + + +    +  +I+ + + + +
                          +-                        +  +- + +        +I+ + + + +
(b) <E G G I W T Q L AL A L V K NL A T L W Q S G D F Q F L G HE C H Y RJ N P T V K RL K W K Y K G K F
```

```
            60                          80                              100
   W C P S W T S I T G R A T K S S R S G A V E H S V RN F VG Q A K S S G L I TQ R QA E QF I S Q YN
          +   +   +                - +   +|        +         +
          +   +   +                - +   +|-       +         - + -
   W C P S W T S I T G R A T K S S R S G A V E H S V RD F VS Q A K S S G L I TE K EA Q TF I S Q YQ
                                                                                            E
```

FIG. 5. Alignment of the sequences of anti-LPS factors from *Limulus polyphemus* (a) and *Tachypleus tridentatus* (b). Identical residues are boxed and the charged residues are indicated by + or − between the two sequences (Aketagawa et al 1986, Muta et al 1987).

the region from Arg-41 to Lys-49 has basic amino acids at every second residue and the region from Arg-61 to Arg-76 is also rich in basic residues—in this region there are positive charges at almost every third residue. The hydropathic profile indicates that the N-terminal region of ALF is highly hydrophobic, rendering this molecule amphipathic.

Quite recently, Hoess et al (1993) reported the three-dimensional structure of recombinant *Limulus* ALF. The crystal structure of ALF reveals a simple tertiary fold with a striking charge distribution and amphipathicity. The molecule has a single domain consisting of three α-helices packed against a four-stranded β-sheet, and is wedge shaped, about 40 Å tall and 28 Å wide at its base. The binding site for LPS probably involves an extended amphipathic loop of ALF and binds the phosphoglucosamine portion of lipid A. An analogous LPS-binding loop also exists for two human proteins that bind LPS—LPS-binding protein (LBP; Schumann et al 1990) and bactericidal permeability-increasing protein (BPI; Marra et al 1992).

ALF inhibits the LPS-mediated activation of *Limulus* factor C zymogen (Tanaka et al 1982, Muta et al 1991, 1993). It also inhibits activation of this factor by synthetic lipid A; thus ALF appears to interact with the lipid A portion of LPS. ALF also shows haemolytic activity against erythrocytes sensitized with LPS (Ohashi et al 1984). As shown in Table 3, human, horse and chicken erythrocytes are susceptible to this haemolysis. The minimal concentration of ALF required for haemolysis depends on the concentration of LPS used for the sensitization. Under optimal conditions, the minimal haemolytic concentration of ALF is 0.48 μg/ml. LPS sensitization is essential for this phenomenon, because ALF has no effect on intact cells. ALF activity can be neutralized by free LPS but not by the polysaccharide portion of LPS. ALF also lyses human polymorphonuclear leukocytes, mononuclear cells and leukaemia cells (K562) which have been exposed to LPS.

TABLE 3 Haemolytic activity of anti-LPS factor on red blood cells (RBC) sensitized with lipopolysaccharide (LPS)

RBC sensitized with	Dilution of anti-LPS factor											
	2	4	8	16	32	64	128	256	512	1024	2048	Control
S. minnesota R595 LPS (Human RBC)	+	+	+	+	+	+	+	+	+	+	−	−
S. minnesota 1114W LPS (Human RBC)	+	+	+	+	+	+	+	+	+	+	+	−
S. minnesota 1114W LPS (Chicken RBS)	+	+	+	+	+	+	+	+	+	+	−	−
S. minnesota 1114W LPS (Horse RBC)	+	+	+	+	+	+	+	+	+	+	+	−
E. coli 0113 LPS (Human RBC)	−	−	−	−	−	−	−	+	+	−	−	−
None (Human RBC)	−	−	−	−	−	−	−	−	−	−	−	−
None (Chicken RBC)	−	−	−	−	−	−	−	−	−	−	−	−
None (Horse RBC)	−	−	−	−	−	−	−	−	−	−	−	−

50 μl of 0.5% RBC sensitized with LPS were mixed with 50 μl of a twofold serial dilution of anti-LPS factor in a microtitre U-plate and incubated at 37 °C for 30 min. The concentration of original solution of anti-LPS factor was 2 mg/ml. +, haemolysis-positive; −, haemolysis-negative. S, *Salmonella*; E, *Escherichia*.

TABLE 4   Effect of anti-lipopolysaccharide factor on bacterial growth

Bacterial strain	Minimal antibacterial dose (µg)
Salmonella typhimurium	
LT2 (S type)	No effect[a]
SR1034 (SR)	No effect[a]
TV149 (Ra)	No effect[a]
TV148 (Rb)	10
SL1069 (Rc)	5
SL1181 (Rd2)	1.3
SL1102 (Re)	1.3
Salmonella minnesota	
1114W (S)	7.45
R595 (Re)	0.12

[a]At 20 µg dose. The following strains were insensitive to anti-LPS factor (20 µg dose): *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13833, *Enterobacter cloacae* ATCC 23355, *Proteus vulgaris* ATCC 13314, *Pseudomonas aeruginosa* ATCC 27853, *Serratia marcescens* ATCC 8100, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* 0111 (B4, 0124, 0113 and UKTB), *Pseudomonas aeruginosa* TE, *Staphylococcus aureus* 209P.

ALF exhibits growth inhibitory activity against some, but not all, strains of Gram-negative bacteria (Niwa et al 1990) (Table 4). Among the strains tested, *Salmonella minnesota* R595 and *Salmonella typhimurium* 1102 are the most susceptible to the antibacterial action of ALF. Because ALF binds to lipid A, it probably neutralizes the biological activity of LPS (Roalansky & Novitsky 1991). In fact, ALF suppresses the pyrogenicity of LPS in rabbits and inhibits endotoxin-mediated activation of cultured endothelial cells and B cells *in vivo* (Siber et al 1992). ALF also reduces mortality when administered before and after LPS challenge or bacterial infection (Alpert et al 1992, Siber et al 1992). Thus, ALF might be clinically useful for removing LPS and suppressing its activity (Wainwright et al 1991).

As mentioned earlier, ALF contains two positively charged regions and an N-terminal hydrophobic region. These two positively charged clusters might provide interaction site(s) with phosphate groups in the lipid A portion of LPS. Once ALF interacts with LPS on the cell membrane through the LPS-binding loop, the membrane structure seems to be perturbed by insertion of the hydrophobic N-terminal region, including up to about the 27th residue. The region appears to be long enough to cross a lipid bilayer-like transmembrane α-helix of the bacteriorhodopsin molecule. The fact that ALF is localized in the L-granules and has strong antimicrobial activities suggests that ALF is secreted during degranulation of the haemocyte induced by LPS and functions as a bactericidal substance following engulfment of the invaders by the coagulation system.

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DISCUSSION

Ganz: How phagocytic are the horseshoe crab haemocytes?

Iwanaga: We haven't studied their phagocytic activity, but Dr Peter Armstrong (1991) has already shown that these haemocytes have phagocytic action, but they are not as active as macrophage cells, for instance.

Ganz: Do you know which of the granule populations degranulates in response to phagocytosis? Which granules enter the phagosome?

Iwanaga: I don't know.

Lehrer: What can you tell us about the proline-rich peptide in the large granule?

Iwanaga: We have sequenced the proline-rich protein, but not the peptide. Some properties of this protein have been published (Shigenaga et al 1993). The proline content is about 20%; we will soon have the whole sequence.

Elsbach: What is the significance in having a high proline content in proteins and peptides? What are the implications of having lots of what are presumed to be very disruptive amino acid residues?

Boman: We are trying to obtain the structure of our proline-rich peptide, PR-39, but we don't yet have an answer. This is quite a long-term project. I learned about the approach we are taking from Edmundson, known for his famous wheel, in

Jerusalem in 1989. The idea is that first we raise a monoclonal antibody against PR-39, which we now have, then we purify enough of the peptide and the antibody for us to be able to co-crystallize the peptide bound to the antibody. Finally, in order to obtain the structure, we will do subtraction crystallography of the complex (in collaboration with Pär Nordlund). This should be relatively easy because we already have X-ray structures for many other monoclonal antibodies.

Iwanaga: I would also like to comment on the proline-rich protein isolated from the haemocytes. It has a molecular mass of about 94 kDa—it is a large protein—and is a very good substrate for the action of transglutaminase located in the cytosol (Tokunaga et al 1993). We don't know the biological function of this proline-rich protein, and we haven't tested its antimicrobial activity.

Lehrer: Was the antimicrobial activity you gave for the defensin-like peptide exerted by the 79 amino acid peptide, or do you think that this undergoes further processing?

Iwanaga: The sequence contains an Arg–Arg motif in the N-terminal portion. I think this dibasic sequence is processed by a KEX-2-type protease or furin. We used the whole S5 sequence to check the defensin activity, and it showed potent antimicrobial activity against Gram-positive and Gram-negative bacteria. However, at the moment we don't know if only the C-terminal portion is required for its antibacterial activity. A protease released from the bacteria might cleave the Arg–Arg motif located in the N-terminal portion of S5.

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Bactericidal permeability-increasing protein in host defence against Gram-negative bacteria and endotoxin

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Abstract. The bactericidal permeability-increasing protein (BPI) is a highly conserved host-defence molecule produced and stored by myeloid cells only and a major constituent of the primary granules of human and rabbit polymorphonuclear leukocytes. The *c.* 50 kDa BPI and a *c.* 23 kDa bioactive N-terminal fragment are cytotoxic only for Gram-negative bacteria. This target-cell specificity reflects the high affinity (apparent K_d: 1–10 nM) of BPI for the lipid A portion of lipopolysaccharide (LPS or endotoxin). Native and recombinant (r) holo-BPI and the N-terminal fragment (rBPI-23) bind with equal affinity to all forms of isolated LPS examined and inhibit the numerous biological effects of LPS *in vitro* (including in whole blood *ex vivo*) as well as in animals. Under the same conditions the antibacterial potencies of holo-BPI and rBPI-23 against Gram-negative bacteria with rough chemotype LPS (whether encapsulated or not) are also the same, but against more resistant smooth chemotype Gram-negative bacteria rBPI-23 is up to 30-fold more potent than holo-BPI. Holo-BPI and rBPI-23 protect a broad range of animals against lethal cytotoxic effects of LPS and in some cases against lethal inoculations with live Gram-negative bacteria.

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The recognition of mammalian endogenous antibiotic proteins as part of host defence against bacterial infection had its origin in the studies of James Hirsch at the Rockefeller Institute in the 1950s (Hirsch 1956). He showed that a broad mix of cationic proteins in crude acid extracts of rabbit polymorphonuclear leukocytes killed both Gram-negative and Gram-positive bacterial species. Further analysis of these acid extracts led to the identification of the first well-defined constituent antibacterial proteins. First, highly purified cathepsin G and elastase isolated from the granules of human polymorphonuclear leukocytes were shown to be microbicidal, independently of their proteolytic activities (Odeberg & Olsson 1976a,b). Thereafter, the non-catalytic bactericidal permeability-increasing protein (BPI) was isolated from human (Weiss et al 1978) and rabbit (Elsbach et al 1979) polymorphonuclear leukocytes. Then, building on the work of Spitznagel and collaborators, who first collected from the acid extracts of

rabbit polymorphonuclear leukocytes a still crude bactericidal subfraction containing protein species of less than 8kDa (Zeya & Spitznagel 1963, 1966), Lehrer and co-workers isolated the defensins, a comprehensively characterized peptide family of *c*. 4 kDa peptides (Lehrer et al 1993). BPI, a novel lysine-rich cationic 50–55 kDa protein, was unusual in that it was toxic only for Gram-negative bacterial species. This highly selective cytotoxicity is attributable to the strong attraction of BPI to the lipopolysaccharides (LPS) that are unique to the outer membrane of the envelope of Gram-negative bacteria. This attraction is also evident toward isolated LPS (endotoxin), classifying BPI as a member of a family of LPS-binding proteins (Schumann et al 1990). BPI stands out among the many other antimicrobial proteins/peptides that have been isolated subsequently because of the potency of its antimicrobial activity towards Gram-negative bacteria. While comparisons of reported activities of different antimicrobial proteins and peptides must be viewed with caution because of dissimilar assay conditions and target microorganisms used by different investigators, the molar concentrations of BPI required for inhibition of the growth of *Escherichia coli* are at least one order of magnitude lower than the concentrations needed of the other peptides and proteins listed in Table 1. Also in the 1980s, Boman and co-workers identified and characterized the first insect bactericidal peptides, the attacins and cecropins (Hultmark et al 1980, Boman et al 1991). More recently, antimicrobial peptides, considered to represent 'antibiotics from within' (Elsbach 1990) have been isolated from a broad range of animal hosts, including the horseshoe crab (Nakamura et al 1988) and amphibians (Zasloff 1992).

While the methodological advances of the past two decades have made possible the isolation of many pure proteins and peptides with antimicrobial properties *in vitro*, leading to important new insights into evolutionary aspects of structure and function of these molecules, much less is known about their actual contribution to antimicrobial defences in the whole organism.

To gain a better perspective of the true biological role of BPI, we have compared its bioactivity as isolated protein and in combination with components of other host defence systems. We find that both in artificial media and in physiological fluids, the concentrations of BPI that are required for inhibition of bacterial growth are reduced up to 50-fold in the presence of concentrations of naturally occurring extracellular and cellular antimicrobial proteins/peptides that by themselves exhibit little or no antibacterial activity (Ooi et al 1990, 1993, Elsbach & Weiss 1993a). The potent bioactivity of added recombinant BPI in the complex environment of plasma and whole blood *ex vivo* (Weiss et al 1992) and of native BPI in inflammatory fluid (Weinrauch et al 1994) supports the ongoing exploration of BPI as a potential therapeutic agent.

In this review, we provide a synopsis of the salient structural properties of BPI and of what we know of its function as a potent antibacterial and anti-endotoxin agent and of its use for the protection of animals. (Other recent reviews of BPI include Elsbach & Weiss 1993a,b, Elsbach et al 1994, Weiss et al 1993.)

TABLE 1   Apparent growth-inhibitory potencies of antimicrobial peptides and proteins towards a laboratory strain of *Escherichia coli*

Peptide/protein	Source	Assay	Minimum inhibitory concentration (μM)
Animal			
Tachyplesin	*Limulus*	Radial diffusion	2–4
Magainins	Amphibians	Radial diffusion	4
Defensin (NP1)	Rabbit	Hypotonic liquid medium	0.5–1
		Radial diffusion	75
β-Defensins	Cow	Radial diffusion	7–7.5
Cyspinecins[a]			
Bactenicins	Cow	Hypotonic medium	1
Indolicin	Cow	Hypotonic medium	7
		Radial diffusion	20–200
p15A	Rabbit	Hypotonic medium	0.2
		Hypotonic medium + NaCl	2
TAP	Cow	Hypotonic medium + 50mM NaF	12–25
Azurocidin/ CAP37	Human	Hypotonic medium	0.02
		Hypotonic medium + NaCl	Micromolar
BPI	Human, rabbit	Hypotonic or isotonic medium	0.001–0.01
Insect			
Defensin-like	Scorpion etc.	Radial diffusion	0.1–1
Attacins	Silk moth	Liquid medium	1–2
Cecropins	Silk moth, pig	Radial diffusion	0.3–0.4
Hymenoptaecin	Bee	Hypotonic medium	1–20
		Radial diffusion	1–20

[a] The term 'cyspinecin' has been coined by Jerrold Weiss (Weiss 1994) and refers to a recently recognized family of antimicrobial leukocyte proteins. Some members of this family share a highly conserved N-terminal region of approximately 100 residues. In two instances, inhibition of Cys proteases has been linked to this region.

General properties of BPI

BPI has been found only in polymorphonuclear leukocytes and is produced during myeloid differentiation at the promyelocyte stage (Weiss & Olsson 1987). The protein is stored in the primary granules of the polymorphonuclear

leukocytes and can also be detected in association with the plasma membrane (Weersink et al 1993).

The primary structures of human, rabbit and cow BPI, derived from the respective isolated cDNAs (Elsbach & Weiss 1993a), show approximately 65% overall identity and a closely similar charge distribution. The N-terminal amphipathic half (proximal of residue 200) carries a strong net-positive charge (human BPI, +17; rabbit BPI, +27; cow BPI, +14); the C-terminal portion of the molecule is relatively uncharged and rich in hydrophobic regions. Whereas BPI is generally resistant to proteolytic attack, a proline-rich stretch separating the two halves is protease sensitive, permitting the separation, by limited proteolysis and subsequent purification, of the N-terminal and C-terminal halves of human BPI (Ooi et al 1987, 1991). Unusually for cytotoxins of comparable size (which normally require the complete molecule for full action) the c. 25 kDa N-terminal BPI fragment, at the same or up to 30-fold lower molar concentrations (Ooi et al 1987, 1991, Weiss et al 1992, Capodici et al 1994), carries all of the known antibacterial and anti-LPS activities of holo-BPI. Studies are in progress to analyse in greater detail the structural requirements for the interaction of the N-terminal fragment with free LPS and intact bacteria. A synthetic peptide stretching from residues 85–99 of human BPI with LPS-binding/inhibitory and growth-inhibitory (E. coli) activities has been identified (Little et al 1994). Other peptides generated by proteolysis or CNBr cleavage displayed no antibacterial activity but did show both LPS-interactive and heparin-binding activity, apparently related to the presence of hydrophobic and basic residues in an arrangement found in other heparin-binding proteins (Little et al 1994). As for small peptide fragments of other antimicrobial proteins of the polymorpho-nuclear leukocytes including cathepsin G and Cap 37/azurocidin (Pereira et al 1993, Shafer et al 1991), molar concentrations are necessary for biological activity that are orders of magnitude higher than the active concentration of the native protein. Therefore, the structural basis for relatively low bioactivity of small fragments of a complex protein may have limited bearing on the structural determinants of activity in the holo-protein. Whether the hydrophobic C-terminal portion has a role as a membrane anchor (Gray et al 1989), in (intra)cellular trafficking (Ooi & Weiss 1992), or in the antimicrobial function of the polymorphonuclear leukocytes, remains uncertain (Ooi et al 1993).

Antibacterial and LPS-neutralizing activities of BPI and its N-terminal fragment

The LPS-binding/neutralizing and antibacterial activities of native and recombinant BPI ('BPI-55') and its N-terminal fragment (BPI 1–193; 'BPI-23') have been studied extensively. Both these proteins, at low nanomolar concentrations, block the biological activities of isolated LPS in a broad range of in vitro as well as in vivo settings (Elsbach et al 1994). The binding affinities (1–10 nM) of BPI-55 and BPI-23 are the same for many LPS species with

polysaccharide chains varying greatly in lengths. They are also the same for the lipid A moiety of LPS that is shared in closely similar form by LPS isolated from many species of Gram-negative bacteria, indicating that lipid A is the site of attraction for BPI (both the holo-protein and the bioactive N-terminal fragment) (Gazzano-Santoro et al 1992). In contrast, binding and potency of BPI-55 and BPI-23 differ for intact Gram-negative bacteria with envelope LPS that contains polysaccharide chains varying in length (Weiss et al 1992, Capodici et al 1994). Both the holo-protein and the N-terminal fragment show the same nanomolar growth-inhibitory concentrations for Gram-negative bacteria with short-chain LPS ('rough'). Higher concentrations are required against bacteria with long-chain LPS ('smooth') and the potency of BPI-23 towards smooth Gram-negative bacteria is up to 30-fold greater than that of BPI-55 (Weiss et al 1992, Capodici et al 1994). We conclude that the attachment sites provided by the clusters of anionic residues in the inner core and lipid A of the tightly packed LPS in the bacterial envelope are not readily accessible to BPI, but more so to the smaller BPI-23. Thus, as in many other settings, host responses to LPS vary not only with differences in the structure of the molecule (particularly polysaccharide chain length) but also with its highly variable physical presentation (cell-free or as part of the bacterial envelope).

In various media, the antibacterial actions of BPI can be separated into two stages. Upon binding of BPI, bacteria stop dividing instantaneously, coincident with alterations that are limited to the outer membrane of the envelope of the Gram-negative bacteria manifest as a discrete increase in permeability for normally impermeant hydrophobic substances and activation of bacterial enzymes that selectively degrade phospholipids and peptidoglycans (Elsbach & Weiss 1992, Weiss et al 1993). However, the bacteria (test organism *E. coli*) retain nearly complete biochemical and structural integrity, permitting resumption of growth under certain conditions (Mannion et al 1990a,b). After longer incubation, additional effects are evident, consisting of loss of energy-dependent biochemical functions that reflect damage to the cytoplasmic membrane (Mannion et al 1990a,b) in parallel with irreversible growth inhibition. The time required for these later, irreversible changes varies with environmental conditions, including pH (Mannion et al 1990a).

Role of BPI in the intact polymorphonuclear leukocyte

The fates of Gram-negative bacteria treated with BPI or ingested by (rabbit) polymorphonuclear leukocytes generally are remarkably similar, allowing rescue of ingested *E. coli*. This implies that phagocytosis, although coincident with growth arrest, does not necessarily result in prompt killing even of avirulent laboratory strains (Mannion et al 1990b). Rapid transition from reversible to irreversible growth inhibition does occur however, when, before treatment with BPI or phagocytosis, the bacteria are exposed to serum concentrations that have

no detectable damaging effect. The serum effect is abolished by depletion of
the late complement component C7. These findings reveal not only an
unexpected limited destructive capability of the polymorphonuclear leukocytes
towards Gram-negative bacteria, in which BPI apparently plays a major
role, but also an integration of extracellular (the late components of complement,
i.e. the membrane attack complex) and cellular (polymorphonuclear leukocytes)
host defence systems that optimizes bacterial destruction (Mannion et al 1990b).

The closely similar effects of isolated BPI and unaided intact polymorpho-
nuclear leukocytes on bacteria appear consistent with a primary role of BPI
in the action of the polymorphonuclear leukocytes on ingested Gram-negative
bacteria. It is also apparent, however, under a range of *in vitro* conditions, that
while the antibacterial effects depend on the presence of BPI, the concentrations
of BPI required for an antibacterial effect may be reduced manifold when other
antimicrobial proteins or peptides are present in amounts that alone do not cause
detectable damage. For example, the newly discovered 15 kDa proteins of rabbit
polymorphonuclear leukocytes, the 'p15s' (Ooi et al 1990, Levy et al 1993), and
the most cationic rabbit defensins NP1 and NP2 act synergistically with both
rabbit and human BPI in artificial media as well as whole blood (Levy et al
1994). Such evidence that various elements of the antibacterial arsenal of the
polymorphonuclear leukocytes act in concert is not necessarily in conflict with
the concept that individual components dominate in the action against specific
target microorganisms. For example, at elevated molar concentrations, proteins
and peptides with low potency and relatively low affinity for the envelope of
a given microbial species, by occupying abundant surface sites (without causing
appreciable damage), may facilitate the interaction of a protein with high(er)
affinity for a limited number of specific sites that must be occupied for its target-
specific action.

Gram-negative bacterial sepsis and death are thought to be the consequence of
exaggerated host responses to LPS which result in tissue-damaging levels of
cytokines, vasoactive nitric oxide and clotting-promoting tissue factor. Consistent
with this view, BPI, by complexing LPS and blocking its effects on host cells, has
now been shown *in vitro* to inhibit LPS-induced release of cytokines (Ooi et al
1991, Weiss et al 1992, Marra et al 1990, 1992, Meszaros et al 1993, Elsbach &
Weiss 1993a, 1993b, Elsbach et al 1994) and monocyte tissue factor (Meszaros
et al 1994), production of reactive O_2 derivatives (Meszaros et al 1993), nitric
oxide (Betz-Corradin et al 1994) and leukotrienes (M. Doerfler & J. Weiss,
unpublished observations) and adhesion of neutrophils to endothelium (Huang
et al 1994) (Table 2). Some of these effects are on cells carrying the lipid-anchored
LPS-binding protein CD14 on their surface. Delivery of LPS to CD14 is
mediated by the acute-phase serum protein LPS-binding protein (LBP), resulting
in dramatic amplification of the cellular response to LPS. BPI, by competing
with LBP for LPS, inhibits the transmission of the LPS signal (Heumann et
al 1993, Dentener et al 1993, Gazzano-Santoro et al 1994).

TABLE 2   n(r)BPI-55 and n(r)BPI-23 inhibit the effects of lipopolysaccharide (LPS) on biological systems *in vitro*

Effect	*Reference*
Priming by LPS of PMN leading to release and metabolism of arachidonic acid after second stimulus (LBP enhances priming by LPS of PMN)	Unpublished observations
LPS-induced and LBP-mediated release of TNF and other cytokines and of reactive O_2 intermediates in whole blood	Meszaros et al 1993
LPS-induced adhesion of PMN to endothelial cells	Huang et al 1994
LPS-induced proteolysis in the '*Limulus*' assay	Ooi et al 1991
LPS-induced expression of clotting-promoting tissue factor by monocytes	Meszaros et al 1994

LPS, lipopolysaccharide; LBP, LPS-binding protein; PMN, polymorphonuclear leukocytes; TNF, tumour necrosis factor.

Protection of animals against LPS and Gram-negative bacteria by recombinant BPI

The large-scale production of recombinant human BPI-55 and BPI-23 by the XOMA Corporation and the demonstration by our group at New York University and the scientists at XOMA that the N-terminal fragment rBPI-23 (residues 1–193) is at least as potent *in vitro* as the holo-protein has led to the testing in experimental animals of the protective capabilities of rBPI-23. Table 3 summarizes the results of such experiments carried out at the XOMA Corporation or elsewhere under their auspices. Two sets of protective effects of rBPI-23 have been studied, against isolated LPS administered intravenously or intraperitoneally and against inocula of live Gram-negative bacteria. BPI inhibits the responses of mice, rats, rabbits and pigs to isolated LPS (Ammons & Kung 1993, Kohn et al 1993, Lin et al 1994). In each species examined, BPI inhibits the lethal effects of LPS and its effects on a range of physiological parameters. Not unexpectedly, considering the highly variable sensitivity and response patterns of different animal species to LPS, the effects of BPI on the different biological endpoints studied also vary. In general, the protection by BPI *in vivo* parallels the inhibition observed *in vitro* on production and release of cytokines, arachidonate derivatives, reactive oxygen radicals, nitric oxide, monocyte tissue factor and on cellular adhesiveness (Table 2). The ability of BPI to block LPS effects *in vivo* is probably also in large measure attributable to its ability to compete with LBP, thereby preventing the delivery of LPS to cellular LPS 'receptors', apparently primarily CD14. The time window of protection is rather narrow, i.e. once a lethal dose of LPS has been injected into the circulation and has had the opportunity to signal its presence to host cells, BPI is less

TABLE 3 **Protection of animals by rBPI-23 against administered LPS or live** *Escherichia coli*

Challenge	Endpoint	Effect of BPI (5–10 mg/kg)
Endotoxin		
Mice	Survival	Increased
	TNF release	Reduced
Rats	Haemodynamics, TNF release and carbohydrate metabolism	Normalized or reduced
(acute focal infection)	Neutrophil accumulation, TNF release and nitric oxide release	Reduced
Rabbits	Haemodynamics, lung changes and fever	Normalized or reduced
(meningitis)	Neutrophil accumulation	Reduced
	TNF release	Reduced
Pigs	Neutrophil activation	Reduced
	Acute lung injury	Reduced
Live bacteria		
Rats (bacteraemia)	Survival	Increased
	TNF release	Reduced
Mice (peritonitis)	Survival	Increased
	IL-6 release	Reduced
	Clearance of bacteria	Increased
(pneumonia)	Survival	Increased
	Clearance of bacteria	Increased

IL-6, interleukin 6; TNF, tumour-necrosis factor.

effective, presumably because maximal (destructive) host responses have already been elicited. However, the administration of a lethal bolus of endotoxin in these experimental animal models differs from natural infections. Exposure to LPS generally follows a focal Gram-negative bacterial invasion accompanied by a more gradual release of envelope constituents, including LPS. Therefore, any protective effects of BPI against inocula of live bacteria are particularly pertinent to the possible therapeutic use of BPI. So far, testing of BPI in experimental animals given lethal inocula of various species of Gram-negative bacteria has been limited (Table 3) (Kelly et al 1993, W. S. Ammons & A. Kung, unpublished observations). It must be recognized, however, that it is not easy to mimic closely the clinical infections that lead to bacteraemia and generalized organ dysfunction. For example, in many animal models of bacterial infection,

the often large inoculum represents a more acute challenge than the clinically
more gradual progression from a localized to a systemic infection. In this
situation it is likely that Gram-negative bacteria and their envelope constituents
are released episodically from the initial nidus of infection (Danner et al 1991,
Bone 1991), perhaps allowing an agent such as BPI that can contribute to both
bacterial growth inhibition and the neutralization of LPS (either shed or as part
of the bacterial envelope) to be protective when infused over an extended time.
Thus, the results of studies in experimental animals do not necessarily predict
the usefulness of BPI in human Gram-negative bacterial infections; this must
await clinical trials that are now in the planning stage.

Acknowledgements

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DISCUSSION

Hultmark: I would like to ask about the extracellular activity of BPI. Are there high enough concentrations of BPI, constitutively or induced, to give a biological activity?

Elsbach: Yes. We have used the peritoneal cavity of the rabbit as a model for eliciting a local inflammatory process *in vivo*. In response to intraperitoneal injection of a sterile glycogen solution, polymorphonuclear leukocytes migrate into the peritoneal cavity and, concurrently, BPI, p15s and defensins accumulate extracellularly, while the so-called ascitic fluid becomes potently bactericidal. The bioactivity of this ascitic fluid is astonishing: very small amounts ($10 \mu l$) kill 10^6 serum-resistant (encapsulated) *E. coli*/ml. In this setting, all antibiotic activity against Gram-negative bacteria is abolished by anti-BPI serum. However, the amount of BPI in the ascitic fluid is not enough to account for the entire activity. The concentration of p15 varies from exudate to exudate, and from animal to animal; this varying amount of p15, in relation to what I said about synergy, can account for the actual activity observed. In other words, we can re-create the bioactivity of native ascitic fluid by combining concentrations of purified BPI and p15 that correspond to their concentrations in the fluid. The defensins are also present in ascitic fluid, but they are present in a high molecular weight complex and, in line with what Tomas Ganz has observed, in an apparently inactive form in this particular setting.

Boman: Is the mechanism of action of BPI stoichiometric or could it be catalytic?

Elsbach: I doubt that any of us can answer this question for any of the proteins and peptides that we have discussed at this meeting.

Boman: How quantitative is your assay for antibacterial activity?

Elsbach: We have binding studies that permit us to actually determine how many molecules of BPI are taken up from a given dose and how that relates to the biological effect. We also know approximately how many molecules are needed on the surface of each bacterium in order to get the bacteria to lose their ability to multiply. The total number of BPI binding sites per bacterium is between 1 and 2 million, corresponding to the number of LPS molecules in the envelope. We tend to view these multiple sites as predominantly non-productive sites. By analogy with the colicins and bacteriocins in the same M_r range as BPI, we believe that only a small subpopulation of sites is involved in the ultimate lethal effect of the protein.

Boman: I don't think there's anything special about BPI. That could happen to a number of molecules that show productive and non-productive binding. The surprising thing is that the number of molecules of BPI required is of the same order of magnitude as found for the helical antimicrobial peptides. Then one wonders what the advantage of having a 55 kDa molecule is when you can do the same thing with a molecule of between 3 and 4 kDa.

Elsbach: A very important aspect, of course, is affinity. If the affinity of agent A is 10- or 100-fold greater than the affinity of agent B, then which one will benefit from the possibility of finding the target depends on the concentration of that agent.

Secondly, the whole issue is very much related to the presence of other antimicrobial systems. None of these agents work in isolation: they are present in a very complex host-defence system in which some of these agents will be antagonistic, some will work together as indicated, and others will have no chance to do anything because they don't get to the target, because other agents have already occupied the available sites.

Boman: The next thing I think is important to pin down is the specificity for the target. Have you tried other lipids, such as cardiolipin or any other negatively charged smaller lipid that can be expected to have similar charge and compete with the binding of LPS? Is there anything that will inhibit the binding of LPS to BPI?

Elsbach: The target specificity of BPI seems well established. I reported that BPI, unlike most other antimicrobial peptides, is not toxic for Gram-positive bacteria or eukaryotic cells at manifold higher concentrations than those that kill Gram-negative organisms.

With respect to your question about agents that will inhibit binding of BPI to LPS: once again, this is an issue of affinities, or more precisely, relative affinities. I would not expect cardiolipin or other negatively charged smaller lipids to have the charge properties to compete effectively with the very strong electrostatic attraction of BPI for the dense cluster of negative charges in the lipid A region of LPS. Moreover, how would one present these lipid molecules in an aqueous milieu in a meaningful experiment? However, not surprisingly, we showed long ago that other highly charged polyanions such as heparin (but not all acid mucopolysaccharides) do inhibit interaction of BPI with *E. coli*. What

happens in a biological setting is clearly exceedingly complex, because targeting by bioactive molecules such as BPI or any antimicrobial polypeptide represents the resultant of multivectorial attractions provided by the relative affinities and concentrations of all the interactive molecules and surfaces in the environment. I hope that the experiments in plasma or whole blood, as well as those in whole animals that I have presented, have convinced you that BPI is not prevented from seeking out both LPS and intact LPS-carrying bacteria, even in the presence of so many potential competing entities.

Kreil: What is the mechanism of the synergism between BPI and phospholipase?

Elsbach: This is intriguing. Our concept is based on the highly asymmetric envelope structure in the outer membrane of Gram-negative bacteria where the phospholipids essentially occupy only the inner leaflet. The stability of the outer membrane depends on divalent cations—either magnesium or calcium, they're pretty much equivalent—that occupy the negative charges on the LPS molecules, preventing electrostatic repulsion among the lipid A regions of the LPS layer. BPI competes with these divalent cations and essentially drives the calcium and magnesium off, destabilizing the LPS layer. One can create conditions where either predominantly calcium or predominantly magnesium is present in the outer membrane. When calcium predominates and is released upon exposure to a membrane-perturbing agent such as BPI or polymyxin, the calcium provides the essential co-factor for strictly calcium-dependent phospholipases. The destabilization of the LPS layer also permits flip-flop of phospholipid molecules and hence formation of phospholipid bilayer regions. This explains why previously impermeant hydrophobic substances now come through (permeability-increasing effect of BPI) and why the phospholipids become accessible to (calcium-dependent) phospholipases.

Interestingly, only some members of the very conserved 14 kDa phospholipase A_2 family act on BPI-treated *E. coli*. We have shown that BPI-responsive enzymes share structural determinants in a variable N-terminal surface domain (residues 1–16). Among BPI-responsive 14 kDa phospholipase A_2s are both extracellular and intracellular host enzymes.

Kreil: But BPI also stimulates the endogenous phospholipase?

Elsbach: Yes, that's also a strictly calcium-dependent enzyme, apparently activated by the BPI-mediated alterations in the outer membrane (Elsbach & Weiss 1991, Weiss et al 1991).

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General discussion III

Mechanism of membrane permeation by cecropin B2

Gazit: The work I am going to describe here was done in the laboratory of Dr Y. Shai at the Weizmann Institute and is part of a collaborative study carried out with Drs P. T. Brey and W. J. Lee of the Institut Pasteur.

The antibacterial peptide cecropin B2, a member of the cecropin family of potent antibacterial peptides (Boman & Hultmark 1987), has been isolated both from immune haemolymph (Teshima et al 1986) and recently from the cuticular matrix of the abraded integument of the silk worm *Bombyx mori* (Brey et al 1993). In order to study the mechanism of action of cecropin B2, we synthesized the peptide chemically and labelled it selectively at its N-terminal amino acid with rhodamine or 7-nitrobenz-2-oxa-1,3-diazole-4-yl (NBD) fluorescent probes. The NBD fluorescent probe allows the localization of the peptide and is also used for binding studies, as its fluorescence spectrum reflects the environment in which it is found (Kenner & Aboderin 1971, Frey & Tamm 1990). The fluorescence emission spectrum of NBD-labelled cecropin displayed a blue shift upon binding to membrane vesicles, reflecting the relocation of the fluorescent probe to an environment of increased polarity. In buffer, NBD–cecropin B2 exhibited a maximum fluorescence emission at 545 nm, which reflects a hydrophilic environment for the NBD moiety. However, when vesicles were added to the aqueous solution containing NBD–cecropin B2, we saw a blue shift in the emission maximum (towards 530 nm) and an increase in the fluorescence intensity of the NBD group in the presence of acidic (phosphatidylserine [PS]/phosphatidylcholine [PC]) or zwitterionic (PC) vesicles. This blue shift suggests a shallow penetration of the N-terminus of the peptides into the phospholipid environment as was observed with the antibacterial peptide dermaseptin (Pouny et al 1992); this is different from the pore-forming polypeptides (emission maximum of 518–526 nm; Rapaport & Shai 1991, Gazit & Shai 1993a,b). This suggests that the NBD group is located at or near the surface of the membrane.

We derived the surface partition coefficients of cecropin B2 from binding isotherms that were obtained by titration of solutions containing NBD-labelled peptide with PS/PC or PC lipid vesicles as described previously (Rapaport & Shai 1991, Schwarz et al 1986, 1987). We found that cecropin B2 is bound much more strongly to acidic phospholipids than to zwitterionic ones, which correlates with its high potency in permeating acidic phospholipid vesicles. The shape of the binding isotherms (straight line with PS/PC and slightly bent upward with PC vesicles) indicates a simple adhesion process to PS/PC or a slight

self-association of peptide monomers in the presence of PC vesicles. The binding isotherms of NBD–cecropin B2 are similar to those obtained with the antimicrobial peptide dermaseptin (Pouny et al 1992), but are very different from those obtained with pore-forming polypeptides such as the antimicrobial peptide alamethicin (Rizzo et al 1987), the neurotoxin pardaxin and its analogues (Rapaport & Shai 1991), the α-5 segment of *Bacillus thuringiensis* CryIIIA δ-endotoxin (Gazit & Shai 1993a) and helix II of *Bacillus thuringiensis* var. *israelensis* cytolytic toxin (Gazit & Shai 1993b). The binding isotherms of the latter bend strongly upward, suggesting a process whereby peptides first incorporate into the membrane and then, once inside the membrane, aggregate to form a pore (Schwarz et al 1986, 1987).

We confirmed the inability of cecropin B2 to associate when bound to acidic PS/PC vesicles by resonance energy transfer experiments with NBD-labelled cecropin B2 (serving as a donor) and rhodamine-labelled cecropin B2 (serving as an acceptor).

Taking all these results together, the mechanism by which cecropin B2 permeates phospholipid membranes seems to be via membrane disintegration caused by the binding of peptide monomers to the surface of the membrane, rather than by the formation of transmembranal pores via a 'barrel stave' (Ehrenstein & Lecar 1977) mechanism. This proposed model, based on biophysical results in model membranes, is consistent with an *in vivo* study that suggests that cecropin A molecules have to be bound in such a way that allows them to form a monolayer on a susceptible bacterium in order to exert their activity (Steiner et al 1988). Recent studies suggest that this mechanism of action may be common to other antibacterial amphiphilic α-helical peptides, such as dermaseptin and magainin (Pouny et al 1992, Bechinger et al 1992). The biological basis for this proposed mode of action might be that non-cooperative binding to membranes allows the antibacterial peptides to diffuse efficiently into the inner bacterial membrane, which is assumed to be the target of antibacterial activity.

Flajnik: How do you couple the NBD fluorescent probe to the peptide?

Gazit: It's coupled by covalent bonding, using the solid-phase peptide synthesis procedure which Bruce Merrifield has developed (see Merrifield et al 1994, this volume), except that in the last step the Boc protecting group is removed, and the succinimide ester of the fluorescence probe is reacted with the peptide, followed by HF cleavage.

Boman: Which residue is it linked to?

Gazit: It's on the N-terminal amino acid.

Boman: I would like to make a general comment on our present state of understanding of the mechanism of action of antimicrobial peptides and the contribution of biophysics to this. It is quite obvious from work first done by Bruce Merrifield that cecropins form channels in artificial membrane bilayers (see Merrifield et al 1994, this volume). This has been shown subsequently for

magainin and defensin. The question is, how far can this kind of experiment be extrapolated to the action of the peptides on live bacteria? I think that the biophysical work is a good start, but the next thing to do is to show that there is specificity. Without a lipid specificity we are very far from the situation in the bacterial membranes. We have to explain—in the case of the cecropins, especially—why these peptides do not attack eukaryotic cells. They don't lyse erythrocytes or insect or mammalian cells in culture. Could this be because of the presence of cholesterol, as has been suggested by several people? Can we find any link between the specificity of the peptides for different bacterial strains and differences in lipid composition in artificial bilayer membranes? Can we find a correlation between the biophysical results recorded on one or other type of vesicle and the lipid composition? Until this gap is closed, I guess we should keep an open mind regarding the interpretations.

If the lytic action is extremely fast, how do we explain other phenomena that seem to occur equally quickly? Especially if your results now show that the cecropins do not show a cooperative effect, then we really have a puzzle. Why does the cecropin P1 lyse bacteria as effectively and as quickly as it does—how are we going to explain this?

Zasloff: That isn't really fair. The cooperativity as measured by fluorescence transfer is not evident, but if you do a simple concentration dependence experiment, and you use the permeabilization of a membrane as an endpoint, you can definitely see cooperativity. The coefficient of cooperativity, the Hill coefficient, can be anywhere between 2 and 4. So, there again, depending on the assay and the means you use to study it, you may or may not see cooperativity, so I don't agree with that interpretation.

Boman: I meant that there is a gulf that has to be filled in from one or another side in order to get a coherent picture of what is going on.

Merrifield: When Steiner calculated that you have about enough peptide to form a monolayer on a bacterial cell, he didn't know at all that such a monolayer exists—the peptide may not all be bound. He just suggested that there might be some explanation there (Steiner et al 1988).

Opella (1990) did solid-state NMR experiments to investigate whether the peptides are on the surface or located perpendicular to the lipid. How much of the opposite orientation can you see in such experiments? The bulk of the material, I assume, is as they find on the surface, parallel, but the few percent you would need to form channels (for pore formation) probably wouldn't be detectable.

Zasloff: That is correct—that's always the criticism.

Boman: You have said before that there may be productive and non-productive binding of cecropin on the surface of bacteria. But at concentrations that give about 50% killing, when you measure binding you will have components from some cells which are fully lysed and some cells which are more or less destroyed, and it's very difficult to tell which part of this bacterial mess

the peptides bind to. So I don't think it's the best system. If one could synchronize the peptide–bacteria reaction in such a way that everything occurred simultaneously, then it would be much better. But at the moment we probably have some cells where repair may be taking place and the point of no return has not yet been passed.

Sahl: With these spectroscopical methods we are simply not able to see the 0.1% or less of peptides which are in a transmembrane conformation and contribute to channel formation. Furthermore, we all have evidence that, *in vivo*, the peptides need a membrane potential in order to act. The good agreement between the planar membrane work and the results we obtained with intact bacterial cells in our experiments gave me confidence that a channel is formed. Using diffusion potentials we could show that you can actually induce channel formation by energizing the membrane and that individual lantibiotics have defined threshold potentials beyond which they are not active. You don't have energized membranes, hence there are no 'active' peptides, when you do solid-phase NMR.

Zasloff: There's a problem here. A lot of us, when we think of gramicidin and alamethicin, think of these beautiful, almost crystal-like, stable structures. With most of the peptides that we have played with—linear α-helical molecules—we don't see discrete channels. We can measure conductances of varying magnitude, which vary with peptide concentration and time. There are other ways to permeabilize membranes with amphipathic helices than through the formation of transmembrane channels; you could imagine peptide 'logs' floating hydrophilic face up, hydrophobic face down, aggregating and spreading to form peptide monolayers, which by their presence would disrupt the permeability of the bilayer. One of the problems that we face in truly understanding the mechanism by which these peptides act is that there isn't a simple technique with which to visualize high-order structures formed by these peptides in membranes. We can 'look' at the individual monomer, but we have a very difficult time catching the larger structures they must form.

Sahl: Yes, because they may also be too short-lived to be visualized. *In vivo*, things may be even more complicated because integral membrane components could take part in opening *holes* in the membrane (as opposed to channels or pores, which are really more defined structures).

Elsbach: There are additional variables that you have to contend with: membrane-perturbing agents (as most of these antimicrobial agents are, including those that do not directly affect the membrane potential) may create physical alterations such as rearrangements of the lipids, especially in asymmetric membranes. This would provide a background alteration against which you have to read whatever else you measured.

Gazit: Accumulating data in Dr Shai's (Pouny et al 1992) and other laboratories (Steiner et al 1988, Bechinger et al 1992) suggest that antibacterial amphipathic helices like cecropin, magainin and dermaseptin exert their activity

by a mechanism different to that used by the pore-forming peptides like alamethicin, δ-toxin or paradoxin, as Michael Zasloff has mentioned. The exact mechanism by which they exert their activity needs to be further characterized. Five or 10 years ago, the mainstream concept was that all the amphipathic helices act by the same mechanism, forming a transmembranal pore; now it seems that there are different mechanisms.

Boman: The biophysical measurements are of course made with a non-living system. If you look at the killing of bacteria, there is a repair mechanism operating. The effectiveness of this may depend on the medium used and, of course, on the target and the rate of synthesis of new targets. The only thing that cannot be repaired is lysis. A lot of other damage can be repaired.

Sahl: This is certainly right. It is pore formation *plus* several other things happening afterwards which makes up the final susceptibility of a given strain towards a given peptide.

I think the alamethicin system is indeed somewhat different, because alamethicin is not a charged peptide—not along the chain. It can be stable as a monomer in a transmembrane orientation without a potential—it can float around. This is not something I would expect from an amphiphilic peptide such as those we have been talking about.

Gazit: It's not charged, but it's amphiphilic in its nature, it's polar.

Sahl: Yes. But the flip-flop mechanism, which was proposed as a model for explaining how the voltage gating takes place, was actually based upon a transmembrane orientation of the peptide aggregates in the membrane in the absence of a potential. When you apply the potential you achieve a parallel arrangement of the helical dipoles, which opens a channel.

Zasloff: A colleague of mine, Bob Guy, has studied δ-lysine, another bacterial toxin (Guy & Raghunathan 1992). This is another amphipathic peptide that permeabilizes membranes. He's done some very pretty modelling work. He envisages this peptide to organize in the following way: the molecules lie as antiparallel arrays floating as if they were logs with the hydrophilic face up and the hydrophobic face buried. He can easily produce these sort of double-raft structures. It isn't inconceivable that in the presence of a voltage gradient, these rafts flip inward, penetrating the inner leaflet. You don't have necessarily a channel, but you have a system with a dipole moment that can be perturbed.

Merrifield: Is this reversible? Can you open and close the holes in the membrane?

Zasloff: I guess it depends on the repair processes.

Merrifield: That sounds more drastic.

Andreu: I would like to complicate the issue a bit further. Any mechanism of action one wishes to propose must also be able to explain the fact that shortened versions of antibacterial peptides, such as those described by Professor Natori and ourselves, are rather effective in killing bacterial cells, even though they cannot possibly span the full thickness of the lipid bilayer. For these 11,

12 or 15 residue peptides, one must certainly envisage some kind of multi-molecular association leading to productive membrane lysis; the mechanism for this event can be similar to that of the parent peptides, or perhaps substantially different. Unfortunately, results from channel-formation experiments with these short analogues are less clearly interpreted than those with larger peptides.

Boman: That was one of our reasons for reducing the size of our hybrid peptides, not only to obtain peptides that are cheaper, but also to try to pin down the minimum length that could still give channels in artificial membranes. The shorter cecropin–melittin hybrids may act preferentially by one mechanism on one bacterium. However, for other organisms there may be several mechanisms going on at the same time.

Sahl: Earlier, you asked why eukaryotic cells are not susceptible to these peptides. Couldn't it just be the differences in membrane potential, or let's say the proton-motive force.

Zasloff: We looked at the sensitivity of certain types of cancer cells. They are normally lysed at low peptide concentrations. However, when the cancer cells are depolarized, they lose sensitivity to the action of magainin (Cruciani et al 1991).

Sahl: I remember experiments where people tried to affect myocytes, which really have quite high potential; they were sensitive.

Hultmark: I've been wondering about the membrane potential. It seems to me like a very plausible explanation for the specificity of membrane-active peptides, but does this predict that nerve cells would be more sensitive to the action of these peptides than other cells?

Sahl: I would say so.

Zasloff: That's not necessarily true—all cells have potentials and nerve cells have regulated gates. Everybody is living with a potential gradient and it's just that these are highly regulated, ligand-gated potentials.

Natori: In the case of bacteria, the majority of the membrane potential is probably due to proton potential. The proton potential is formed during respiration, since the bacterial membrane has a respiration system. This situation is quite different from the membrane potential formation in eukaryotic cells. It is likely that the membrane potentials of eukaryotic cells are formed by various cations.

Sahl: But we depolarized the bacterial cell by adding a protonophore, CCCP (*M*-chloromethoxycarbonylcyanide phenyl hydrazone), and re-energized the cells by adding valinomycin, to induce K^+ efflux and get a diffusion potential. K^+ is getting out, the anions stay back, so you get a diffusion potential, and that was enough to promote the activity of the peptide. So it cannot be a specific proton potential—it's just the electrical potential.

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Potential therapeutic applications of magainins and other antimicrobial agents of animal origin

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Abstract. Magainins are a family of linear, amphipathic, cationic antimicrobial peptides, 21 to 27 residues in length, found in the skin of *Xenopus laevis*. They kill microbial targets through disruption of membrane permeability. They exhibit selectivity, on the basis of their affinity for membranes which contain accessible acidic phospholipids, a property characterizing the cytoplasmic membranes of many species of bacteria. Magainins are broad-spectrum antimicrobial agents exhibiting cidal activity against Gram-negative and Gram-positive bacteria, fungi and protozoa. In addition these peptides lyse many types of murine and human cancer cells at concentrations 5–10-fold lower than normal human cells. Because of their selectivity, broad spectrum, low degree of bacterial resistance and ease of chemical synthesis, magainins are being developed as human therapeutic agents. The most advanced candidate is MSI-78, a 22-residue magainin analogue. This peptide is currently in human Phase IIb/III clinical trials in studies intended to evaluate its efficacy as a topical agent for the treatment of impetigo. Preclinical studies have demonstrated that analogues of magainin exhibit activity *in vivo* against malignant melanoma and ovarian cancer cells in mouse models. Intravenous administration of several magainin analogues has been shown to treat effectively systemic *Escherichia coli* infections in the mouse.

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Animals are engaged in a constant battle with microorganisms. Over the past several years we have come to appreciate the rich diversity of simple substances—peptides in many instances—utilized by animals to kill microbes. Antibiotics are used in a variety of different physiological settings. The phagocytic cells of many animals contain antibiotic peptides within intracellular granules, providing the cell with a non-oxidative microbicidal capacity (Lehrer et al 1991). The epithelia of certain vertebrate and insect organs express abundant antibiotic peptides, protecting the epithelial surface from microbial invasion

(Zasloff 1987, Moore et al 1991, 1992, Diamond et al 1991, 1993, Jones & Bevins 1992, Oullette et al 1989, Samakovlis et al 1991, Brey et al 1993). In some animals, such as the horseshoe crab, the haemocyte, a circulating cell invested with clotting properties reminiscent of the mammalian platelet, aggregates at the site of a wound releasing antibiotics along with clotting proteins, simultaneously sterilizing and occluding the breach (Shigenaga et al 1990). The many different antibiotic agents and physiological systems in which they are expressed in large part constitute the division of an animal's host defence system described as 'innate immunity' (Zasloff 1992).

Our efforts have involved studies of antibiotic substances from several animal species, including frogs (Zasloff 1987), mammals (Diamond et al 1991) and, recently, sharks (Moore et al 1993). We have been involved both in basic studies of these classes of antibiotics and their biological systems as well as in their application as potential human therapeutics.

In 1987 the discovery of magainin was reported (Zasloff 1987). For several years we had been using the *Xenopus* oocyte for studies in the field of RNA expression. After surgical removal of its ovaries, the African clawed frog heals in the setting of the aquarium in a remarkably benign fashion, its sutured wounds closing without significant inflammation, with rarely any evidence of clinical infection. The healing process is strikingly different from the robust inflammatory response that might be expected in an animal capable of mounting inflammation. This observation initiated a series of studies that resulted in the identification in the skin of *Xenopus laevis* of two abundant antibiotic peptides, magainin 1 and 2. Analysis of the mRNA encoding these peptides predicted the existence of a polyprotein precursor containing several magainin segments and requiring endoproteolytic processing to yield the mature peptide (Zasloff 1987, Terry et al 1988). Magainin, along with several other functionally related antimicrobial peptides, was shown to be produced and secreted from the specialized neuroepithelial cell called the 'granular gland' (Bevins & Zasloff 1990, Soravia et al 1988, Gibson et al 1986, Giovannini et al 1987, Andreu et al 1985).

Since the initial discovery of magainin, several thousand synthetic analogues have been produced and studied *in vitro* and *in vivo*. Because of certain properties (chemical stability, ease of synthesis, potency against certain pathogens, etc.) several have advanced into various stages of drug development.

Antibiotics or host-defence agents?

Although magainins, as well as other antibiotic peptides from animals, are generally isolated on the basis of an antibiotic assay, it should be noted that many have been found to exhibit other biological activities. Properties such as the promotion of wound healing (Jacob & Berkowitz 1991, H. P. Ehrlich & S. F. Kelly, unpublished paper, 3rd Int Symp Tissue Repair, 1989, Kudryashov et al 1990, Murphy et al 1993), inhibition of adrenocorticotropic hormone

(ACTH)–receptor interaction (Zhu et al 1988), inhibition of protein kinase C (Nabayubashi et al 1990), stimulation of monocyte chemotaxis (Territo et al 1989) and modulation of G protein-linked signal transduction pathways (Mousli et al 1990) have been reported. We believe that several of these biological properties, unrelated to antibiotic activity, might be utilized in the complex biological milieu where these agents are normally expressed. In addition, these properties suggest that antibiotics from animal sources might exhibit interesting and potentially valuable constellations of pharmacological responses when administered as drugs. For instance, under certain circumstances it would be advantageous to utilize an antibiotic with wound healing promotion properties.

A brief overview of the mechanism of action of magainin

Magainin has a very broad antibiotic spectrum, exhibiting biocidal activity against many species of Gram-positive and Gram-negative bacteria (Zasloff 1987, Zasloff et al 1988, Levison et al 1993), fungi (Zasloff et al 1988) and protozoa (Zasloff 1987, Zasloff et al 1988, Gwadz et al 1989, Huang et al 1990, Schuster & Jacob 1992). In addition, early studies demonstrated the cytotoxic activity of magainin and synthetic analogues against a variety of human and murine malignant cells of haemopoietic and solid tumour origin (Cruciani et al 1991, Ohsaki et al 1992, Peck et al 1993, Baker et al 1993). Magainin was shown to kill rapidly bacterial (Zasloff 1987, Cuervo et al 1988) and malignant cells (Cruciani et al 1991), compatible with a mechanism involving disruption of normal membrane function. However, despite its membrane-disruptive properties, magainin and several of its related family members in *Xenopus* do not lyse human red cells nor circulating peripheral lymphocytes up to concentrations many fold above that at which bacteria, fungi, cancer cells or protozoa are lysed or killed. This selectivity distinguishes this class of peptide from other promiscuously lytic membrane-active peptides, such as melittin (Zasloff 1987).

Over the past several years, a general picture of magainin's mechanism of action has taken form. In aqueous solution magainin exists as a randomly shaped peptide (Marion et al 1988, Chen et al 1988, Williams et al 1990, Jackson et al 1992, Matsuzaki et al 1991). Because of its overall cationic charge, a result of the lysines scattered through the sequence, the peptide will bind electrostatically to a membrane displaying accessible anionic phospholipid head groups such as phosphatidylglycerol, phosphatidylserine, cardiolipin and phosphatidylinositol (Matsuzaki et al 1991). After binding to the membrane, magainin undergoes a dramatic secondary structural transition into an amphipathic α-helix (Marion et al 1988, Chen et al 1988, Williams et al 1990, Jackson et al 1992, Matsuzaki et al 1991), with one face populated by the side chains of hydrophobic amino acids and the other by the hydrophilic residues, including lysines. On interaction with the membrane, the peptide helix lies

parallel to the plane of the membrane (Bechinger et al 1991, 1992, 1993, Shon et al 1991, Milik & Skolnick 1993). The peptides do not, however, appear to disrupt alkyl chain packing in the hydrophobic phase of the membrane, suggesting that they do not bury themselves within a leaflet to any extent (Williams et al 1990). Precisely how these helical peptides, once associated with the membrane, organize into higher order structures, remains controversial. The peptides, visualized as 'logs', might aggregate into 'rafts' which grow increasingly larger with time and displace one leaflet of the bilayer, forming a 'peptide monolayer' (Guy & Raghunathan 1992, Durrell et al 1992). Indeed, *in vitro*, under conditions which favour hydrophobic interactions, magainin will spontaneously form macroscopic birefringent fibres, reflecting the propensity of this peptide to form higher-order structures (Urrutia et al 1989). As yet, no evidence exists that magainins form classical transmembrane channels as suggested for certain model ionophoric peptides (Lear et al 1988). However, it is still possible that a small number transiently orient this way in the setting of a membrane (Cruciani et al 1992, Duclohier et al 1989).

Regardless of the precise design of the higher-order structure that forms within the membrane, these peptides disrupt permeability (Westerhoff et al 1989a,b, Juretic et al 1989, Juretic 1990, de Waal et al 1991). Discrete ion channels form at low peptide concentrations, but seem to grow larger with time and increasing concentration of peptide in the membrane (Cruciani et al 1992, Duclohier et al 1989). The data suggest that magainin and its analogues simply form a continuous spectrum of 'holes' or imperfections, of sizes ranging from those of ions to small molecules, rather than a discrete series of precisely organized ion-sized pores.

The mechanism by which magainin kills its target cells almost certainly depends upon this membrane-disruptive property. Thus bacterial cells are depolarized almost immediately on exposure to these peptides (Westerhoff et al 1989b), as are cancer cells (Cruciani et al 1991). After a brief exposure of bacterial cells to peptide the effect can be reversed (Juretic et al 1989), indicating that the membrane permeability has been altered without actual disruption of the cellular membrane. Mitochondria are not physically lysed, but rather oxidative phosphorylation is uncoupled (Westerhoff et al 1989a,b). A similar effect is seen on exposure of spermatozoa to magainin (de Waal 1991). The precise event which causes cell death is not clear, but must be a consequence of the disruption of the permeability properties of the cell membrane, affecting the cell's potential gradient, osmotic regulation and transport functions.

The basis of selectivity—that is, the reason a bacterial cell is more sensitive than an erythrocyte to the action of magainin—is a reflection of the profound differences that exist in the design of the membrane surrounding the bacterial cytoplasm from that of plasma membrane surrounding 'normal' cells like the human erythrocyte. Bacteria, for reasons unclear, concentrate anionic phospholipids in their membrane within the outer leaflet of the bilayer (Voelker

1985). In addition, bacterial membranes lack cholesterol, a steroid which reduces the affinity of magainin for a phospholipid bilayer (Williams et al 1990). The erythrocyte membrane by contrast, contains very little anionic phospholipid; that present is localized principally in the inner leaflet of the cytoplasmic membrane oriented towards the cytoplasm (Voelker 1985). In addition, the vertebrate membrane contains a considerable proportion of cholesterol. Many types of cancer cells have been shown to exhibit 'bacterial-like' lipid organization in their cytoplasmic membrane (Utsugi et al 1991), thus we favour the hypothesis that an 'abnormal' membrane is the basis of these eukaryotic cells' sensitivity to magainin and other cationic antibiotic peptides. We speculate that all sensitive microbes, including fungi and protozoa, will share these commonalities in membrane design. In essence, antibiotic peptides such as magainin represent some of the simplest molecules which permit an animal to distinguish microbes from their own cells.

Consistent with the mechanism proposed, magainin retains its activity and selectivity when configured out of all-D-amino acids (Wade et al 1990, Bessalle et al 1990), in which case it forms a left-handed α-helix. Since neither loss of activity nor selectivity occurs with this enantiomer, it appears that magainin does not function by interacting with a chiral centre such as an enzyme or classical receptor. These experiments support a model in which self-interacting monomers organize into a higher-order structure leading to membrane permeabilization. Of particular importance to the development of these peptides as therapeutic agents, the all-D-peptides are not susceptible to proteolytic cleavage (Wade et al 1990, Bessalle et al 1990). Thus they will not be degraded in the body of an animal or in the gastrointestinal tract, obviating two major difficulties facing development of peptide antibiotics as drugs.

Biology of the magainin peptide family in *Xenopus laevis*

Magainin and other related peptides are stored and secreted from the granular glands present in the skin of *Xenopus laevis*. The amphibian granular glands are large multinucleated cells which share a common cytoplasm filled with large rice-shaped granules. These neuroepithelial structures accumulate many biologically active peptides and biogenic amines that have counterparts in mammalian neuroendocrine cells (Bevins & Zasloff 1990, Erspamer & Melchiorri 1980). When the granular gland is discharged, which occurs upon injury, if the animal is administered an α-adrenergic agonist or when stressful stimuli are imposed, its contents are emptied through a duct onto the surface of the skin. The granules, each of which contains all of the antibiotic peptide and neuropeptides synthesized by the structure (Moore et al 1991, 1992), undergo osmotic lysis on contact with the water bathing the skin surface. The skin is thus covered with a hydrophobic gel interspersed with peptides and hormones (M. Zasloff, unpublished results 1994). An endopeptidase ('magaininase') is also liberated

which inactivates the antibiotic peptides at some time after secretion (Resnick et al 1991).

The granular gland in *Xenopus* is present not only on the dorsal and ventral surfaces of the skin, but also within the epithelium which lines the animal's digestive tract (Moore et al 1991, 1992, Reilly et al 1994a,b). Scattered throughout the upper and lower digestive tract are cells ultrastructurally similar to the classical granular gland. They are the sites of synthesis, storage and secretion of the identical antibiotic gene products expressed by the outer skin glands. They appear to represent the amphibian version of the mammalian Paneth cell, a granular cell which lies within the crypt of the small bowel and has recently been shown to express antibiotics of the defensin class in mouse (Oullette et al 1989) and human (Jones & Bevins 1992). Thus, as has been suggested for the Paneth cell, the antibiotic-producing cells of the frog's gut appear to be designed to control microbial growth both in the lumen of the bowel and within the bowel wall itself, a function that might be required after injury to the bowel lumenal epithelium.

The magainin peptides are first expressed in abundance normally during metamorphosis (Reilly et al 1994b, Clark et al 1994). Their explosive expression in the animal is a reflection of the appearance of granular glands at this stage in development (Bevins & Zasloff 1990). Premature induction of metamorphosis by treatment of tadpoles with thyroid hormone results in the appearance of granular glands expressing antibiotic peptides (Reilly et al 1994b).

The epithelium as an active defensive barrier

The presence of a system producing abundant antibiotics from the epithelium that covers the external skin and lines the gut of *Xenopus* demonstrates that a vertebrate with a fully developed complex immune system (Du Pasquier 1982) invests its epithelial surfaces both externally and internally with cellular equipment designed to deliver high concentrations of very broad spectrum antibiotics locally. Almost every microbe or transformed cell that comes into contact with the concentration of antibiotic released will be killed. Furthermore, the system is in part regulated by controls exerted over secretion, an event modulated by adrenergic stimulation. It is therefore clear that in this animal the epithelium is protective not only by serving as a physical barrier, but through its investment with potent microbicidal agents. The epithelium must be regarded as actively 'defensive', capable of killing potential invaders. Similar expression of antimicrobial agents from epithelia is seen in the trachea of the cow and in the small intestine of both mouse and human, and probably will be observed in many types of epithelial cells in both plants and animals. Precisely how these systems are regulated is the subject of intense investigation.

Molecular diversity of amphibian antibiotic peptides

Over the past several years, we and others have explored the diversity of antibiotic peptides from various species of frogs (Zasloff 1987, Mor et al 1991, Gibson et al 1991, Simmaco et al 1991, Mignogna 1993). Of particular interest has been the observation that no two species of frog have yielded the same peptides. Even within *Xenopus*, the family of linear amphiphilic antibiotic peptides share little primary sequence homology at either the amino acid or nucleic acid sequence level (Bevins & Zasloff 1990). They are all cationic amphipathic membrane-active peptides, are all expressed in this animal within the granular gland, and are all processed by an enzyme that recognizes secondary structure in preference to precise details of primary sequence (Resnick et al 1991). The diversity of primary sequence of antibiotic peptides suggests that evolutionary pressure has been exerted on function rather than on sequence.

Although many species of frog express linear peptides, *Rana* appears to represent an exception. Several species, including *Rana esculenta* (Simmaco et al 1993), *Rana brevipoda* (Morikawa et al 1992) and *Rana catesbeiana* (Clark et al 1994), express peptides which contain a single disulphide bond, producing a peptide with a linear N-terminal segment linked to a C-terminal loop consisting of seven amino acids (including the two cysteine residues). The bullfrog peptide, which we termed 'ranalexin', exhibits homology in both primary and secondary structure with polymyxin, the membrane-active antibiotic peptide produced by a species of bacteria and used for many years as a therapeutic agent. The structural similarities between these two antibiotics suggest that parallel evolutionary processes have invested both bacteria and animals with similar selective membrane-disruptive agents.

Development of magainin as a potential human therapeutic agent

Because of the relative simplicity of the magainin peptide sequence, it has been possible to synthesize analogues readily by standard solid-phase techniques. Through considerable refinement of structure based on evaluation of anti-bacterial activity and selectivity, numerous analogues have been prepared which exhibit considerably enhanced activity when compared to magainin 2, with retention of almost comparable selectivity. In this section we shall describe several peptides which exhibit biological properties *in vivo* that suggest their potential utility as human therapeutic agents.

We will introduce four applications: as a broad-spectrum topical agent; as a systemic antibiotic; as a wound healing stimulant; and as an agent against cancer.

MSI-78: development as a broad-spectrum topical antibiotic agent

MSI-78 (GIGKFLKKAKKFGKAFVKILKK amide) is a 22 amino acid analogue of magainin 2, which evolved through extensive structure–activity studies of the natural peptide (W. L. Maloy & J. P. Kari, unpublished results 1994). This molecule exhibits potent antibiotic activity against a wide variety of Gram-positive, Gram-negative, anaerobic and fungal pathogens. When assayed against a range of dermal isolates recovered from several geographically distinct clinical centres, the potency appears to be uniformly excellent (Table 1).

In October 1992, MSI-78, formulated as a topical preparation, entered Phase I clinical trials, a phase of evaluation in which human safety is established. A good safety profile emerged and in February 1993 Phase II studies were initiated. These studies were designed to determine whether the formulated preparation of MSI-78 could effectively reduce dense bacterial flora indigenous to the perineal area from healthy human volunteers and to measure the effect of concentration of antibiotic on the response. In a study involving 45 subjects, application of peptide at concentrations ranging from 0.5% to 2.0% significantly reduced all species of bacteria and yeast by several orders of magnitude by 1 hour after application (Leyden et al 1993). The antibacterial effect of a single application persisted through 24 hours. On the basis of these findings, MSI-78 entered Phase IIb/III clinical trials in June 1993, to be evaluated as a topical preparation for the treatment of impetigo, a serious skin infection commonly seen in children. These trials are still in progress and will determine the clinical efficacy of several concentrations of the peptide formulated in a cream base.

TABLE 1 *In vitro* **activity of MSI-78 against key dermal pathogens**

Pathogen	MIC_{50}	MIC_{90}	Range	No. of strains
Gram-positive				
Staphylococcus aureus (including MRSAs)	4	8	2–16	30
Staphylococcus epidermidis (including MRSEs)	2	4	2–4	31
Group A β-*Streptococcus (Streptococcus pyogenes)*	4	4	2–4	23
Gram-negative				
Pseudomonas aeruginosa	8	16	2–16	37
Xanthomonas maltophila	4	8	2–16	18
Acinetobacter baumanii	4	4	2–8	12

Minimum inhibitory concentrations (MICs) are expressed in μg/ml and were determined as reported elsewhere (Leyden et al 1993). Bacterial strains were obtained from several clinical centres within the USA and were derived from infected dermal wounds. MRSA, methicillin-resistant *Staphylococcus aureus*; MRSE, methicillin-resistant *Staphylococcus epidermidis*.

Depending on the outcome of this study, future studies will evaluate the efficacy of this agent in other skin infections characterized by complex flora, such as infected diabetic ulcers, decubitus and venous stasis ulcers.

Magainin peptides as systemically administered antibiotics

It is now generally accepted that the emergence of bacterial resistance against many widely used antibiotics is posing an ominous threat (Cohen 1992). The existence of new classes of antibiotics of animal origin offer the hope that some might be developed into agents that can be used against organisms of medical importance. The extensive clinical use of polymyxin, a selective membrane-active peptide, clearly demonstrated that molecules which act by discriminating pathogens on the basis of differences in membrane design could be developed into systemically administered therapeutics. Unfortunately, polymyxin is associated with certain unacceptable toxicities, such as renal tubular damage, a result in part of our inability to metabolize this peptide (Storm et al 1977).

We and others have begun to explore the efficacy of animal-derived peptide antibiotics as systemically administered agents. In these experiments, bacterial infections are introduced in mice and the animals subsequently treated intra-venously administered antibiotics. Several antibiotic peptides have exhibited activity in the *in vivo* infection model and will be described in detail elsewhere (T. Williams et al, unpublished results 1994). The development of peptide antibiotics as systemic therapeutics will naturally lag behind their development as topical agents because of the extensive evaluation of toxicity required for this human application.

Magainin has been shown to act synergistically with certain 'standard' antibiotics in the chemotherapy of Gram-negative bacterial infections (Darveau et al 1991). In one published study, mice rendered immune-compromised with cyclophosphamide were infected with *Escherichia coli*. Magainin 2, which by itself exhibited no parenteral efficacy in this setting, enhanced the efficacy of cephalosporin, whose activity had been reduced in this compromised animal. Synergy between these two antibiotic agents was hypothesized to result from the permeabilizing effect of the peptide which disturbed the integrity of the bacterial outer membrane and facilitated entry of the antibiotic into the bacterial cell. The authors suggest that administration of an antibiotic peptide such as magainin might find use along with a traditional antibiotic in the treatment of infections in clinical settings where immune function has been depressed.

Because these peptides, like polymyxin, interact with bacterial endotoxin (Rana et al 1990, 1991, Rana & Blazyk 1991) they have an additional therapeutic property of potential value. Thus it is possible to rescue a mouse from endotoxin shock by administration of a peptide intravenously (T. Williams et al, unpublished results 1994). As human therapeutics, peptide antibiotics will be developed which exhibit both antibiotic and endotoxin-neutralizing activity, a

combination of properties that might be beneficial in the chemotherapy of bacterial infections.

Antibiotic peptides as wound healing stimulants

Numerous reports exist in the literature of antibiotic peptides of animal origin promoting wound healing (H. P. Ehrlich & S. F. Kelly, unpublished paper, 3rd Int Symp Tissue Repair, 1989, Kudryashov et al 1990, Jacob & Berkowitz 1991, Murphy et al 1993). A major challenge for drug development is to translate this property into a drug for human use. Several studies in animals have encouraged our interest in this area. In a study involving cortisone-treated rats, administration of magainin 2 intramuscularly at a site distant from a wound promoted significant acceleration of collagen deposition, recovery of wound strength and rate of wound closure (H. P. Ehrlich & S. F. Kelly, unpublished paper, 3rd Int Symp Tissue Repair, 1989). The mechanism remains unexplained.

Recent studies have suggested that certain magainin-related peptides promote corneal re-epithelialization in a well-established *in vivo* rabbit eye model (A. Weber et al, unpublished results 1994). In this model, corneal epithelium is chemically damaged by local administration of a long-chain alcohol. The rate of re-epithelialization is then monitored by use of a standard fluorescein method to visualize denuded areas. In animals treated solely with topical application of saline or with a biologically inactive peptide, 80% of the cornea re-epithelializes by 12 hours as is normally seen in untreated rabbits. In animals treated with an hourly application of MSI-420, a 14-residue peptide composed of all-L-amino acids, 80% re-epithelialization was observed by about 4 hours, representing a significant promotion in the otherwise efficient process. Administration of an all-D-peptide resulted in a similar response, suggesting that the acceleration of re-epithelialization was not a consequence of a receptor-mediated event akin to the interaction of a hormone with its receptor. In this animal model, the effects of peptide administration are too rapid to be explained by mechanisms involving cell proliferation. Rather, we propose that events such as cell spreading, epithelial cell recruitment and epithelial cell migration are the most likely processes stimulated. If wound healing promotion can be demonstrated in humans these antibiotics will clearly offer new therapeutic opportunities. One could imagine their use in clinical settings where they would be administered both systemically and topically. Future clinical studies will be designed to evaluate their potential as wound healing stimulants in humans.

Antibiotic peptides as anticancer agents

Magainin and several analogues have been shown to kill a variety of human and murine tumour cells *in vitro* (Cruciani et al 1991, Ohsaki et al 1992, Peck et al 1993, Baker et al 1993). The effect is rapid, comparable to the

bactericidal effect, and is best explained as a consequence of selective membrane damage.

Several animal studies have been conducted to evaluate the use of magainin peptides in cancer-bearing animals. These studies have determined the efficacy of these agents administered directly to the site of tumour, and represent models in which success or failure was not dependent on adequate delivery systemically of effective drug concentrations to the tumour cell mass.

An ovarian cancer murine model was evaluated to determine if magainin or an analogue would be effective in extending survival (Baker et al 1993). In this model, cells are introduced into the peritoneum of a mouse. Several days later, after the tumour has begun to proliferate within this compartment, a solution of peptide is introduced directly into the peritoneal cavity. The concentrations achieved locally are sufficiently high to lyse the tumour cells. Within minutes of introduction of peptide into the belly, tumour cells drawn from the peritoneum can be shown to have lysed and absolute numbers are reduced. After two injections, greater than 99% of tumour is eliminated from the peritoneum. Survival in this model compares with Adriamycin®, administered at its maximal tolerable dose. While Adriamycin caused pronounced myopathic damage in the treated mouse, the magainin analogue appeared to cause mild local peritoneal fibrosis. All-D-peptides resistant to degradation by proteases released on breakdown of existing tumour exhibited enhanced efficacy compared with their all-L counterparts.

Magainin analogues have been evaluated in animal models of human melanoma (M. Herlyn et al, unpublished results 1994). In this model, human melanoma was introduced subcutaneously into athymic nude mice. After approximately two weeks, when tumours of about 1 mm in diameter appeared at the site of inoculation, a small volume of MSI-511, a 14-residue peptide composed of all-D-amino acids, was injected at a concentration of 50 mg/ml in saline directly into the tumour. The logic underlying the experiment is simply that the melanoma, shown to be sensitive to lysis by MSI-511 *in vitro*, was exposed to supralytic concentrations of this peptide. The tumour would be expected to be destroyed to a greater extent than the more resistant surrounding tissues. Within several days of injection the area surrounding the tumour exhibited clinical evidence of local tissue damage, along with disappearance of the initial melanoma tumour. In about a month, the lesions healed without consequence in six out of nine animals. The animals were free of tumour locally and had no evidence of metastases. In contrast, in untreated animals the implanted tumour had grown to about a 200 mm³ mass with extensive metastatic disease evident.

These experiments highlight the relative sensitivity of tumour cells compared with normal tissue. The challenge facing drug development is to translate the interesting and provocative effects seen on local administration to a systemically delivered agent, since most human tumours metastasize and require systemic

chemotherapy. However, magainins and several other analogues might yet have utility in local chemical ablation of cancerous tissue, for example, in the treatment of basal cell skin carcinoma.

Discovery strategy

After the report of the discovery of magainin in 1987 our laboratory set out to search for antibiotics in other vertebrate species. We were guided by two very simple hypotheses:

   (1) Antibiotic substances might well be utilized in defence of the epithelium of vertebrates. At sites of contact between the epithelium and the outside environment, microbial insult is constant. A wound would become a portal for invasion. Since defence by neutrophils in mammals is usually associated with inflammation, in mucosal sites where we see little inflammation such as the tongue, GI tract, or the cornea, defence might well be complemented by expression of antimicrobial agents.

   (2) Antibiotics might be produced by vertebrates with 'primitive' immune systems, for example elasmobranchs and jawless fish. The work of Boman (1991) and others clearly demonstrated that invertebrates such as insects, which lack equipment of classical vertebrate immunity such as immunoglobulin and lymphocytes, do well in the fight against microbes. They possess a complex array of antimicrobial agents including cecropin and defensin-related antibiotic peptides (Boman 1991). In many instances these peptides circulate within the internal body fluids of the insect, induced by injury upon bacterial invasion. Might certain 'primitive' vertebrates utilize such 'systemic' agents as if they were making their own 'penicillin'?

   Driven by the first hypothesis, we began a search of mucosal surfaces of *Xenopus*, resulting in the discovery of an antibacterial system comparable to the skin (Moore et al 1991, 1992, Reilly 1994a). In addition, with Charles Bevins and Gill Diamond, we began to search for antibiotics in the tracheal epithelium of the cow, resulting in the discovery of TAP (Diamond et al 1991). Other work led to the discovery of defensins in the crypts of the human small intestine (Jones & Bevins 1992).

Squalamine

Guided by the second hypothesis, we began to look for the presence of antibiotic agents in a common species of shark, *Squalus acanthias*, and in 1993, we reported the discovery of squalamine (Moore et al 1993). The shark was chosen for study in part because of its reputation of being exceedingly hardy to infection (Sigel et al 1972), rarely succumbing to infection following surgery, its low reported prevalence of cancer and its unique gestational behaviour. The females harbour their pups for about two years after conception. The fetal sharks derive

nutrients from a yolk sac and are not in vascular communication with the mother. Perhaps on a daily basis, this animal evacuates her oviduct, eliminating fluid and waste surrounding the pups; she subsequently pulls in sea water, replacing her oviductal fluid. Thus her reproductive tissue as well as the fetal passengers are exposed to microbially contaminated sea water. This process suggested the existence of an extraordinary immune mechanism protecting both the adult, her reproductive tract tissues and her immune-naive fetuses. Furthermore, the shark exhibits a classical immune system considerably less responsive and 'plastic' compared with our own (Du Pasquier 1982); it appears to make only one class of immunoglobulin, namely, IgM (Marchalonis & Schulter 1990, Marchalonis et al 1993) and might not have T lymphocytes (Smith & Davidson 1992). It is exceedingly difficult to stimulate a specific antibody response with an administered vaccine (Sigel et al 1972). The immunoglobulin genes are organized as cassettes of single variable and constant domain genes not requiring the recombination that occurs in teleost fish and tetrapods, which provides a component of the antigen recognition diversity observed in these animals (Hinds & Litman 1986). The shark has very high circulating concentrations of so-called low affinity 'natural' antibodies, presumably reflecting the expression of these pre-rearranged immunoglobulin genes (Marchalonis et al 1993).

Thus, on the one hand the animal is clinically robust; on the other, it has a seemingly 'compromised' immune system compared with ours. It was our hypothesis that systemic antibiotics existed in this animal to complement its known immune equipment.

We searched various tissues of the dogfish shark for the presence of antibiotic agents, focusing first on the stomach, since we and others had successfully identified antibiotic peptides in the gastrointestinal tissues of *Xenopus* and several mammals (Oullette et al 1989, Jones & Bevins 1992, Lee et al 1989, Agerberth et al 1991). The antibiotic activity was purified by chromatographic methods and its structure deduced by fast atom bombardment mass spectroscopy and nuclear magnetic resonance spectroscopy (Wehrli et al 1993). Chemical synthesis subsequently confirmed its chemical structure (Moore et al 1993). Squalamine was shown to be a novel steroid (Fig. 1).

Squalamine is characterized by a 'flat' steroid ring system, a consequence of the A–B *trans* ring junction. It has hydroxyl groups on C-7 and C-24, the latter esterified by a sulphate, steroid modifications seen in the bile salts of many species of fish, amphibians and reptiles. Perhaps the most unique feature of this molecule, however, is the presence of the spermidine condensed to the C-3 position on the steroid backbone, yielding a steroid without precedent in vertebrates. Of the known steroidal natural products, the most closely structurally related substances are a family of cationic steroids isolated from certain medicinal plants and used therapeutically as antiprotozoan agents (Moore et al 1993).
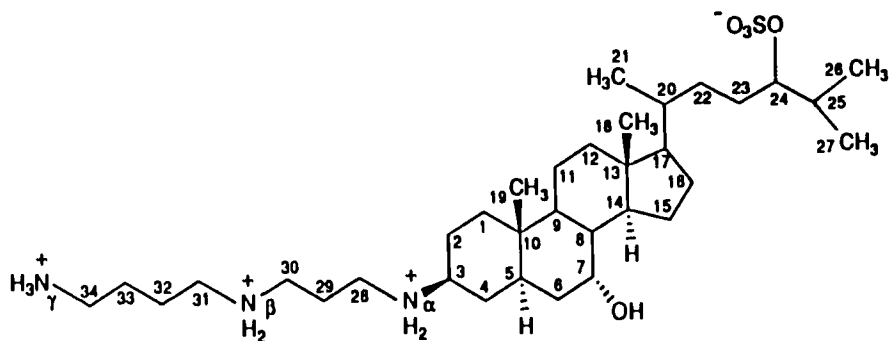
FIG. 1.   The structure of squalamine.

Although the mechanism of action of squalamine has not yet been determined, it is almost certain that it functions in part by disrupting the membrane permeability of the organisms it kills, since it rapidly lyses protozoa. As observed for the antibiotic peptides from animals, squalamine exhibits selectivity, killing microbes at higher concentrations than those required to lyse human red cells. Thus, we believe that squalamine discriminates sensitive and resistant cells on the basis of membrane design, very much like the mechanism proposed for the cationic membrane-active antibiotic peptides. The spermidine moiety of squalamine permits it to bind electrostatically to anionic phospholipid head groups of lipids disposed on the outer leaflet of a cell, resulting in the initial 'docking' of the steroid. Once on the membrane, squalamine is designed chemically to undergo a curious secondary structural transition. Molecular modelling demonstrates that the terminal amino group of spermidine and the sulphate attached to the steroid side chain can pair through a salt bridge to form a handle overriding the steroid scaffold. In this configuration the molecule has an amphipathic character, spatially segregating polar and hydrophobic atoms, reminiscent of the amphipathic α-helical structure adopted by magainin, for example, once it has associated with a membrane. In this 'basket' structure, squalamine can float within the membrane and disrupt permeability.

Squalamine can be isolated from many different tissues of the shark. Its richest source appears to be the liver and gall bladder (4–7 μg/g tissue), followed by spleen and testes (2 μg/g), stomach (1 μg/g) and gills (0.5 μg/g). As yet, we do not know the biosynthetic pathway of squalamine, nor the route by which it arises in each of these tissues.

Although the biological functions of this molecule are still to be elucidated, we have speculated that squalamine could be functioning within the shark as a true systemic antibiotic serving as an *in vivo* tissue 'preservative' capable of inhibiting a great variety of microbial invaders. Such a system would be expected to be regulated by both synthesis and metabolism. This substance, along with other simple antibiotics, would complement the existing immune system. Since

squalamine exhibits a very broad spectrum of antibiotic activity, killing bacteria, fungi and protozoa, this single substance could provide protection necessary for defence against a surprisingly diverse array of pathogens sharing little commonality.

Because of the nature of the steroid–spermidine condensation, we also suggest that steroid–polyamine antibiotics might be found in other vertebrates. A search for steroids with these properties or the enzymes involved in their biosynthesis is underway in our laboratory.

Squalamine represents an exciting candidate for drug development, a process which began after its recent discovery. The molecule is protease and acid stable, guaranteeing its survival in the upper gastrointestinal tract and offering opportunities for oral administration. Because squalamine is tolerated to substantial concentrations when administered orally to mice, and exhibits activity against *Helicobacter pylori* and certain pathogenic protozoa, it is being evaluated for the treatment of lumenal gastrointestinal infections. It exhibits broad-spectrum antifungal activity *in vitro*, encouraging its development as an antifungal agent. Because squalamine resembles a bile salt structurally, we suspect that it might be trafficked like a bile acid and thus be absorbed from the distal small intestine, opening the possibility of its development as an orally active systemic agent.

Phylogenetic exploration

Only a small fraction of the vast diversity of existing species, both plant and animal, has as yet been explored with regard to host defence systems. We expect that since every living thing must have equipment to defend itself from microbial consumption, many species will be found to express antimicrobial chemical entities, be they peptides or simpler molecules, and some will be suitable for drug development. To this end, a surprising number of small organic molecules with potent antibiotic activity have been isolated recently from marine animals, such as echinoderms (Service & Wardlaw 1984) and ascidians (Davidson 1993). Attractive antibiotics are those which exhibit limited toxicity, which are available in the substantial amounts necessary for preclinical studies, and which demonstrate therapeutic efficacy in appropriate animal models. Ideally, they should have chemical structures that permit chemical synthesis of analogues to allow development of molecules which optimize certain properties required in a drug. As the search for antibiotic agents in animals widens, we expect to see a broadening in our knowledge of the strategies used by animals in defence. We speculate that these studies should provide insights into as yet unrecognized mechanisms upon which mammals depend. Hopefully, these investigations will also yield chemicals that can be developed into drugs for the treatment of human disease.

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DISCUSSION

Ganz: The early developers of cationic antibiotics were concerned about nephrotoxicity. Has that been a problem when you have used these peptides in whole organisms?

Zasloff: That is obviously of great interest to us. We have decided to be very safe, so the first of the agents that has been widely tested, MSI-78, is not absorbed terribly well through the skin. Its structure is peppered with so many lysines that it really doesn't live very long in the bloodstream. Polymyxin, in contrast, a peptide antibiotic widely used as a systemic drug some years ago, is not degraded in the human. As a result it accumulates and leads to cellular membrane damage at various sites in the body, such as the kidney. When we begin to explore the systemic activity of these peptides we will see if we run into the same problem. I don't think we will, because many of the peptides we have are degradable, but as we begin to use all-D molecules, that will become an issue.

Andreu: What type of administration are you using for MSI-78?

Zasloff: MSI-78 is being developed as a topical agent, to be applied directly to open lesions. We have several other peptide antibiotics in preclinical development that exploit other plausible routes of administration. An antibiotic peptide, under development in collaboration with Colgate, has been shown to

inhibit plaque formation effectively when administered orally to Beagle dogs, demonstrating activity in the oral cavity. Another topical use being considered for one of our potent broad-spectrum antibiotic peptides is as a vaginal microbicide, for prevention of sexually transmitted diseases. Furthermore, as I discussed in my presentation, direct introduction of certain peptides into the peritoneum of mice can effectively treat ovarian cancer within the peritoneum. This application is a form of topical therapy, as well.

Elsbach: You have obviously a number of potential applications in mind for therapeutic use of your very numerous analogues. This implies an enormous spectrum of scanning and screening of different endpoints for different analogues. How do you systematically arrive at that kind of selection?

Zasloff: It isn't as intellectually challenging as you might imagine. MSI-78 was an analogue that emerged that had a broad spectrum which, when applied to rabbit skin, did not cause irritation. Once that molecule appeared in our hands we developed it; that was about two and a half years ago (subsequently, we have found many molecules that share that property). This molecule is also chemically stable, and can be made by solid-phase chemistry, solution-phase chemistry and by recombinant methodologies. That's very important, because you can't make an entity into a drug if it costs thousands of dollars a gram. At present, MSI-78 is being synthesized for something in the order of a few hundred dollars a gram. It will be produced at $50 gram, and by recombinant methods we hope to bring it down to $10 gram. That's $10 a gram for a 22 amino acid peptide. This has involved an enormous amount of work, and Bruce Merrifield has helped us in this. Several peptides, like 511, which is an all-D molecule, turned out in screening to have very effective *in vitro* activity. Many molecules were tested for systemic activity and we picked those that worked at a reasonable dose. Why they worked and others did not is not clear.

Ham: How does MSI-78 compare with existing treatments for similar problems?

Zasloff: That's a very important question: why waste your time developing a new drug if adequate therapies exist? For cancer that's not an issue. For complex wounds like diabetic ulcers, where there is no effective therapy, in many cases—where a single agent with this spectrum doesn't exist—people in the dermatology and infectious disease community are looking forward to being able to use this. But you could question whether or not it pays to develop this as a systemic antimicrobial drug. On the other hand, if five years from now we start to see patients with *Staphylococcus aureus* infections which are resistant to all existing drugs, then the situation may be different. This is beginning to happen in the United States. Some bacteria are completely multidrug (and vancomycin) resistant. Multidrug resistance in *Enterococcus* is approaching 18% (Cohen 1992).

Sahl: *Enterococcus* is vancomycin resistant, but so far *S. aureus* is not.

Zasloff: In Europe, doctors in France are scared to death about vancomycin resistant staphylococci—they have discovered a vancomycin resistant plasmid that is transferred from *Enterococcus* to staphylococci (J. M. Bader, personal communication). There may not be a lot published on this, nor is vancomycin resistance rampant yet, but regardless, resistance is emerging in many strains.

Boman: Once resistance has been picked up and is on a plasmid, it's going to spread throughout the microbial community, as has been happening for other drugs. If the antimicrobial peptides from animals can be shown to have multiple targets or multiple mechanisms of action, then the likelihood of resistance emerging is much reduced. The mutation rate in one target may be 10^8–10^9 and the likelihood for two independent mutations in two different targets is 10^{16}–10^{18}; this simply does not happen.

Andreu: Could anybody speculate about the appearance of resistance to peptides such as those Michael Zasloff has described? Suppose that the clinical trials are successful and that MSI-78 starts being administered as a human therapeutic: how likely is it that resistance might emerge within a few years?

Zasloff: This is a very complex problem. As a drug starts to hit human populations you start to screen and subject all sorts of flora to the agent. There is very limited resistance to polymyxin, and MSI-78 is similar to polymyxin in many ways. With nisin, for example, very little resistance exists.

I should also point out that there really are a limited number of antifungal drugs that exist, and there is a place, I think, for intravenous antifungal therapy. Amphotericin has terrible toxicity, although advances are being made in the delivery of that drug.

Kreil: You mentioned that you have used some of these analogues to combat infections in the oral cavity. I was told that farmers in Southern Italy put a frog, *Bombina variegata*, into their mouths when they have an oral infection. I think this is one of the reasons why Maurizio Simmaco, Donatella Barra and their colleagues at the University of Rome started working with this species.

When the Italian scientists isolated and characterized some of these bombinins, it turned out they are very active against *Staphylococcus aureus* and other staphylococci (Simmaco et al 1991).

Boman: It is my opinion that the notion of a pathogen is one that has been invented by doctors. In Nature there are a few obligate intracellular pathogens which cannot grow outside the cell, like *Rickettsia* or *Chlamydia*, for instance. But there are many microorganisms that belong to the natural flora and these are important. When a natural organism, for some reason, begins to grow in a niche where it isn't supposed to be, it can easily become a pathogen. Many pathogens also have avirulent forms, and they may not have evolved as animal parasites. Just as antibiotic resistance is often plasmid borne, there are many virulence factors located on plasmids. If such a plasmid is lost, you are back again to an avirulent strain. The normal flora of bacteria on the skin, mouth and gut must somehow be kept at a steady state. My view is that antimicrobial

peptides in animals play an important role here. It is also clear that, in many cases, resistance to immune mechanisms is the single most important property for creating a pathogen. Infections are always dynamic affairs. They are races between the rate of synthesis of the immune substances versus the growth rate of the pathogens—the fastest process wins. One of the advantages of the peptide antibiotics is their fast rate of synthesis. They also have simple and efficient control mechanisms if you compare them with those needed for immunoglobulins or the complement system. Another important thing is that peptide antibiotics require a minimum number of genes; maybe only 3–4 structural genes are needed for the peptide and its processing and amidation. I don't know how many genes are involved in the synthesis of squalamine, but I should imagine it's in the order of 10–20.

Zasloff: Yes, but we do have considerable amounts of cholesterol and spermidine around readily available as precursors. Thus the synthesis of squalamine might only require the action of the few enzymes needed for steroid modification and polyamine condensation.

Boman: But if you consider the efficiency, that is, the cost to the organism of producing a defence system, this favours the antimicrobial peptides. We have learnt from immunology the importance of recognizing self and non-self, and to sort out self-attacking cells by one or another procedure. A simpler way to avoid self damage is to direct activity against a target that is not present in self. Another advantage is the size: peptides are small molecules with faster diffusion rates than larger molecules like immunoglobulins or whole cells, even if chemotaxis of cells can make up for part of this difference.

Flajnik: Has anyone tried to make bugs resistant to these peptides in a classical way?

Zasloff: This has been studied extensively by Eduardo Groisman at the Washington University, St Louis. He has, by applying insertional mutagenesis to *Salmonella* strains, identified certain loci that increase an organism's sensitivity to the action of cationic antimicrobial peptides. These loci include a gene that encodes a protein that looks like an ATP-dependent peptide transporter, such as pheromone transporter STE-6 (Parra-Lopez et al 1993). In addition, Groisman and colleagues had shown previously that mutations within the *PhoP* locus in *Salmonella* are associated with hypersensitivity to magainins, cecropins and defensins, along with decreased virulence *in vivo* (Groisman et al 1992). This locus encodes a transcription factor that regulates an operon activated when an organism is exposed to certain types of environmental stress, such as low pH. The precise gene product responsible for antibiotic resistance in this operon has not been identified. In addition, changes in the structure of the outer membrane of *Salmonella* have also been shown to confer some level of resistance (Rana et al 1991).

Boman: You can increase sensitivity, but that is not the same thing as resistance. Inga Siden showed that in *E. coli* there is a locus that affects solely

sensitivity to cecropin D, which is the least potent of the cecropins (Siden & Boman 1983). This suggests that the action of cecropin D is different to that of cecropins A and B.

Casteels: We have one *E. coli* mutant which I picked up on the basis of its resistance to bee apidaecin. This mutant is resistant to one type of apidaecin, but is still sensitive to the other type. We picked it up looking at the mutation frequency, which was around 10^{-8} for *E. coli*.

Boman: I think that makes sense, because if your D-form is inactive, it is likely that you have a target for the L-form, which is a protein. Then, by a relatively large probability, a single-step mutation can change that target in such a way that peptide binding is sufficiently reduced to give resistance. But something like that is unlikely to happen for a lipid target, I would say.

Sahl: We have done some experiments concerning development of a resistance mechanism for the lantibiotic Pep5, but I really don't know if one can generalize these results. When we treated 10^9 bacteria in broth with a fairly high amount of Pep5, the number of colony-forming units rapidly decreased to 10^3–10^5. We incubated these further and observed bugs growing up again after 20–30 h. This means we didn't kill all the bacteria and a few survived. They started growing again and reached a population size of 10^9–10^{10}, roughly. If you take these bugs and treat them again with Pep5, they are not sensitive. But when you do several passages on a plate, and then treat them with Pep5, they are sensitive again. Initially, when you plate them, the colonies behave differently from what you would normally expect. They are somewhat slimy. We looked at them under the electron microscope and to me it seemed like they had made a surface layer of protein. They lose this when there is no selection pressure (Sahl et al 1987).

Zasloff: As part of the necessary bacteriology that went into the characterization of MSI-78, extended passage subculture studies of this type were done on this molecule. These were done in cultures of *Staphylococcus aureus*. Cultures were maintained at some fraction of the minimum inhibitory concentration, repeatedly subcultured for seven days and no resistance emerged. If you do this with gentamycin, for example, you generally see resistance emerge as you would expect for a mutation.

Boman: We have done the same thing for PR-39, but our interpretation is slightly different. After growth with PR-39 we saw no colonies at all, but we saved the plates and left them in the corner of the lab. A couple of days later, colonies began to reappear. From these colonies a few clones were isolated and tested for genetic markers and for resistance. They were all like the parent strain.

Our interpretation was that either the reaction is reversible and the peptide diffuses away after dilution, or the cell has a repair mechanism. From the medical point of view, lytic agents offer an advantage, because with lysis there is a point of no return for a bacterium. With non-lytic mechanisms there are clearly points of return.

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Cammue: In trying to develop an antimicrobial agent like MSI-78, would it be logical to try to generate a resistant pathogen before one emerges in Nature, so that you can stay a step ahead and study the resistance mechanism?

Zasloff: When you screen clinical isolates broadly, and you see no isolates which exhibit resistance, that ultimately makes you happy; if you see a great deal of resistance you should be concerned. It would clearly help us understand a great deal more about how these molecules work to discover resistant strains, so as a scientist it's unpleasant not to have resistance, but when one is trying to develop a therapeutically useful antibiotic, the absence of resistance is very favourable. The real truth will come when this drug is put on a great many different people and when it is exposed to the organisms in the environment.

Cammue: Have you tried to generate resistant strains?

Zasloff: We've tried in *E. coli* and *S. aureus*, and have failed.

Kreil: I have noticed that many of these amphipathic antimicrobial peptides contain glycine as the N-terminal residue. The amino group of glycine at this position has a lower pK value than other amino acids (Wilcox & Eisenberg 1992). I assume that in your studies on numerous magainin analogues you have also changed the N-terminal residue, which is glycine in the natural peptide. What is the biological activity of magainins containing not glycine but other amino acids at the N-terminus?

Zasloff: I don't know what the patterns would be.

Ganz: One possible mechanism for resistance is that the lipid composition of the bacterial membrane might change. For example, in eukaryotic cells, you can change widely the lipid composition of the membrane by feeding the eukaryotic cells in culture with different things.

Zasloff: I believe that's why *Serratia* as a species is so resistant to many of our peptides. Very few of our peptides can handle *Serratia*. *Serratia* has very little anionic phospholipid in its membrane; it has a much more animal-like membrane (Kates 1986).

Boman: But *Serratia* also makes quite a number of proteolytic enzymes.

Zasloff: So does *Pseudomonas*.

Boman: We have mutated *Serratia*, and that changed the susceptibility to cecropins.

Zasloff: Are cecropins active against *Serratia*?

Boman: Yes, but that was a special strain of *Serratia* isolated from sick *Drosophila*.

Kreil: I have a question concerning the peptide analogues containing only D-amino acids. Are there any known bacteria that can use all-D-peptides as the sole carbon and nitrogen source? In other words, do enzymes exist that would degrade such a peptide? I remember reading a paper on a D-amino acid-specific aminopeptidase (Asano et al 1989), but I do not know whether endopeptidases that can degrade all-D-peptides have ever been found.

Ganz: Are there D-oxidases too?

Kreil: Yes, there are D-amino acid oxidases, but as far as I know, these enzymes only degrade free amino acids. We are talking about peptides containing 20 or more amino acids, all in the D form. I just wonder whether there are microorganisms that could grow in a solution that contains just all-D-peptides.

Natori: Does the 511 peptide show cytotoxicity *in vitro*?

Zasloff: Yes, and it lyses a pretty broad range of melanomas at about 1–2 μg/ml.

Elsbach: With respect to squalamine, does the polyamine tail make it less hydrophobic? In other words, where is it found in the tissue—is it part of the membranes?

Zasloff: That's a very hard question to answer. The polyamine tail does not make this molecule more hydrophobic, it actually makes it more hydrophilic. Interestingly, the sulphate makes the molecule more hydrophobic (the presence of the sulphate causes this molecule to elute more hydrophobically from an HPLC column). It's also interesting that this molecule elutes precisely in the same position as magainin, defensin, cecropin and tachyplesins from our HPLC columns. So it has physical properties very similar to other antimicrobial peptides.

Elsbach: Do you think that the salt bridge is broken on the HPLC?

Zasloff: No, I think it's retained.

Ganz: Is squalamine actually a peptidomimetic, and is there a way of taking any one of the peptides that we've talked about and converting them logically to a peptidomimetic? Is there such a thing as a peptidomimetic that can be made by design, from any of the peptides that we have talked about?

Zasloff: That's a wonderful question.

Squalamine is an example of a peptidomimetic version of an antibiotic peptide. As a cationic amphipathic molecule it recognizes targets similar to those recognized by antimicrobial peptides. Its chemical structure suggests that it should exhibit no secondary structure in water, but in a hydrophobic environment, in contrast, it should adopt a basket-like structure, in which the steroid side chains bearing anionic and cationic groups form a hydrophilic handle overriding the hydrophobic steroid basket. In this configuration, the molecule would possess a strong amphipathic structure, segregating hydrophilic and hydrophobic groups, and conferring membrane-disruptive properties. Until squalamine finds and interacts with a receptive membrane, however, its detergent character would still be 'latent'. I don't believe we could have ever logically conceived this chemical entity. Nature's creativity is simply astounding.

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Primitive vertebrate immunity: what is the evolutionary derivation of molecules that define the adaptive immune system?

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Abstract. The adaptive immune system is capable of responding to an infinite number of antigens with the antigen-specific receptors immunoglobulin (Ig) and the T cell receptor (TCR). Ig binds soluble antigens while TCR recognizes antigen bound in clefts of polymorphic self-encoded major histocompatibility complex (MHC) class I and class II molecules. All of these molecules are wholly or partially composed of Ig superfamily domains. TCR and Ig use V-set Ig superfamily domains, always in heterodimeric forms, in antigen recognition. Although the ways in which TCR and Ig bind antigen are fundamentally different, the structure of the heterodimeric V domains is probably identical. The antigen-binding cleft of MHC proteins has a structure unlike Ig superfamily domains, although several investigators have proposed that this cleft is evolutionarily derived from Ig domains. We believe the MHC cleft is a primitive structure, perhaps related to the peptide-binding domains of intracellular chaperone proteins. A model is proposed whereby chaperone proteins were the primordial MHC molecules, presenting peptides derived from invariant proteins residing inside cells for recognition by lymphocytes with minimally diverse receptors. Such a system may be reflected today by the epithelial immune system, apparently governed by monomorphic MHC molecules and lymphocytes with unconventional antigen-specific receptors.

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The defining molecules of the vertebrate adaptive immune system enable the body to recognize an enormous number of pathogens (reviewed in Paul 1993). Lymphocytes, classified as T cells and B cells, use as their antigen-specific receptors the T cell receptor (TCR) and immunoglobulin (Ig), respectively. The two molecules have a similar structure, with Ig superfamily V domains (Williams & Barclay 1988) associating as heterodimers to recognize antigen. TCR and Ig also possess Ig superfamily domains of the C1 set that are involved in either effector functions or biosynthesis.

    The major difference in antigen recognition by Ig and TCR is that the former binds to antigens in solution while the TCR can only bind antigens that are physically associated with self major histocompatibility complex (MHC) proteins. Thus, Ig is the most important component of the humoral immune system, binding to pathogens in extracellular fluids, and TCR guides cytotoxic lymphocytes of the cell-mediated immune system to destroy self cells harbouring intracellular pathogens. One idea is that TCR proteins coevolved with their MHC recognition structures such that they became unable to bind soluble antigen. The alternative hypothesis is that TCR and Ig are both derived from monomorphic cell surface receptors that first became capable of recognition of a polymorphic ligand and later evolved to become secreted after lymphocyte stimulation by antigen (Davis & Bjorkman 1988).

    The MHC encodes two classes of cell surface proteins (class I and class II) with very similar tertiary structures. Both types of MHC molecules have a membrane-distal cleft composed of a floor of β-strands upon which rest two long α-helices, and two membrane-proximal domains that are members of the C1 set of the Ig superfamily (Bjorkman et al 1987). There have been several recent reports of high-resolution structures of MHC molecules bound to particular peptides (Matsumura et al 1992, Madden et al 1993). Class I molecules, ubiquitously expressed and, in general, presenting antigen to CD8^{+} killer T cells, tightly bind peptides of eight or nine amino acids. Seven extremely well-conserved amino acids at either end of the cleft 'lock in' the peptide by hydrogen bonding with backbone atoms of the octamer or nonamer (Madden et al 1991). Class II molecules, found on the surfaces of B cells and antigen-presenting cells, present antigen to CD4^{+} regulatory cells. Peptides associated with class II molecules are not bound tightly at their N- or C-termini and can therefore stick out on both ends of the cleft (Brown et al 1993). Class II molecules bind peptides in cellular endocytic compartments, while class I molecules bind peptides early in their folding pathway in the endoplasmic reticulum (ER) (Germain & Margulies 1993). In summary, class I and class II molecules are practically identical in their tertiary structure and the function of both molecules is to present antigenic peptides to T cells, but the tissue distribution, the types of T cells that employ one or the other as restriction elements and the cellular compartments where they acquire peptides are quite distinct. Another property shared between the classical class I and class II molecules is the extreme polymorphism found within a species. Almost all of the diversity between alleles is found in those amino acids whose side chains point into the cleft to interact with peptide.

Phylogenetic distribution of Ig, TCR and MHC

Ig and MHC molecules have been described in organisms as evolutionarily primitive as cartilaginous fish, suggesting that the basic components of the adaptive immune system arose at least 450 million years ago (Kasahara et al 1992).

The only extant representatives of the most primitive class of vertebrates, lampreys and hagfish, are very poorly characterized for adaptive immune function (Papermaster et al 1964), and have not been reported to possess any molecules that are members of the Ig superfamily—not to mention Ig, TCR or MHC. Proteins from hagfish previously described as antibodies are either poorly characterized or have been found to be other molecules, e.g. the complement component C3 (Fujii et al 1992). Thus the adaptive immune system arose either with the ancestor of cartilaginous fish and all other vertebrates, or in an earlier ancestor whose extant descendants have not yet revealed their secrets. This puts comparative immunologists in the unfavourable position of so far not being able to study molecules or mechanisms that might be intermediates during the formation of the adaptive immune system.

MHC and chaperones

Over the past four years, we have been analysing the Ig and MHC genes of ectothermic vertebrates. The first class I molecule isolated from the amphibian *Xenopus* was found to have sequence similarity in the putative peptide-binding region to members of the heat shock protein 70 (Hsp70) family (Flajnik et al 1991a). Hsp70 molecules were first discovered by their induction after heat shock or other types of stress to the cell (reviewed in Gething & Sambrook 1992); it was subsequently found that injection of denatured protein into cells could induce Hsp70 expression, suggesting that the role of these proteins might be to prevent aggregation of cellular proteins in times of stress (Pelham 1988). Later, constitutive homologues of the inducible proteins were found in the cytoplasm (Hsp70) and in the ER (BiP or Grp78). The prevailing paradigm is that the Hsp70 family members act as 'chaperones' capable of associating with proteins either to assist indirectly in their folding or to escort them across membranes.

Hsp70 molecules have been found in all organisms so far studied, including prokaryotes and eukaryotes. They are composed of at least two functional domains: an N-terminal region that binds ATP and a C-terminal part believed to interact with protein substrates. In addition, Hsp70 can clearly bind to short peptides (Flynn et al 1989). The similarity we detected with the *Xenopus* class I cleft sequence was in the region of Hsp70 believed to interact with proteins. Subsequent analyses of hydrophobicity and secondary structure predictions, and mapping of the Hsp70 homologous region onto the crystal structure of class I, further supported the idea that the peptide-binding regions of Hsp70 and MHC class I molecules might be homologous (Flajnik et at 1991b). We proposed that the peptide-binding region of MHC was a preexisting structure that had evolved to interact with other proteins. We further suggested that if the previous statement were true, class I molecules were likely the first MHC molecules to appear in evolution since the peptide-binding regions of both Hsp70 and MHC class I, in contrast to class II proteins, are formed as intramolecular dimers.

Four pieces of evidence support the idea that the peptide-binding regions of class I and Hsp70 molecules are homologous. First, inducible forms of Hsp70 are linked to the MHC in mice (Gaskins et al 1990), humans (Sargent et al 1989) and *Xenopus* (Salter-Cid et al 1994). Thus, the genetic association of the two gene families extends back at least 300 million years. We suggested that the *Hsp70* genes could have donated exons encoding the peptide-binding region to an Ig superfamily domain exon to give rise to primordial class I genes. Second, peptide binding studies with the chaperone BiP have suggested that, like MHC class I, peptides with a minimal size of seven amino acids are likely to bind to BiP in an extended conformation (Blond-Elguindi et al 1993). Unlike class I, there are no 'anchor residues' found in peptides that bind to BiP, but there is a propensity for certain amino acids to be included or excluded from the binding site. Since Hsp70 molecules must bind to regions of whole proteins, whereas MHC most likely can only bind to short peptides, it is not surprising that the peptide-binding region has diverged considerably in the two types of proteins. Third, chaperones, including Hsp70, derived from tumour cells, can prime mice to reject tumours (Srivastava & Maki 1991), suggesting that there may be a 'relay line' of peptide-binding proteins that transfer peptides from cytosol to the ER, where class I molecules are finally charged and enabled to complete their folding. Such results have prompted Srivastava and colleagues to propose that there is a 'certain symmetry' between molecules involved in peptide transfer to class I molecules; they incorporate our finding of Hsp70/MHC similarity in their theory to suggest that all chaperones in the relay line might share a peptide-binding ancestor. Fourth, until recently, all known members of the Hsp70 family were clearly intracellular. However, a sperm receptor on the cell surface of sea urchin eggs has now been discovered that shows definitive homology to Hsp70 (Foltz et al 1993). The region of homology encompasses most of the ATP-binding domain and what, in our model, would be one half of the peptide-binding region, ending precisely on the major turn at the beginning of the other side of the peptide-binding region. This sperm-binding molecule is proposed to have both carbohydrate and protein specificities and is postulated to signal the onset of fertilization (Foltz & Lennarz 1993). This result is exciting and certainly fulfils one requirement of our model: that Hsp70 molecules can be recruited evolutionarily to be extracellular, transmembrane proteins; it does not take much more imagination to propose that MHC proteins could have arisen in a similar fashion. However, because the sperm receptor only has half of the proposed peptide-binding region, the 'class I-first' argument is called into question, although binding to a cell surface ligand on the sperm head may require a more 'open' binding site for interactions.

In summary, there are several pieces of circumstantial evidence to support the idea that the peptide-binding regions of Hsp70 and MHC molecules are homologous, including the binding of peptides by both molecules in an extended conformation, the capacity of both proteins to shuttle peptides intracellularly,

a genetic linkage of the two families and a newly found member of the Hsp70 family that is present extracellularly. Thus, my collaborators and I would stand by our assertion that members of the Hsp70 family are indeed forerunners of the MHC molecules (Flajnik et al 1991b).

MHC class I plasticity

In addition to the polymorphic class I and class II molecules described above, there are in all vertebrates so far studied non-classical class I molecules, called class Ib molecules. The genes encoding these proteins may or may not be encoded by the MHC; they may either be ubiquitously expressed like the classical class I or have a limited tissue distribution; and they can vary greatly in number in even closely related species (Hedrick 1992). Class Ib proteins are also capable of binding peptides, although not always in the same manner as polymorphic class I. The one characteristic (so far!) that distinguishes class Ib unambiguously from classical class I molecules is that there is no selection for changes in amino acids whose side chains point into the cleft to interact with peptide. In fact, it has been proposed that there is actually a selection against change in this region, suggesting that the non-classical class I molecules might then have a propensity to bind relatively conserved peptide ligands (Teitel et al 1994).

This prophecy has been fulfilled for at least one class Ib molecule. The non-polymorphic M3 class I molecule was discovered because of its ability to bind to a mitochondrial-derived peptide termed Mta (maternally transmitted antigen; Fischer-Lindahl et al 1980). Further work demonstrated that M3 could only bind peptides containing *N*-formyl methionine, a property of the N-terminus of bacterial and mitochondrial proteins. Subsequently, M3 was shown to present peptides from intracellular bacteria (*Listeria*) to cytotoxic T cells (Kurlander et al 1992, Pamer et al 1992). This was the first definitive example of a class I molecule apparently designed for a particular function.

Results obtained over the past five years suggest that several of the class Ib proteins are expressed in epithelial surfaces such as the skin, gut, uterus and lung, and that these class Ib molecules are involved in 'first-line defence' against pathogens using these common routes of admission into the body. Consistent with this hypothesis is the finding that these class Ib molecules apparently bind conserved peptides derived from pathogens, specifically peptides contained in evolutionarily conserved heat shock proteins. Furthermore, Hsp peptides can influence the biosynthesis of non-classical class I molecules, probably by stabilizing class I proteins during their biosynthesis (Imani & Soloski 1991). Binding to such conserved regions of pathogens is consistent with the hypothesis that the non-classical class I binding site is not selected for diversity and suggests that a peptide displayed by a class Ib molecule can be from a region of an indispensable protein that cannot change its primary sequence, thus making it impossible to avoid generating an immune response. These class Ib/peptide complexes are apparently

recognized by T cells with very restricted TCR repertoires (Janeway 1992), again consistent with the idea that this entire 'first line of defence' evolved to deal with conserved bits of pathogens.

Finally, there is some evidence that non-classical class I molecules might even present self peptides to such non-conventional T cells (Teitel et al 1994). This result implies that even in the absence of infection, T cells are capable of recognizing and lysing cells that have been exposed to stress. This ascribes to the immune system an interesting role: destruction of stressed self cells in epithelial surfaces in the apparent complete absence of foreign antigen. The peptide ligands for these interactions are not known, but one might implicate peptides derived from highly conserved stress-induced proteins.

Putting all these preliminary data together, we propose the following speculative scenario outlined in Fig. 1, borrowing greatly from a model proposed by Janeway (1992). This 'first line of defence' in the vertebrate epithelial immune system may be where non-adaptive and adaptive immune systems coalesce, i.e. the adaptive immune system may have evolved from such a strategy, employing killer lymphocytes with restricted repertoires to recognize peptide fragments of conserved chaperone proteins bound to monomorphic 'MHC' molecules upon stress to epithelial cell layers (step 1). From this point in evolution, certain class I isotypes may have coevolved with TCRs of minimal diversity to expand the repertoire of the non-adaptive immune response (step 2). At this stage, self tolerance became essential. Finally, MHC proteins became diverse within species to bind to many different combinations of peptides (step 3); here, T cells with diverse repertoires would have been required in order to recognize the practically infinite combinations of MHC/peptide that could be generated within a species. Thus, a special organ, the thymus, became necessary for the fine-tuning of the differentiation of T cells recognizing polymorphic ligands. We have previously argued that the thymus evolved specifically as a differentiation site, directing the positive selection of T cells with receptors for self MHC proteins (Houssaint & Flajnik 1990). In the primitive case, the immune system had to rely on the presentation of invariant stress proteins upon infection of the cell. In the case of the most advanced system, T cells with diverse repertoires became capable of recognizing a plethora of foreign antigens in association with the self MHC proteins. In both cases, the same effector systems for killing could have been used; it is only the recognition phase that became more complex.

Polymorphism first?

An alternative view of MHC evolution is that the polymorphism of cell surface MHC-like proteins preceded the ability of these proteins to present peptides and that a diverse set of receptors was educated to tolerate self but reject polymorphic ligands. Such a system may exist in the famous fusion/rejection reactions of colonial tunicates in which colonies can be induced to fuse if one
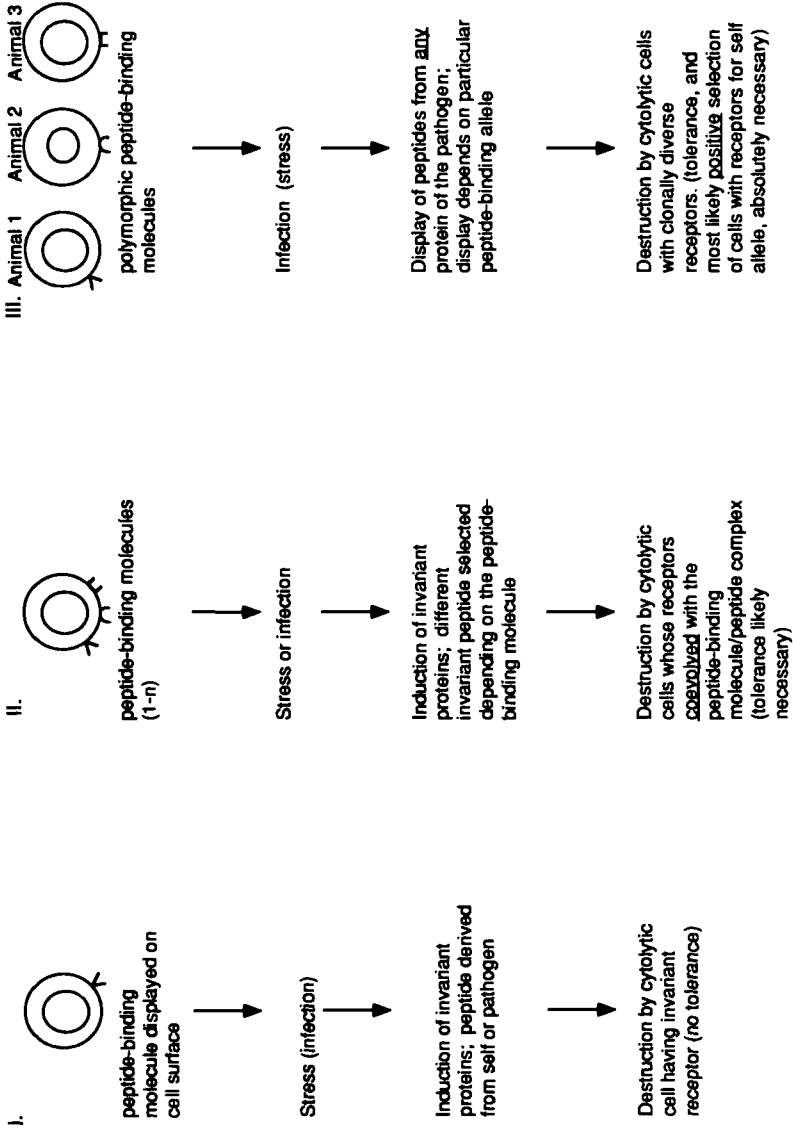
FIG. 1. Speculative view of an adaptive immune system arising from a non-adaptive recognition system. Details provided in the text.

polymorphic locus is shared, whereas rejection ensues if no allele is in common (Oka & Watanabe 1957). Fertilization is governed by the converse rule: fusion of egg and sperm only occurs when the gametes differ in the polymorphic alleles. No biochemical basis for these phenomena has been discovered yet, but it has always been attractive to implicate polymorphic MHC-like molecules in directing these reactions. We feel that any similarity of the colonial tunicate fusion reactions with the MHC in the vertebrate immune system could be entirely through convergence.

Conclusions

Clearly, we favour the idea that non-polymorphic cell surface molecules related to Hsp70, which could bind conserved peptide ligands, were the first self proteins to direct cell-mediated immunity in a non-adaptive immune system, but as yet such speculations are based on few data. It is difficult to incorporate all of the diverse bits of information into a cogent theory about evolution of the immune system. It is, however, becoming more and more clear that evolutionarily conserved heat shock proteins play a major role in the epithelial immune system; whether such proteins actually provided the nucleus for the emergence of the adaptive immune system is a problem that will keep us on the edge of our seats.

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DISCUSSION

Boman: Most people would agree that the function of the immune system is to prevent infections. This again raises the question of the assays related to that function. Immunologists sometimes think that the higher the specificity is, the higher the scientific interest is. I don't quite agree. There may have been situations when Nature has forced evolution to increase specificity as a consequence of the complexity of the system, but, with less complexity, a simple system would seem to be superior.

Flajnik: Well, I've been subjected to a very rude awakening. When I was in Basle as a post doc, we were not really taught that the immune system has anything to do with fighting pathogens. All we were really interested in was finding new mechanisms of cell–cell interactions or another genetic mechanism for making an antibody. We were not really taught that the immune system has anything to do with defence.

Boman: I know the Basle school of thinking. There are now other schools of thinking in immunology, stemming from biochemistry and molecular biology. Thinking of David Baltimore and his work on the regulatory mechanisms that control the immune response, are there molecules similar to NF-xB or I-xB in *Xenopus* and the shark?

Flajnik: I would be very surprised if there weren't. In *Xenopus* there are, but I think that the fact that you can find them in *Drosophila* and mammals suggests that they're going to be found in the shark also. We actually found the x genes themselves in the shark (Greenberg et al 1993). It was thought that x hadn't diverged at this phylogenetic level yet, but that's not true. We haven't analysed the regulatory regions, but I would be very surprised if it turned out that they lacked the NF-xB binding sites.

Boman: When I started working on insect immunity, around 1970, it was quite clear to me that I should not use the standard methods of immunology at the time; that I should assay bacterial concentration and insect killing as a way to follow the course of an infection. I don't think I would have got anywhere, otherwise. Many people tried to look for immunity in insects by conventional methods, such as lysis of erythrocytes, and they didn't work.

Mike Zasloff, how does a shark avoid infection? Is it by means of the conventional immune system, is it through squalamine or does it have many other defence mechanisms yet to be found?

Zasloff: Are you asking for a religious answer? I have a bias which may not be rooted in reality. On the basis of what we know about the shark, I approach it as a physician would approach a highly immunocompromised individual. As Martin Flajnik has said, the shark makes a restricted group of immunoglobulins, IgM. They are by and large of low affinity and they clearly exhibit relatively low diversity. You can't find classical T cells in the shark, and so on. Despite that, the animal is about as hardy as you could imagine. What really struck

me when I saw these animals for the first time, just a few years ago, was that the female couldn't care less about the microorganisms in the ocean, because she keeps her pups in her fallopian tubes for about two years (they are essentially oviducts) and she flushes these tubes with sea water. These babies are not connected to her through a circulatory connection; they are feeding from egg yolk and they are still fetal. She has all her reproductive tract exposed to the outside world. It presents a very peculiar scenario—on the one hand you see an animal with a relatively sluggish-looking immune system, and on the other hand you see an animal that couldn't care less about microbes. I approached it from the perspective that if this were a human being, with something like a hyper-IgM syndrome, this person would have a real hell of a time with all sorts of fungal and bacterial infections. We could probably keep this person alive if we gave them a whole cocktail of antibiotics. So, in my simple-minded approach, I imagined that the shark was making its own penicillin, cecropin, magainin, sapecin, BPI and so on. Penicillin can take care of a lot of different bacteria, none of which resemble each other in significant ways. Amphotericin can knock off half of the population of fungi that we encounter. So, discovering squalamine, which exhibited all these properties, was a surprise: squalamine can kill all of the major fungi we have exposed it to, it handles almost all the bacteria (including *Proteus* and *Serratia*) and it knocks off protozoa. It's present in the shark at concentrations that exceed its minimum inhibitory concentration (MIC). We also now know that squalamine is one of a dozen relatives; it's the most abundant, but there are a good dozen.

My long-winded semi-religious answer to your question is that the shark has got the immune system that Martin Flajnik described, and then there are these chemicals. It's like it's making its own pharmacy.

We have also looked at hagfish. The hagfish gut is loaded with antibiotic peptides, some of which are being characterized, but I haven't found steroid antibiotics yet.

Flajnik: Do you find the same things in mammals? It seems stupid to lose them during evolution.

Zasloff: I'm sure they are not lost.

Lehrer: Your paper was very focused on the machinery of the adaptive immune system. You could also approach it by looking at its purpose. Its primary purpose is to combat infection. One of the concerns I have when I come to meetings like this is that phagocytes do not get their due. An important function of the adaptive immune system, with respect to infection, is to make phagocytes more efficient by providing opsonins so that organisms are more efficiently taken up by the phagocytes. Once they are within a phagocyte, they are exposed to these ancient but still effective molecules we have been hearing about, at high concentrations, in mixtures (not just one at a time), and in conjunction with an array of potent oxidants.

Flajnik: I agree. Srivastava & Heike (1991) have shown that one can isolate intracellular 'chaperones' from tumours (Hsp70 and another chaperone called gp96 that's found in ER) and use them to induce tumour-specific immunity. If you immunize mice with the chaperones, they are protected from a subsequent tumour challenge, the idea being that cell surface receptors of the phagocytes bind the chaperones which are bound to peptides found in tumours. After pinocytosis, the peptides charge class I and class II molecules; thus an antitumour response can be generated just by immunizing with the chaperones. I know it sounds too good to be true, but here's a case where you are taking two very primitive things, the conserved chaperones (which are found in all organisms) and the phagocytes (which are found in most eukaryotes), and with them you can generate a potent adaptive antitumour response.

Lehrer: In oncology, things generally turn out to be too true to be good!

Ganz: What does a primitive immune system, such as that found in the shark, get you, in terms of fighting infection? What does it facilitate, and how does it kill bacteria?

Flajnik: The IgM response in sharks may only be a first-line defence, so that it's good enough to provide opsonization or complement fixation, but in terms of increasing affinity of the adaptive response, it just doesn't happen with the *bona fide* immunoglobulin. We think that everyone has been studying the *bona fide* immunoglobulin for all these years and they have overlooked the new molecule that we found—the one that's undergoing extensive somatic mutation and generating great diversity in complementarity-determining region (CDR) 3. We don't have a clue about what the main effector function of this molecule is. But, of course, anything that increases in affinity for specific antigens is of value to the organism. It will bind to the pathogen more tightly, and should clear the system of the pathogen faster.

Ganz: This is a recognition system; what is the effector system it interacts with?

Flajnik: I don't know yet.

Hultmark: I want to straighten out a conceptual confusion. I don't think it's very productive to compare antibacterial peptides with immunoglobulins and T cell receptors, because these things are doing entirely different things. On the one hand, we have effector functions that kill bacteria, which T cell receptors or immunoglobulins never do. What they do is to recognize bacteria and to direct effector mechanisms to kill bacteria. Organisms need both of these functions for a successful defence system. This is true both for vertebrates and for invertebrates.

Boman: How large is the need for recognition, really? Recognition is something immunologists have put very high up on their scale of scientific values.

Ganz: I can try to give a peptide answer to this. Recognition allows you to use peptides that would otherwise would be unusable—peptides which would be very toxic if they were released non-specifically. Recognition allows you to confine events to a phagocytic vacuole or to an interface of a particular cell or a particular target.

Boman: If you have effector molecules directed to a target that is absent in the host, you avoid the whole complexity of self recognition.

Ganz: But then you allow the pathogen simply to avoid this defence by becoming more and more like the host.

Boman: But the question is, which bug really wants to be a pathogen?

Bevins: Along those lines, higher organisms have evolved so as to allow a certain population of microorganisms to exist at mucosal surfaces as the 'normal flora'. A specific immune response may allow the higher organism selectively to kill a subset of microorganisms without killing the normal flora.

Flajnik: I agree with Dan Hultmark that it is a bit silly to compare the adaptive and non-adaptive immune systems. Both systems have been selected and obviously both are important for protection of the organsim. My personal opinion is that the adaptive immune system is activated whenever a pathogen slips by everything that the non-adaptive immune system has in its arsenal to combat it.

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Antimicrobial proteins with homology to serine proteases

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Abstract. The azurophil granule, a specialized lysosome of human neutrophils, contains a family of antimicrobial proteins with structural homology to serine proteases, the serprocidins. Three members of this family are serine proteases (cathepsin G, elastase and proteinase-3) and one is a proteolytically inactive homologue (azurocidin). They are synthesized as preproteins with a characteristic leader peptide and a propiece, both of which are removed by processing enzymes to yield the mature protein. The functional genes for three serprocidins (elastase, proteinase-3 and azurocidin) are grouped in a single genetic locus on chromosome 19 and are coordinately expressed and regulated during haemopoietic differentiation. Multiple and sometimes overlapping biological functions are a feature of this family, yet they all seem to pertain to host immunity. The structural requirements for the function of one member of this group (azurocidin), particularly its antibiotic function, are under investigation.

Well before adaptive immune mechanisms evolved, 'innate' mechanisms served to prevent infections. Antimicrobial proteins are common agents of innate immunity. They play a central role in immune defence in the lower phyla and they are also increasingly recognized as part of the host defence mechanisms in the animal kingdom. In mammals, they can serve both as a first line of defence before adaptive immunity is mobilized and subsequently as a back-up to our adaptive immune strategies. Proteolytic enzymes and other molecules structurally related to proteases also play a role in innate immunity and are often found in close association with antimicrobial proteins. Protease cascades contribute to the immobilization and killing of invading microorganisms, as in the complement cascade (Esser 1991) or in the clotting of the *Limulus* amoebocyte lysate (Iwanaga 1993, Iwanaga et al 1994, this volume). Proteases participate in the processing and activation of various cytotoxic molecules (Valore & Ganz 1992, Zanetti et al 1990, Resnick et al 1991). Proteases from natural killer cells

and cytolytic T cells are thought to allow cytotoxic molecules to gain access to their targets—virally infected or tumour cells (Hudig et al 1993). Others can play a direct cytocidal role. Such is the case with the serprocidins, a family of serine proteases with antimicrobial activity found in the azurophil granules of human polymorphonuclear leukocytes (Campanelli et al 1990a).

Polymorphonuclear leukocytes and intracellular granules

By the late 19th century, phagocytes were recognized as essential for the killing of ingested microorganisms, hence for host defence; consequently, many of the studies on antimicrobial mechanisms concentrated on these cells (Metchnikoff 1905). In the late 1950s, the cellular response of the neutrophil to microbial invasion was broken down into three steps: chemotaxis, phagocytosis (with its associated burst of metabolism) and degranulation. The latter step was marked by the fusion of lysosomal granules with the phagocytic vacuole and resulted in the delivery of granule contents to the newly formed phagolysosome (Hirsch & Cohn 1960a,b). This event led to the eventual death and degradation of ingested microorganisms. Pioneering work in the identification of endogenous antibiotics included the discovery by Hirsch (1958) of an antibacterial substance (phagocytin) associated with neutrophil granules. The subsequent characterization of a number of endogenous antibiotics followed, initially the bactericidal permeability-increasing protein (BPI) and the defensin family (see Elsbach 1994, Bevins 1994, Ganz 1994, this volume).

At least three morphologically and functionally distinct populations of granules exist in mature neutrophils, namely azurophil, specific and tertiary granules (Bainton 1992). While specific or tertiary granules are viewed mainly as secretory organelles that release their contents to the plasma membrane and the extracellular milieu upon activation, the azurophil granules are thought to release their contents primarily within the confines of intracellular phagosomes. Table 1 lists the contents of these various granule populations. Azurophil granules sequester a complex mixture of antimicrobial proteins and microbicidal enzymes, which is capable of delivering a lethal hit to the invading organisms. Thus, this organelle appears to play an important role in the overall antimicrobial activity of the polymorphonuclear leukocytes.

Subcellular fractionation and localization of antimicrobial proteins

A technical advance in cellular fractionation provided the means for an improved separation of cellular compartments and, in particular, neutrophil granules (Borregaard et al 1983). The granules obtained by this method (nitrogen cavitates of polymorphonuclear leukocytes subjected to Percoll gradient density sedimentation) could be separated into well-defined populations (specific and azurophil granules) and appeared intact. These pure populations seemed to be

TABLE 1   Constituents of granules from human neutrophils[a]

Azurophil granules	Specific granules	Other intracellular compartments
Microbicidal enzymes		
Myeloperoxidase		
Lysozyme	Lysozyme	
Bactericidal perme-		
ability-increasing		
protein (BPI) or		
CAP 57		
Defensins		
Serprocidins		
Elastase		
Cathepsin G		
Proteinase 3		
Azurocidin or CAP 37		
	Collagenase	Alkaline phosphatase
	Gelatinase	Tetranectin
Acid hydrolases		
β-Glycerophosphatase		
β-Glucuronidase		Gelatinase
N-Acetyl-β-		
glucosaminidase		
α-Mannosidase		
Cathepsin B		
Cathepsin D		
	Lactoferrin	
	Vitamin B_{12}-binding	
	proteins	
	Plasminogen activator	
	Histaminase	
	Cytochrome *b*	
	β_2-microglobulin	
	Receptors	Receptors
	FMLP[b]	Fc RIII
	CR3 (C_3bi)	CRI
	Laminin	
	CD 11b/CD18	CD 11b/CD18
	Vitronectin	

[a]Subpopulations may exist without these two basic granule types.
[b]FMLP, *N*-Formyl-methionyl-leucyl-phenylalanine.
Reprinted with permission from Bainton (1992).

ideal for an extensive screening of the cell antimicrobial arsenal. This approach led us to the findings that: (i) the majority of the antibacterial activity against *Escherichia coli* resides in the azurophil granule of neutrophils; and (ii) it is associated with a pelletable material upon granule disruption, however, apparently not a granule membrane component (Gabay et al 1986). An acid

extract from these granules released ten major antimicrobial proteins, including three previously unrecognized species on the basis of N-terminal sequence analysis: a novel defensin HNP4, azurocidin and proteinase-3 (PR-3, previously named p29b) (Gabay et al 1989). It is possible to review the antimicrobial proteins from azurophil granules as follows: two are unique in primary structure (lysozyme and BPI), while the remaining eight fall into two families of four members each: the defensins on the one hand and cathepsin G, elastase, PR-3 and azurocidin on the other, collectively named serprocidins.

The serprocidins

Members of this family are cationic glycoproteins of similar size (25–29 kDa) and relative abundance (1–2 μg/10^6 polymorphonuclear leukocytes) showing extensive homology to serine proteases and broad-spectrum antimicrobial activity. Cathepsin G and elastase were originally identified on the basis of their neutral esterase activity. The former is a chymotrypsin-like enzyme with the ability to degrade connective tissue components such as cartilage proteoglycan and collagen type I and II and whose role *in vivo* is unclear (Gerber et al 1974). The latter is an enzyme of greater specificity and potency than cathepsin G, capable of digesting underlying matrix components of lungs, arteries, skin and ligaments (elastin and collagen type IV; Travis et al 1980). Thus, this protease has been implicated in several inflammatory diseases with abnormal tissue injury, such as emphysema, adult respiratory distress syndrome and rheumatoid arthritis (Malech & Gallin 1987). In addition to proteolytic activity, both proteins displayed antibiotic activity, although to a lesser extent for elastase (Blondin & Janoff 1976, Blondin et al 1977, Odeberg & Olsson 1976). Interestingly, the antibacterial activity of the two proteins appeared independent of their proteolytic activity (Thorne et al 1976). An internal heptapeptide derived by hydrolysis of the cathepsin G molecule was isolated and showed some antimicrobial activity *in vitro* (less than 5% of the activity of full-length cathepsin G) (Gangalore et al 1990). Studies with synthetic peptides spanning the full-length sequence of the cathepsin G protein and averaging twenty amino acid residues in length identified three which exhibited antipseudomonal activity (Shafer et al 1993). However, the activity associated with the most active peptide represented only a small fraction of the bactericidal activity of full-length cathepsin G. The limitations of the synthetic peptide approach to identify antimicrobial domains in large molecules are discussed at the end of this paper. We and others contributed to the characterization of two novel members of the serine protease family in polymorphonuclear leukocytes with high homology to elastase: azurocidin and PR-3.

Proteinase-3

The primary sequence of the mature protein is predicted to consist of 228 amino acids with a M_r of 25 000 for the polypeptide backbone (Campanelli et al
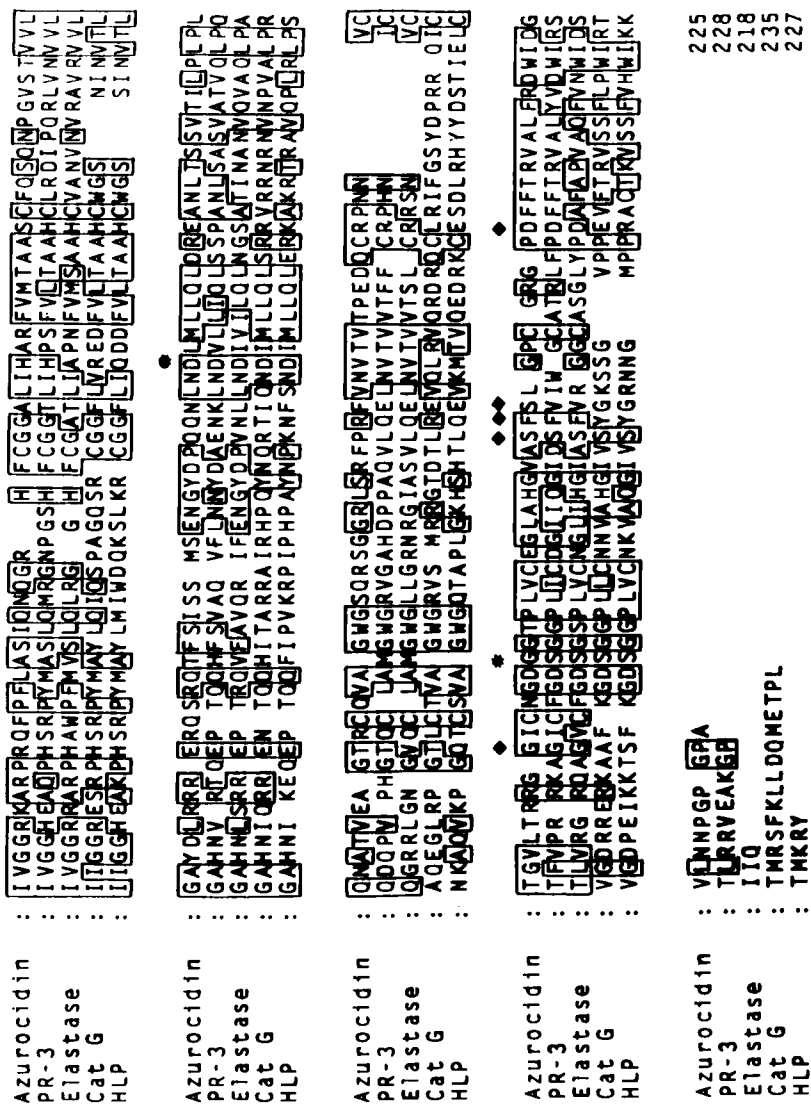
FIG. 1. Comparative sequences of serprocidins (azurocidin, proteinase-3 [PR-3], elastase and cathepsin G [Cat G]) and human lymphocyte protease (HLP). Conserved residues are boxed. The histidine, aspartic acid and serine residues constituting the catalytic site are indicated by asterisks. Residues lining the substrate-binding pocket of serine proteases are marked by diamonds. Amino acids are represented by one-letter code.

1990b). Two potential *N*-linked glycosylation sites (Asn–X–Ser/Thr) are present. Eight cysteine residues (five clustered in the C-terminal third of the molecule) suggest that there may be four intramolecular disulphide bonds as in elastase. It has the active site triad His–Asp–Ser typical of serine proteases.

The N-terminal half of PR-3 contains six regions, averaging 17 residues each, in which there is a marked periodic alternation of hydrophilicity and hydrophobicity, reminiscent of the amphiphilic properties of many antimicrobial peptides. The protein is predicted to contain three short stretches of α-helix (8.3%) and 10 predominantly hydrophobic β-sheets (40%).

The PR-3 sequence is homologous to that of elastase as well as to those of a number of immune cell-derived proteases (Fig. 1). The isolated cDNA clone for PR-3 encodes a preproprotein with a hydrophobic signal peptide and a dipeptide N-terminal extension requiring cleavage to generate the mature, active protease. Northern blot analysis suggests that PR-3 expression is primarily confined to the promyelocytic/myelocytic stage of bone marrow development (Sturrock et al 1992). The PR-3 gene falls into a cluster of elastase-like genes and the possible implication of this finding will be discussed below (Zimmer et al 1992).

Proteinase-3 combines multiple functions. PR-3 is the third neutral serine proteinase identified in the azurophil granules of polymorphonuclear leukocytes (Dewald et al 1975). PR-3 has a proteolytic profile and a specific activity similar to that of elastase, which also suggests a role in the pathogenesis of inflammation: (i) it cleaves connective tissue proteins, in particular collagen IV and elastin (Rao et al 1991); (ii) at mildly acidic pH it is more active against insoluble elastin than human leukocyte elastase; and (iii) it is a potent inducer of emphysema in hamsters (Kao et al 1988).

We have found that PR-3 is a potent broad-spectrum antibiotic of human polymorphonuclear leukocytes and that this activity is distinct from its enzymic activity (Campanelli et al 1990b). The antimicrobial activity of PR-3 *in vitro* is greatest at acid pH (5.5), a pH optimum that apparently differs from those of BPI and defensins. Similarly to most granule-associated antimicrobial proteins, the antimicrobial activity is inhibited in a dose-dependent fashion but not abrogated by NaCl, CaCl$_2$ and serum. This would certainly limit a potential antimicrobial function for antimicrobial proteins in the extracellular milieu. The mechanism of action of PR-3 is unknown. Many of the antibiotic proteins studied so far carry a positive charge and, because many bacterial surfaces are anionic, the initial contact between antimicrobial proteins and the target cell is electrostatic. In contrast, PR-3 is not a very highly charged molecule (pI of 7.9) and it is unclear whether the binding of PR-3 to its target may involve another type of interaction.

In addition, granule-associated serine proteases of neutrophils are the targets of autoantibody formation. Antibodies that bind cathepsin G, elastase and PR-3 have been detected in the sera of patients suffering from chronic inflammatory

diseases (Kallenberg et al 1991). In Wegener's granulomatosis, autoantibodies against PR-3 are regularly found as an obligate feature of this autoimmune disease (Niles et al 1989). Furthermore, PR-3 appears to regulate the growth and terminal differentiation of the myelomonocyte lineage (Bories et al 1989).

Azurocidin

Azurocidin (also known as cationic antimicrobial protein 37 [CAP 37] or human heparin-binding protein [hHBP]) is a 29 kDa glycoprotein with antimicrobial activity (Gabay et al 1989, Flodgaard et al 1991, Shafer et al 1984). The complete primary sequence of azurocidin was determined (Pohl et al 1990) and shows the following: the 221 amino acid polypeptide contains eight cysteine residues (likely paired via disulphide bonds) and three potential glycosylation sites. Azurocidin is highly homologous to the serine protease family, particularly elastase and PR-3. However, two mutations can be found in the putative active site of this serine protease homologue which are sufficient to explain its lack of proteolytic activity. Interestingly, another 'pseudo' serine protease (factor D) has been recently identified in the *Limulus* system (Iwanaga 1993). The majority of the basic residues are present in the first 70 amino acid residues of the molecule and most are predicted to be exposed on the surface.

The cDNA for azurocidin encodes a preproprotein with a signal sequence and a seven amino acid propeptide that are cleaved to produce the mature protein (Almeida et al 1991, Morgan et al 1991). Since the primary and cDNA-derived sequences differ at their end point, it is also likely that a processing event occurs at the C-terminal end of the molecule. The peptidase(s) involved in the N- and C-terminal processing of the azurocidin molecule have not been identified. The functional gene for azurocidin is grouped in a cluster of elastase-like genes (the AZU-PR3-NE locus) within a 50 kb region on chromosome 19pter (Zimmer et al 1992). The three genes are expressed at high levels in the neutrophil and monocyte lineage and down-regulated during haemopoietic differentiation. Thus it appears that the high-level, lineage-specific gene expression of the serprocidins is developmentally restricted to the promyelocytic stage of phagocyte maturation (a time when the microbicidal storage granules are formed and assembled). This supports the hypothesis that the three protein products of these genes have biological functions that pertain to host defence against invading micro-organisms.

Azurocidin is a potent antibiotic against Gram-negative bacteria and a major contributor to the overall antimicrobial activity of the polymorphonuclear leukocyte azurophil granule *in vitro* (Campanelli et al 1990a, Gabay et al 1989). At somewhat higher concentrations, azurocidin also kills Gram-positive bacteria and fungi. Synergies between azurocidin and two other serprocidins (elastase and cathepsin G) have been demonstrated in the killing of oral bacteria. Azurocidin alone exhibited no antimicrobial activity against oral bacteria.

However, it synergized the bactericidal activity of enzymically active elastase or cathepsin G against these bacteria (Miyasaki & Bodeau 1992). Thus enzyme-dependent interactions between serprocidins may occur.

The mechanism of azurocidin killing has not yet been elucidated; as for many antimicrobial proteins, an initial event may be the binding of this basic molecule to the anionic bacterial surface, and in particular to lipopolysaccharide (LPS). The nature of the LPS present on a particular strain influences the antimicrobial activity of azurocidin against that strain. A direct functional interaction between azurocidin and LPS is supported by the fact that azurocidin binds lipid IVA (an intermediate in LPS biosynthesis) and that the addition of lipid IVA inhibits approximately 50% of the antimicrobial activity of this protein (Gabay et al 1990). Azurocidin exhibits no apparent structural homology with BPI or LPS-binding protein (LBP), two members of the family of LPS-binding proteins. The basic nature of the azurocidin N-terminal domain may promote an electrostatic interaction with LPS.

Do small, specific domains within this protein contain the active site(s) for antimicrobial activity? Only a fraction of azurocidin activity is retained by a synthetic peptide corresponding to residues 20–44 which contains a disulphide conserved among the serprocidins (Pereira et al 1993). The availability of cDNA clones in expression systems will facilitate the structure–function analysis of the azurocidin molecule.

In addition to an intracellular role in the killing of invading microorganisms, azurocidin may have at least two extracellular functions. Pereira et al (1990) showed that 90% of intragranular azurocidin can be released in the extracellular milieu upon neutrophil activation and that azurocidin is a strong chemotactic agent for monocytes *in vitro* at nanomolar concentrations. Other reports present evidence that this protein mediates reversible contraction of fibroblasts and endothelial cell monolayers and stimulates monocyte survival and thrombo-spondin secretion (e.g. Ostergaard & Flodgaard 1992).

Conclusions

Proteases and protease analogues are important components of the antimicrobial arsenal of phagocytic cells. They are found in storage organelles that are traditionally involved in microbial killing or that may be brought into contact with microbes upon lysosome fusion. They may have a number of roles: they may exert a cytotoxic effect through their proteolytic activity or independently from it, and they may contribute to the digestion of previously killed target cells. They may act alone or in combination with other antimicrobial agents. In addition, they may convert other cytotoxins from inactive to active forms. Thus proteases and analogues in many different ways can arm the cell to achieve its full potential in host defence.

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DISCUSSION

Ganz: I have a question about the methodology that is used to define these smaller peptides within a larger molecule. My concern is that this method may be difficult, because the specific termini of the small peptides matter. You cannot simply divide a protein into little segments, because a shift of one or two amino acids at either end may completely change the properties of the peptide you get. Does anyone have a solution to this problem? I just don't think that this method will work.

Ham: But it is a commonly used technique.

Ganz: I agree. The question is, will it provide a meaningful answer?

Elsbach: I have very similar reservations about dissecting a molecule into pieces, especially when you have a difference in molar concentrations of several orders of magnitude in terms of finding any given bioactivity in relation to what the whole protein does. One has to be awfully careful not to draw too many conclusions from what you can find out using just a piece of the molecule. Clipping a molecule and retaining the activity at least of the same order may be one thing, but I'm very much less convinced by this sort of arbitrary overlapping peptide business. I do think results like those of Dr Natori (1994, this volume) are more encouraging, where the peptide that he was working with really had activity that was comparable to the whole molecule to start with, especially if you then make additional modifications that enhance activity. This seems to lead in the right direction, but I still think that Nature makes large molecules in certain situations for very good reasons, that may have a bearing on delivery and so on.

Gabay: You have a very large molecule (BPI) that can actually be split, and then further split (Elsbach et al 1994). This is where the issue of large molecules being designed only for one purpose or for a number of purposes arises. We are interested in is defining domains of activities. How big are these domains within a given molecule? That's something that we're working on.

Rees: There are two things you could do. First, you could look at the 3D structure in solution. That would give you confidence that you're looking at the same sort of electronic stearic configuration that the whole protein has. For example, Dr Natori told me that the α-helix of sapecin that he described (Natori 1994, this volume) has the same 3D structure when isolated as it does in the complete sapecin molecule. Secondly, you could confirm the key features necessary for activity by doing deletion mutagenesis or substitution mutagenesis around that site and see if you can obliterate all the activity of the parent protein.

Gabay: This is true, but 3D structures are usually hard to get. One advantage that we have here is that because the serprocidins are a family of proteases, we have at least got a model to work from. Elastase, which is a serine protease highly homologous to azurocidin and proteinase 3, has been crystallized and there is a 3D structure for it (Navia et al 1989) which can be used as a structural model for azurocidin.

Elsbach: Have you generated an antibody against serprocidin?

Gabay: No, but that's something we're intending to do.

Ganz: One simple way of finding out if the peptides are meaningful is to make sure that they're somewhat boundary insensitive—that the choice of where you make the cut off is not terribly important, so if you make it a little longer it will not drastically change its properties.

Gabay: Yes, but one of the limitations of this approach is that it is not possible to synthesize very long peptides. As I understand from discussing with David Andreu, the upper size limit for synthetic peptides is around 40 residues.

Ganz: When the biological properties of the peptide fragment are insensitive to the exact choice of termini, it reassures me (at least a little bit) that the peptide reflects some local structure.

Sahl: Lysozyme acts as an enzyme, but there have been reports which clearly show that you can inactivate lysozyme by boiling and it still has some antimicrobial activity. Presumably, this is because it triggers autolysis, pretty much in the same way that we see occurring in the case of the lantibiotics (Sahl 1994, this volume). So you may be looking at two completely different activities.

Gabay: This is a very good point. Serprocidins resemble lysozyme in this regard. For example, proteinase-3 acts as an enzyme but it is possible to inhibit almost completely its proteolytic activity and it still has antimicrobial activity. As for azurocidin, although it is structurally related to serine proteases, it is enzymically inactive. The antimicrobial activity of azurocidin operates clearly non-enzymically.

Natori: We raised an antibody against sarcotoxin I (which is a cecropin of *Sarcophaga*). Using affinity-purified antibody, we did an immunoblotting experiment with a pupal extract of *Sarcophaga*. We found two immunoreactive proteins in this extract. One was, of course, sarcotoxin I, but the other protein was clearly larger than sarcotoxin I. We purified this protein to homogeneity and found that it is a serine protease from its partial amino acid sequences. In fact, this protein contained proteinase activity. We haven't tested the antibacterial activity of this proteinase, but it might have a similar antibacterial activity to azurocidin.

Ganz: Adding to the list of antimicrobial substances that are enzymes, one should not forget the ribonucleases of the eosinophil granules, eosinophil cationic protein and eosinophil-derived neurotoxin.

Gabay: It may be that azurocidin molecules are more widespread than previously has been appreciated. Their exact function in the defence system will be interesting to elucidate.

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Antimicrobial peptides as agents of mucosal immunity

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Abstract. Mucosal surfaces are continually exposed to a wide range of potentially pathogenic organisms, yet the incidence of infectious disease resulting from these encounters is relatively low. This suggests the presence of highly effective defence mechanisms in these tissues. Antimicrobial peptides have recently been discovered in mucosal tissues and may play a significant role in host defence. Several mucosal peptides (andropin, magainin, tracheal antimicrobial peptide, enteric defensins and PR-39) all fulfil minimal criteria for a role in mucosal host defence, including potent *in vitro* antimicrobial activity and accumulation at the mucosal surface. Most of these mucosal peptides are encoded by members of large gene families that contain members found in other biological contexts more classically associated with antimicrobial defence. The abundance, activity and evolutionary history of several epithelial peptides suggest that antimicrobial peptides play a key role in host defence at mucosal surfaces.

1994 Antimicrobial peptides. Wiley, Chichester (Ciba Foundation Symposium 186) p 250–269

A critical biological boundary exists at mucosal surfaces. An animal encounters not only hazardous chemical and physical agents at these sites, but also a biological environment often rich in microbes. To combat these threats, animals utilize diverse strategies, which presumably have been retained through the selective pressure of evolution. Many effector molecules contribute to host immunity at mucosal surfaces, including immunoglobulin A, lectins and lysozyme (Kraehenbuhl & Neutra 1992, Newhouse & Bienenstock 1989, Sastry & Ezekowitz 1993). This paper considers one group that may also play a significant role—the antimicrobial peptides.

Antimicrobial peptides are host-defence effector molecules attracting increasing interest. Recent pioneering efforts by a small group of independent investigators have converged on elucidating this widespread, but previously unrecognized, mechanism of animal host defence (for reviews, see Boman 1991, Lehrer et al 1991, Zasloff 1992). The numbers of identified antimicrobial

peptides are increasing rapidly. One way to classify these peptides emphasizes anatomical sites of origin: (i) peptides released into haemolymph or other internal fluids; (ii) peptides of circulating phagocytic cells; and (iii) peptides of mucosal surfaces. Initial efforts in the field of antimicrobial research were focused largely on the first two of these classes, which will be referred to here as 'classical' antimicrobial peptides. This review will describe antimicrobial peptides of mucosal tissues.

Classical peptides and epithelial counterparts

Two lines of investigation have served as cornerstones for establishing a role of classical antimicrobial peptides in general host defence. First, insect anti-microbial peptides are induced upon infection and released into haemolymph at concentrations sufficient for antimicrobial activity. This is part of a coordinated host response, where several defensive peptides and proteins combine to eliminate offending organisms. Second, high concentrations of peptides with potent antimicrobial activity are found in circulating phagocytes, such as neutrophils. These cells, which are highly specialized for host defence, are charged with the task of engulfing and killing potentially pathogenic micro-organisms. Antimicrobial peptides are found in phagocytes across a range of phylogenetically diverse species, from insects to crustaceans to mammals. Abundance of these peptides in phagocytic cells is consistent with an anti-microbial function. Together, these two lines of investigation offer compelling arguments for the participation of antimicrobial peptides in host defence. The discovery of epithelially derived peptides, with both comparable antimicrobial activity and similar structure to the more classical peptides (Table 1), has fuelled an accelerated effort to understand better the biological role of the epithelial counterparts.

Several criteria should be met before a newly discovered peptide may be considered to have a role in host defence *in vivo*. The minimal criteria include: (1) potent specific activity against microbes *in vitro*; and (2) expression of sufficient concentrations of peptide *in vivo* to enable antimicrobial action.

TABLE 1   Selected epithelial antimicrobial peptides and their more classical counterparts (see text for references)

Epithelial peptide	*Organ system*	*'Classical' counterpart*
Andropin	Reproductive tract	Cecropins, sarcotoxins —induction upon infection
Cryptdin	Intestinal tract	Defensins —abundant in phagocytes
Tracheal antimicrobial peptide	Respiratory tract	β-Defensins —abundant in phagocytes

Several epithelial peptides may prove to fulfil these criteria (Agerberth et al 1991, 1993, Diamond et al 1991, Lee et al 1989, Moore et al 1991, Samakovlis et al 1991, Selsted et al 1992, Zasloff 1987). Other biological properties can support a role in host defence, including modulated expression by infection and microbial by-products, and expression in cells devoted to host defence. Five mucosal peptides are selected to address these points.

Andropin, a cecropin-related peptide in the reproductive tract

The insect cecropins, the first well-characterized family of antimicrobial peptides, were initially identified by Boman and co-workers in the giant silk moth, *Hyalophora cecropia* (Steiner et al 1981). The cecropins are a group of membrane-active cationic peptides with broad-spectrum antimicrobial activity; they are discussed in more detail elsewhere in this book (Hultmark 1994, Natori 1994, this volume). Cecropins and other insect antimicrobial proteins are strongly up-regulated in response to infection, treatment with lipopolysaccharide (LPS) or simply injury to the body wall (Boman et al 1991, Brey et al 1993). The molecular basis of this gene induction appears to involve the binding of a transcription factor related to the mammalian enhancer protein NF-xB to a consensus sequence in the 5'-flanking region of the defence-peptide genes (Ip et al 1993, Kappler et al 1993, Kobayashi et al 1993, Reichhart et al 1992, Sun et al 1991). This particular aspect of gene regulation is addressed below and is covered in greater detail elsewhere in this symposium (Hultmark 1994, this volume). This regulated expression of cecropin peptides, together with their specific activity, offers compelling evidence for a key role in host defence.

Andropin, an epithelially derived cecropin, was recently discovered by Hultmark and colleagues (Samakovlis et al 1991). These investigators screened a cDNA library to identify transcribed genes from the *Drosophila* cecropin locus. One clone had an open reading frame of 57 codons and the 23 amino acids at the N-terminus of the putative prepropeptide are strikingly similar to the signal peptides of the other cecropins. However, the remainder of the sequence had significantly lower similarity in primary structure and is much less basic than other cecropins. Nevertheless, the putative mature andropin peptide at the C-terminus is predicted to form two amphipathic helices, similar to cecropins (Fig. 1). Synthetic andropin has moderate antibacterial activity against Gram-positive bacteria in standard assays, but unlike the other cecropins, little activity against Gram-negative bacteria. Curiously, in assays using plates with higher ionic strength, andropin had significant activity against the Gram-negative bacterium *Escherichia coli*. This observation may have particular significance when the ionic composition of the medium in which this peptide functions is determined.

Andropin mRNA is detected only in a distinct tissue of the adult male abdomen. *In situ* hybridization and RNase protection assays demonstrate that

VFIDILDKVENAIHNAAQVGIGFAKPFEKLINPK   NPVSCVRNKGICVPIRCPGSMKQIGTCVGRAVKCCRKK

ANDROPIN   **TAP**

GIGKFLHSAKKFGKAFVGEIMNS   LRDLVCYCRSRGCKGRERMNGTCRKGHLLYTLCCR

MAGAININ-2   **CRYPTDIN-1**

RRRPRPPYLPRPRPPPFFPP RLPPRIPP GFPPRFPP RFP-NH2

PR-39
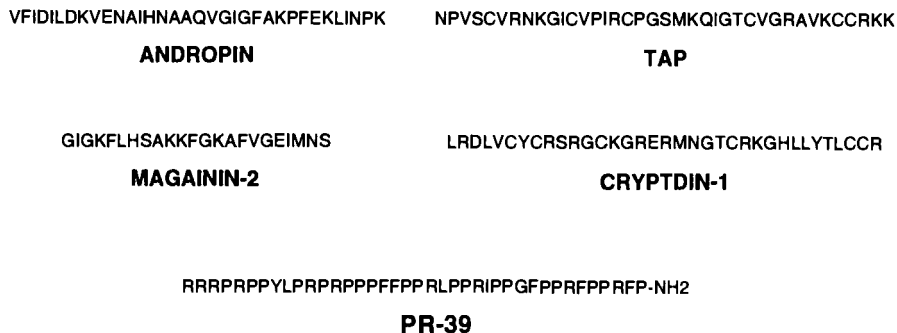
FIG. 1. Primary structure of selected antimicrobial peptides isolated from mucosal tissues (see text for details and references).

the andropin gene is transcribed in cells of the ejaculatory duct and expression is distributed throughout its length. Extracts from the male genital tract have several antimicrobial components; one shows comparable migration in acidic polyacrylamide gels to synthetic andropin.

Interestingly, andropin expression is regulated differently than other genes in the cecropin locus. In contrast to the cecropin genes, levels of andropin mRNA are not elevated in response to bacterial infection. Rather, andropin mRNA levels are induced several hours after mating, suggesting that the reaccumulating seminal fluid is replenished with andropin peptide.

In summary, andropin may be considered a male-specific cecropin-like peptide expressed in ductal epithelial cells of the reproductive tract. This peptide has antimicrobial activity and patterns of regulated expression that are distinct from the other members of the cecropin gene family. One may speculate that this peptide offers antimicrobial protection for semen and the male reproductive tract—and perhaps also the female tract—after semen is deposited upon mating.

Magainin, a peptide from vertebrate wet mucosa

A family of peptides was isolated from the granular glands of the skin of *Xenopus laevis* (Zasloff 1987). While specifically investigating possible mechanisms of mucosal immunity in this vertebrate species, Zasloff isolated peptides, named magainin 1 and 2 (Fig. 1), that have a broad spectrum of potent antimicrobial activity, encompassing bacteria, fungi and protozoa. In *Xenopus*, over a dozen antibiotic peptides structurally related to the magainins have been identified (for review, see Bevins & Zasloff 1990). The genes encoding these peptides are clearly members of a large gene family; one feature of their gene structure worth noting is a highly conserved nucleotide sequence in the 5'-most exon. Like cecropins, the magainin peptides are membrane active. Interestingly, two of the peptides, PGLa and magainin, show dramatic synergy upon

combination. The increase in activity was seen at nearly equimolar concentrations of the two peptides, with this mixture being manyfold more potent than either peptide alone (cf. Bevins & Zasloff 1990). Since the frog peptides are released onto the skin surface in copious amounts in response to adrenergic stimulation and skin injury (Giovannini et al 1987, Zasloff 1987), it is reasonable to conclude that the magainin family of peptides probably have an important role in anti-microbial host defence of this mucosal skin surface. However, it has not yet been possible to test directly this assertion.

The magainin peptide family is also highly expressed in cells of the gastric mucosa (Moore et al 1991, 1992) and intestinal tract of *Xenopus* (Reilly et al 1994a,b, Sadler et al 1992). Molecular studies indicate the genes expressed in the digestive tract are the same as those expressed in the skin (Moore et al 1991). The peptides are packaged in intracellular granules, resembling the granules containing them in the skin (Moore et al 1992, Reilly et al 1994a). The working hypothesis is that the peptides are released into the lumen of the gastrointestinal tract, but this needs to be verified.

The expression of this peptide family in the skin and in the digestive tract may form the basis of a more general amphibian mucosal defence system. Although there has been no report offering direct data, one reasonable prediction is that this family might be also expressed in circulating phagocytic cells, on the basis of parallels found in other antimicrobial families.

TAP, a peptide from mammalian respiratory mucosa

As a result of interest in host-defence mechanisms of the respiratory tract, we have proposed that antimicrobial peptides might be present in the tracheal mucosa of mammals. Acid extracts of the bovine tracheal mucosa were found to contain an abundant peptide with potent antimicrobial activity (Diamond et al 1991). The peptide, named tracheal antimicrobial peptide (TAP; Fig. 1), was isolated by a combination of chromatographic separations using antimicrobial activity as a functional assay. TAP exhibits a broad spectrum of activity when assayed *in vitro* against several different strains of microbes, including *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Candida albicans*. This indicates that it is active against both bacteria and fungi. To our knowledge, TAP is the first mammalian antimicrobial peptide of epithelial origin to be isolated.

Analysis of RNA isolated from various bovine tissues indicated that TAP is expressed specifically in the respiratory tract (Diamond et al 1991, 1993). *In situ* hybridization studies localize TAP mRNA to airway epithelial cells. The cDNA and gene encoding TAP were cloned. There is a consensus recognition site for NF-xB 181 nucleotides upstream from the transcriptional initiation site (Diamond et al 1993). This bears striking resemblance to the cecropin genes, as discussed above. In light of these findings, we tested the hypothesis that LPS

induces TAP expression in cultured airway epithelial cells. Preliminary studies have shown a dramatic up-regulation of TAP mRNA following exposure of the cells to LPS (Diamond & Bevins 1994). We suggest that LPS, by way of airborne bacteria, is among the factors that modulates TAP expression in the respiratory tract.

Southern blot and genomic sequence analysis support the hypothesis that the *TAP* gene is a member of a larger family of antimicrobial peptide genes. We noted that many antimicrobial peptide gene families maintain high nucleotide identity in the 5′ region of the mRNA. To determine if this might be true for *TAP*, a Southern blot containing bovine genomic DNA was hybridized with a probe corresponding to the *TAP* 5′ mRNA sequence. Within the bovine genome, there are numerous bands that hybridize to this sequence. The same blot when subsequently hybridized with a probe from the mature peptide-coding region of *TAP* showed a single band, indicating a single copy of the *TAP* gene in the cow genome. The multiple bands obtained with the 5′ probe are consistent with the presence of a group of *TAP*-related sequences in the bovine genome. This was validated upon sequence analysis of several genomic clones obtained as follows. A bovine genomic library was sequentially screened with both the 5′ and the 3′ TAP probes. One phage strongly hybridized with both probes and was found to encode TAP. Preliminary sequence analysis indicates that several of the clones hybridizing to the 5′ probe, but not the 3′ probe, encode TAP-like family members (G. Diamond & C. L. Bevins, unpublished results). In further support of a large *TAP*-like gene family, thirteen antimicrobial peptides closely related to TAP, called β-defensins, have been recently identified by Selsted et al (1993) in bovine neutrophils.

Thus, TAP has potent broad-spectrum antimicrobial activity and is an abundant peptide in the respiratory mucosa. Its expression *in vivo* may be induced by bacterial LPS. TAP is a member of a large family of antimicrobial peptides, some of which are highly abundant in phagocytic cells. These findings support our proposal that TAP, an epithelial peptide of the mammalian respiratory tract, plays an important role in host defence.

Enteric defensins (cryptdins), peptides of mammalian intestinal mucosa

Defensins are cysteine rich, cationic peptides that have been characterized largely through investigations led by Lehrer, Ganz and Selsted (for reviews, see Lehrer & Ganz 1992, Lehrer et al 1991). They were originally isolated as a component of the non-oxidative antimicrobial activity of rabbit lung macrophages (Selsted et al 1983) and now have been isolated from myeloid-derived cells of several mammalian species. Defensins are also membrane active and have *in vitro* antimicrobial activity against bacteria, fungi and enveloped viruses (Lehrer & Ganz 1992). These peptides are 30–35 amino acid residues in length and are distinguished by eleven conserved residues within the sequence, including six

cysteines that participate in intramolecular disulphide bonds. In addition to antimicrobial activity, certain defensins have other biological activities, including monocyte chemotaxis, adrenocortical suppression and eukaryotic cell cytotoxicity (Lehrer & Ganz 1992).

Investigations led by Oullette and colleagues have shown that defensins are expressed in the mouse small intestine. Using a differential hybridization technique, the amount of defensin-related mRNA was shown to be increased in the small intestine soon after birth (Ouellette & Cordell 1988). One isolated cDNA encoded a defensin whose putative protein product was named cryptdin. Cryptdin mRNA was detected throughout the small intestine and was further localized by *in situ* hybridization to the Paneth cells (Ouellette et al 1989). Several enteric defensin peptides subsequently have been isolated from the murine small bowel (Ouellette et al 1992, Selsted et al 1992, Eisenhauer et al 1992). The most abundant enteric defensin, named cryptdin-1 (Fig. 1), corresponds to the previously identified cryptdin cDNA. Interestingly, this peptide has antimicrobial activity towards an avirulent strain of *Salmonella typhimurium* that has a mutation in the *pho P* virulence locus. In contrast, the wild-type, virulent strain of *S. typhimurium* (with an intact *pho P* locus) is resistant to this peptide (Selsted et al 1992). The demonstration that for at least one pathogenic bacterium both *in vitro* resistance to cryptdin and *in vivo* virulence are associated with a single bacterial gene locus suggests enteric defensins have a role in host defence in the mammalian intestine.

In humans, defensins constitute 5–10% of protein in human neutrophils and contribute to the non-oxidative killing of microorganisms by these cells. Four myeloid-derived human defensins have been isolated and characterized (reviewed by Lehrer & Ganz 1992). Studies in our laboratory suggest the family of human defensins is much larger (Jones & Bevins 1992). Our group explored the diversity of the human defensin gene family, using a strategy similar to that discussed above for TAP. We noted a highly conserved nucleotide sequence at the 5' end of defensin mRNA and designed a probe for Southern blot hybridization and for screening a phage library of human genomic DNA. We obtained evidence that the human defensin family is large and two new members were shown to be expressed in Paneth cells of the small intestine (Jones & Bevins 1992, 1993). The co-localized expression in Paneth cells (cells which have been implicated in intestinal host defence by various studies, e.g. Satoh et al 1986 and references therein) of mouse cryptdins and human defensins 5 and 6 suggest these molecules all have similar physiological roles. *In vitro* activity of the human peptides has not yet been tested.

Two possible physiological roles of enteric defensins of the Paneth cell have been suggested (Jones & Bevins 1992, Selsted et al 1992). First, the defensins might regulate the level of luminal microbiological flora. Second, the defensins could be important in mucosal defence from microbial invasion.

PR-39, a proline-rich peptide of the mammalian gastrointestinal tract

One of these peptides serves as a focal point for discussion of proline-rich antibacterial peptides. Agerberth et al (1991) isolated a 39 amino acid peptide from the porcine proximal small intestine, which was rich in proline (49%) as well as arginine (24%) and was therefore named PR-39 (Fig. 1). Two other proline-rich peptides have been identified in bovine neutrophils, named Bac5(PR-42) and Bac7(PR-59) (Frank et al 1990). Certain structural features, such as the presence of Pro–Pro and Pro–Arg–Pro motifs, suggest an evolutionary relationship of the pig intestinal and the cow neutrophil peptides. This possible relationship may be clarified when the structures of the genes encoding these peptides are determined.

PR-39 has antibacterial activity against several strains of both Gram-positive and Gram-negative bacteria, including one strain of *E. coli* pathogenic in pigs. However, some bacteria were resistant. Interestingly, *Proteus vulgaris* and *Pseudomonas aeruginosa* were resistant to PR-39, just as they were resistant to Bac5(PR-42) and Bac7(PR-59) (Gennaro et al 1989).

The mechanism of PR-39 bactericidal activity was recently investigated (Boman et al 1993). In contrast to the killing by membrane disruption seen with most other classes of antimicrobial peptides, Boman and colleagues found that the bacteria were killed by PR-39 through a mechanism that stops protein and DNA synthesis. Kinetic data suggest induced proteolysis in the target bacteria may be important for the bactericidal activity. In addition, it was concluded that the outer membrane of the bacteria served as a penetration barrier, which led to a lag phase of several minutes prior to bacterial killing. It will be important to establish the mechanism of killing by other proline-rich antimicrobial peptides; if the mechanism is distinct from that for the many membrane-active peptides, synergy between these classes of peptides may be observed.

Concluding remarks and future directions

This review is focused on antimicrobial mucosal peptides. Investigations on these mucosal peptides have revealed several recurrent themes in this emerging field. The peptides are generally cationic, amphipathic and membrane active, although their structures vary widely. PR-39 is a notable exception in that it is not membrane active. The peptides are usually members of large families, with some family members expressed in circulating phagocytic cells and others in epithelial cells. Apart from a few consensus residues or short structural motifs, the primary sequences of the mature antimicrobial peptides vary widely even within a family. Surprisingly, the 5' portion of mRNA often contains the highest levels of nucleotide conservation within an antimicrobial gene family. This phenomenon remains to be explained in functional terms, but has facilitated discovery of new family members.

These studies, however, lead us to many new unanswered questions. Might these peptides have other biological activities *in vivo*? What molecular pathways modulate expression of these peptides *in vivo*? Most of the evidence supporting a role in host defence for epithelial antimicrobial peptides, although intriguing, is circumstantial. What studies would directly test this asserted role? Genetic evidence would be invaluable. Identification of a mutation in an antimicrobial peptide gene that renders the organism susceptible to infection would offer a compelling argument. Such a mutation could be spontaneous or introduced through genetic engineering. Two animal model systems that may lend themselves well to these studies are mice and *Drosophila*.

Do epithelial antimicrobial peptides hold promise for therapeutic applications? These peptides are designed to work at epithelial surfaces. Therefore, direct delivery of appropriate peptides to the eye, respiratory tract, gastrointestinal tract or reproductive tract may be a feasible therapeutic modality. The concept of synergy was discussed in two contexts above. In principle, one could capitalize on peptide interactions by creating synergistic mixtures *in situ* through a combination of exogenous and endogenous agents. In addition, it might be possible pharmacologically to modulate levels of endogenous peptides. For example, therapeutic up-regulation of these systems might tip the balance in favour of the host as opposed to the pathogen in some clinical infections.

Several investigators in the field have suggested that the peptides now identified and characterized represent the tip of a large iceberg. In-depth investigations on selected peptides, together with more comprehensive searches for new molecules, will contribute to a much better understanding of this component of host defence.

Acknowledgements

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DISCUSSION

Lehrer: It is striking that each animal we've heard about has a different array of antimicrobial peptides. Have you looked in any animal besides the cow for TAP?

Bevins: When we first identified TAP, we immediately screened human lung cDNA libraries to identify a human homologue. We were unsuccessful. We decided to put off investing more time in this until we understood a little bit more about the biology of TAP, thinking that this might give us some clues. One aspect of TAP biology that may be relevant is the gene's inducibility. This may offer a reasonable explanation for why we didn't find a human homologue: the TAP gene may not have been turned on. We went back this fall to try a different approach which involved cloning at the level of the human genome; in other words, to take our probes and screen a genomic library and determine if there are any human genes or pseudogenes. We have identified some positive clones and we're now in the process of characterizing these clones.

Lehrer: What do you know about the delivery of TAP? Is it constitutively secreted? Is it secreted when a bacterium lands on the tracheal surface?

Bevins: In terms of processing and delivery, which are extremely important questions, our efforts have been hampered because we don't have a good antibody to TAP. We're quite frustrated by not being able to carry out these studies.

Zasloff: Was I right in thinking that there are 12 mouse cryptdins expressed in the Paneth cell?

Ganz: I spoke to Andy Ouellette (Boston, USA) and more have been discovered: it may be close to 20 by now (Huttner et al 1994).

Zasloff: Are each of these encoded by a specific gene?

Ganz: Yes. There is a cluster of multiple genes.

Zasloff: Has each been assigned a discrete DNA sequence?

Ganz: I can't say that each of the individual cryptins has been assigned a sequence, but there are certainly many genes. Nor can I say that there aren't some instances of alternative splicing or some other mechanism that increases the number further beyond the number of genes.

Bevins: It does appear that for defensins, each of the many identified peptides has a corresponding cDNA. Many of the genes encoding those cDNAs have been cloned, but the possibility of RNA editing has not been excluded. Mike Selsted has identified 13 β-defensins in the cow neutrophil (Selsted et al 1993), and at least one of the β-defensin genes that we've isolated encodes one of the neutrophil peptides.

Lehrer: I have a question about the PR-39 work that you have done. Have you localized which cells in the intestine contain this peptide?

Boman: We have tried, but we have not tried very hard. We have had many more problems with studying the localization of cecropin P1. We are relatively

close to finishing the porcine gene sequence for PR-39, and then we will have suitable probes for Northern and *in situ* hybridization experiments. The techniques we have used so far are immunohistochemical and I think that in these studies the fixation is crucial. What are the chances of finding a small peptide with an antibody, if fixation could cause cross-linking that sterically hindered antibody binding? The surprising thing is that these techniques work as often as they do.

Lehrer: There is a problem. We have purified either PR-39 or a very close homologue of PR-39 from white blood cells. Mature white cells will be present in the intestine. So, the question is: is PR-39 also found in the intestinal epithelial cells or might you be using the intestine as a source of white cells? If you rely only on the *in situ* hybridization you will not see it in the white cells because these will be mature cells that are no longer synthesizing RNA. You really have to use a combination of the antibody technique and RNA *in situ* hybridization.

Boman: Storici & Zanetti (1993) recently showed that PR-39 is present in the blood cells and may be being synthesized in the bone marrow.

Hultmark: I'm intrigued by the possible involvement of β-like sites and Rel proteins in the activation of the target peptides. I would like to draw your attention to the possible involvement of similar mechanisms in the cell-specific expression in myeloid cells. Recently, I noticed that MyD88, an early marker for differentiation in myeloid cells, is a third member of the same family as the interleukin-1 receptor and the *Toll* receptor in *Drosophila* (Hultmark 1994). If MyD88 sends signals to members of the Rel family of transcription factors, the implication is that Rel proteins may also be involved in cell-specific expression in the early differentiation of neutrophils and maybe also in the expression of antibacterial peptides in these cells.

Bevins: Have you seen any NF-xB recognition sites within the haemopoietic defensin genes?

Ganz: Not any that were strict consensus. The only way to see if you have NF-xB sites that do not conform to strict consensus is to do binding studies or, even better, to do footprinting with the appropriate cloned protein. But even then it is not reliable because sometimes binding factors will bind sites that have no biological relevance. So you have to do both the biological studies of induction with the appropriate stimulus and the footprinting studies (possibly even *in vivo* footprinting studies) to confirm that the binding actually occurs.

Hultmark: One should be keep in mind that there are several different Rel proteins. They have presumably slightly different specificities for DNA, so it's hard to predict exactly how this xB-like site should look. Also, the Rel proteins are not just involved in the induction by LPS and other such substances. For instance, in the B cell, NF-xB is involved in cell-specific expression but not induction; that may also be the case in the neutrophil. You need not necessarily expect induction.

Ganz: One feature that emerges from looking at the genes encoding these proteins is that the actual microbicidal proteins have been highly variable through the course of evolution. The conserved parts may have more to do with targeting or the folding of the mature protein, an aspect that we don't fully understand yet. The cryptin family of mucosal peptides also shows this. There is a side branch of the cryptin family in which the signal peptide and propiece are conserved, but there is an entirely different mature peptide attached to these in each case. The same is true for the cathelin and defensin families.

Boman: One should keep an open mind on what the function of the propiece may be. I was never convinced by the claim that cathelin was a protease inhibitor. Günther Kreil showed very nicely that, for melittin, the propiece is a time-delay device. Melittin is extremely toxic; consequently, the propeptide is gradually chopped up in a process that serves to deliver the peptide whilst preventing any self damage.

Kreil: This is turning up in a number of situations. We have cloned two of the precursors of opiate peptides from closely related species of South American frogs. Again, the signal peptide is very similar, but the propieces are extremely conserved. There are a few point mutations in the stretch of 60 nucleotides that forms the propiece and then when it comes to the mature part, there are 30 mutations in seven amino acids or something like this—it's bizarre. So you have the feeling that there is something going on with efficiency of processing, targeting or transport.

Flajnik: Murphy (1993) has examined the rate of mutation in defence proteins versus other proteins. Defence proteins evolve three times faster than any other proteins, apparently because there is a constant battle between pathogens and defence molecules. Thus the propeptide and the leader peptides evolve at a constant rate, but the defence molecule is evolving much faster because it's under strong selection. That's probably why in *Rana* and *Xenopus* it's hard to find the orthologous proteins. It's the same in the adaptive immune system with these non-classical MHC class I clusters. You would think if they were performing an essential and evolutionarily old function that they would be very conserved, but they're not. So there are only a few characteristics that you have to conserve to maintain the structure of the defence molecule, but the rest of it can change rapidly, and in fact you want it to change rapidly.

Bevins: This conservation that we talk about in the amino acid sequence of the putative signal and propeptide regions for some of these families is actually less striking when compared with that seen at the nucleotide level. The nucleotide conservation might reflect selective pressure exerted on either the mRNA or DNA. To give a specific possibility, nucleotide conservation at the mRNA level could result from some sort of mechanism of translational regulation. There are well documented examples of tight regulation of mRNA translation mediated by conserved sequence motifs in the 5' end of mRNAs. Another, more speculative possibility is at the DNA level: the highly conserved nucleotide region

may serve as a 'flag', tagging each member of a cluster of defensive genes. This flag could perhaps bring in cellular machinery to mediate mutation in the DNA encoding the mature peptide. In other words, hypermutability could be targeted slightly downstream from this highly conserved sequence. This might offer a mechanism to promote diversity in these specific genes through evolution.

Boman: A recent paper on *Bombyx* immunity (Brey et al 1993) showed that in the induction of cecropin synthesis there is control also after the level of transcription and in the final processing. This is something we can expect to find in more cases. Conclusions based only on Northern blots or analysis of RNA can be misleading as to what is actually happening in the living organism.

Zasloff: This is an anecdote, so it's probably worthless, but over the years as we were studying the stomach and gastrointestinal tract of *Xenopus*, when we had no problem isolating RNA, there were times when we could identify peptides and there were times when we couldn't. I came to the conclusion that there was regulation taking place at the translation level that we just couldn't put our finger on. It is our impression that this goes on. We have yet to prove it.

The reason we were excited by the possibility of translational control was that you could imagine a scenario in which you stored large concentrations of a relatively benign substance—a message—and then triggered its translation into a pretty tough substance to deal with when the time came. This would permit you to decrease the response time of the system.

Bevins: The conservation we see in the introns is relatively low; about what you might expect for random drift through mammalian evolution. Whereas in the 5' region, the conservation is significantly higher. It really suggests positive selection. Conversely, in other regions, there is an intermediate degree of conservation. I think the various portions of the gene are under quite different sorts of constraints.

Ganz: What is the evolutionary relatedness between the α- and β-defensins?

Bevins: Initially, when we isolated TAP, its structural properties and cysteine array were closer to those of the α-defensins than we would have liked, because we were interested in identifying *new* families of peptides. Now, after looking at several bits of data, I've concluded that although these two classes are clearly related, they are distinct. How far back they share an ancestor, or if this is instead an example of convergent evolution, isn't yet clear.

On the one hand, if you look at the gene structure, epithelial α-defensins have two exons similar to TAP. On the other hand, within the defensins the 5' nucleotide sequence is very highly conserved in all the genes that have been isolated, even across species. There is no nucleotide sequence similarity when you compare the α-defensins with TAP and the other β-defensins.

Secondly, the cysteine array, which is highly conserved for each of these two families, is clearly distinct. Furthermore, the human α-defensins map to chromosome 8 and in the mouse they map to a syntenic region on mouse chromosome 8. The cow β-defensin locus appears to map to a non-syntenic

region. Together, all of this evidence suggests that α-defensins and β-defensins are distantly related, if they did indeed share a common ancestor.

Lehrer: Recently, we have been working on chicken leukocytes and have isolated and sequenced completely three antimicrobial peptides (Harwig et al 1994a,b). We call them gallinacins, because the chicken is *Gallus gallus*. Gallinacins are β-defensins, suggesting that the β-defensin family goes back at least 250 million years, before the avian and mammalian lineages diverged. Thus far we have not found classical defensins in the chicken. As far as I know, the cow, which has all of these β-defensins, also lacks classical defensins. It would be interesting to see if we could find an animal that has both families, but for the moment it's either/or.

Bevins: If I was pushed to make a prediction, it would be that in most mammalian species we're likely to find at least remnants of each of these two families in the genome; it's possible that some of the genes simply exist as pseudogenes and are no longer expressed. But I do think you're going to find some evidence for the coexistence of both α-defensins and β-defensins throughout all species of mammals.

Lehrer: We have had another interesting experience with respect to protegrins, a peptide family that we described a few months ago (Kokryakov et al 1993). These are peptides that have four cysteines, and in many ways they resemble tachyplesins more than defensins. Like tachyplesins, protegrins have an amidated C-terminus. When we did sequence analysis, we were really surprised to find in the peotegrins a stretch of 10 amino acids that contained eight residues that were identical to those in one of the defensins (rabbit NP-3A), whereas the other two were very conservative substitutions. We didn't know what to make of this.

Subsequently, Storici & Zanetti (1993) reported the cDNA cloning of protegrin-2 and showed that, like PR-39 and other proteins from pigs and cattle that we've heard about at this meeting, they're made on a cathelin-like precursor, perhaps evidence that they're from a different family.

If so, then we have the disconcerting problem of explaining why the primary sequence homology to NP-3A occurred. NP-3A has been called corticostatin 1 by Solomon and his associates because it has the peculiar ability to bind reversibly and with high affinity to the adrenocorticotropic hormone receptor (Hu et al 1993, Zhu & Solomon 1992). There are many things about this work that bother me. Nevertheless, if protegrins and NP-3A are not of the same family, why does the common sequence exist? We recently found a possible explanation for this, because the protegrins turned out to be LPS-binding peptides that are about as active as polymyxin B in binding LPS. When we went back and tested the various defensins, we found that NP-3A had the best LPS-binding activity amongst the defensins. This might be a very nice example of convergent evolution, converging towards LPS-binding properties. However, other possibilities exist and we are still working on this.

Elsbach: In this new family, which involves the protegrins, Bac5 and a number of others, there is this highly conserved region that the p15s seem to share to some extent as well. Members of this family have been claimed to contain two distinct domains, one that recognizes LPS and Gram-negative bacteria, and another region that people have tried to relate to Cys-protease inhibitory activity (Verbanac et al 1993). What is intriguing is the initial impression that there is this correlation between antiprotease activity and LPS recognition, in one protein. This suggests that there are bifunctional aspects that may have an important role in host defence. There are obviously structural divergences that are not at all related to function, as far as we know. Unfortunately, this provocative notion is now in disrepute, because cathelin, first reported to be a Cys-protease inhibitor by Vito Turk, turns out to have been contaminated by a structurally unrelated protein that is the actual protease inhibitor (Lenarčič et al 1993).

Lehrer: It's interesting to me that you refer to this group as a family. It's not a protein family in the sense used traditionally, where you look for primary sequence homology in the mature protein or peptide product.

Elsbach: There is the primary sequence homology to justify the term family.

Lehrer: In the precursor?

Elsbach: Yes, although p15 doesn't include a precursor. But it does have 30–35% homology with members of the family.

Boman: I have an unorthodox view on the evolution of the antimicrobial peptides. I have had a chance to talk to some of the participants in this symposium about this, and I know that they disagree. For me, it is not a major problem to see a relationship between all the peptides with four or more cysteines. That's why I would put them in one group irrespective of whether there is also an α-helix added on one side of the β-sheet or the other. There is a recent review by Doolittle & Bork (1993) about the evolutionarily mobile modules in proteins. They talk about domains and say that evolution could move domains as exons or by mobile elements. However, these domains are large and are based on sequence homology. Still, when I suggested to Bruce Merrifield that we should make cecropin–melittin hybrids, I believed that the domains could be quite small and also useful.

This is, of course, synthetic work, but I am sure the same thing can happen in Nature. There are many mobile DNA elements in higher animals and there are many retroviruses that could move information for small domains.

With my background as a microbiologist, I learned early on about how plasmids can carry antibacterial resistance and pathogenicity and I saw how easily plasmids and mobile pieces of DNA can pick up information and carry it to other places.

Does anyone believe that the β-sheet peptides, say defensins and insect defensins, have a common origin and constitute a homologous family?

Ganz: I don't believe that. Basically we only have three choices: proteins can be mostly helical, mostly β-sheet, or mostly random—all proteins are going to fall into one of those three categories. This doesn't mean that the proteins within each category are related, necessarily.

Lehrer: Neither do the proline-rich peptides necessarily have a common ancestor.

Ganz: That may be another choice. The proline-rich peptides may have a different helix.

Boman: But there are obviously small peptides with only two cysteines. They form loops and the loops can never turn into β-sheets because they are too small. As far as I know, there are no glycines in the loop which could provide an extra possibility.

Zasloff: We cloned a relative of the esculentin family from *Rana catesbeiana*. This is a peptide of about 20 residues, and it has a heptapeptide loop formed through a disulphide bond at the end of the molecule. It has a group of basic amino acids within that loop. This molecule is a structural analogue of polymyxin (which is an antibiotic peptide made by *Bacillus*). If you look at the heptapeptide loop produced within the polymyxin molecule, you see the same array of basic amino acids. The loop is not formed by a disulphide bridge, but by an intramolecular amide bond. Instead of the N-terminal hydrophobic region in polymyxin, which consists of a fatty acid of about nine carbons, you see a corresponding α-helical, hydrophobic stretch of amino acids which extends about the same length as would an extended carbon chain. Here's an example of two membrane disruptive molecules, which look terribly similar in many ways, one produced by a bacterium, the other produced by a frog.

Kreil: I'm not so sure. We worked with polymyxin in the 1970s. If I recall correctly, in the ring of polymyxin there are both L- and D-amino acids.

Zasloff: Yes, there's a D-valine. So clearly there are differences.

Kreil: And polymyxin has a long fatty acid chain at the N-terminus. In our hands at that time, polymyxin was like melittin on a molar basis, extremely aggressive and lytic. So you really have to check these peptides from different *Rana* species as to what extent they really do the same thing as polymyxin does.

Fagerhol: I would like to draw your attention to a protein that we (Fagerhol et al 1980) described as the leukocyte L1 protein. When we found that it was antimicrobial (Steinbakk et al 1990) and that it bound Ca^{2+}, the name calprotectin was suggested. Its structure, tissue distribution and biological properties have recently been reviewed (Fagerhol et al 1990).

Calprotectin constitutes more than 60% of the soluble protein in the cytosol fraction of neutrophils. As judged from immunohistochemistry, similar amounts are found in the squamous epithelial cells of mucous membranes. We have hypothesized that it consists of two types of polypeptide chain, $L1_H$ (heavy) and $L1_L$ (light) with M_r 13 300 and 8200 respectively. Each of the chains carry one typical and one modified EF hand for Ca^{2+} bindidng. The intact protein

can bind a maximum of six Ca^{2+} ions and has a M_r of about 36 000. On the basis of these results, the most likely structure of the protein is a complex of two heavy chains and one light chain held together by non-covalent bonds.

Among its striking properties is its resistance to heat and proteolysis in the presence of Ca^{2+}: it will only fall apart at temperatures above 90 °C and partial proteolysis in preparation for peptide mapping is difficult unless excess EDTA is added to the buffer.

We have found that calprotectin is antimicrobial against yeast in concentrations of 0.1–4 μM, while somewhat higher concentrations are needed to prevent the growth of bacteria.

Plasma calprotectin levels in healthy adults are about 0.5 mg/l, while increases of 20–100-fold are seen in many types of disease, such as infections, rheumatoid arthritis, cancer and circulatory disturbances. The available data suggest that plasma calprotectin concentrations reflect the neutrophil turnover rate in the body.

This protein is also released from monocytes in culture after the addition of immune complexes, endotoxin, mitogens, ionophores or phorbol ester.

Calprotectin is also present in all biological fluids and excretions. Particurlarly high concentrations are found in stools. In healthy adults the average level is about 2 mg/l, but in response to gastrointestinal cancer or inflammatory diseases of the bowel, in particular Crohn's disease, the concentration may be up to 5 g/l (Røseth et al 1993). Increased stool concentrations are also found in patients with productive purulent cough. Presumably, calprotectin in swallowed neutrophils survives the passage through the gastrointestinal tract. In fact, calprotectin is so stable in stools that measured levels are unaffected by storage of stools for more than seven days at ambient temperature.

We have hypothesized that calprotectin in stools derives from neutrophils which have migrated towards chemotactic substance in the gut lumen, and that this protein may contribute to the regulation of the microbial flora on mucous membranes and, in particular, in the gut.

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Closing remarks

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In the following, I have made an attempt to summarize the state of affairs of antimicrobial peptides:

(1) Many antimicrobial peptides have been found in a variety of animals (and also in plants). More will certainly be discovered.

(2) On a chemical/functional basis, there are already five families of these peptides.

(3) The genes encoding these peptides are of two types: those that are one-peptide-copy genes and those that are multicopy genes.

(4) The antibacterial activity of the peptides is generally of broad-spectrum type. D-amino acid peptides are resistant to proteolysis and in most cases are as active as their natural counterparts.

(5) The targets of most of these peptides are membranes. Target specificity avoids host damage in most cases.

(6) Peptides offer size and rate advantages when compared to immunoglobulins, the complement system and whole cells.

(7) In insects these peptides are the main humoral immune factors. In mammals they kill microorganisms inside phagocytic cells. They may control the natural flora on the skin, in the air ways and in parts of the digestive and reproductive systems.

When I made an estimate last autumn, there were about 50 antimicrobial peptides. This may be nearly 100 by now, and this number is rapidly increasing. They are found in a variety of animals, plants and microbes. More will certainly be discovered. On a biochemical functional basis, there are already five peptide families, and whether Hans-Georg Sahl would like to place his antimicrobial peptides from bacteria into one of these five or into a separate family I will leave up to him. What is important is that the genes for these peptides, as far as they are known, are of two types. There are surprisingly many where there is only one peptide copy in each gene and there are other sequences (without open reading frames) before and after. But there are two cases where although the genes have not yet been worked out, we can conclude from the mRNA that we have multicopy genes. This might be a magnification mechanism, as Peter Casteels has pointed out. If this is true, then we have to explain why this mechanism isn't used more often. Is it just because the evidence is difficult to

find, and that multicopy genes for antimicrobial peptides are in fact abundant, or is there something in the biology that has caused one solution to be favoured over another?

D-enantiomers of antibacterial peptides are very useful tools. They're always trypsin resistant but not always antibacterial. There are a growing number of peptides that have a target. For most of these peptides, the membranes are the target, and they are then equally active when you make the D-enantiomer. I would like to stress that the target specificity avoids host damage in most cases. For circulating peptides it seems to be absolute. For peptides in phagocytic cells it is more relative, but on the other hand the confinement of the peptides to these prevents host damage.

Peptides offer size and rate advantage when compared to conventional effectors of classical immunology—the immunoglobulins, the complement system and the T and B cells. In insect cells, the evidence is fairly compelling that these peptides are inducible immune factors. In mammals it is also very clear that they kill microorganisms inside phagocytotic cells. In big animals, like cows or pigs, we have weaker evidence, but there is an abundance of antimicrobial peptides and you can infer from their localization that they do have an important role. They may control the natural flora and the contaminants of mucosal surfaces.

Concerning control mechanisms, sequences like the xB-binding element are clearly involved in both mammalian and insect immune responses and are perhaps also involved in the control of maturation of blood cells.

To finish, I would like to be slightly provocative. What should we do next? If there are now 50–100 antibacterial peptides, what will we learn from the next sequence that we did not know before? When you are isolating a new peptide, you don't know what you will get, so obviously you are sometimes going to find peptides which will not teach you anything, but it's still something to think about. Can you think of a place where you are likely to find something new?

There is certainly much more to understand about the genes that encode these peptides: very few are so far known. We have learnt this the hard way: when we isolated the first insect cecropin gene in 1988, we could draw almost no conclusions from the sequence. In order to understand the function of genes, you need information from many others for comparison.

Not all antimicrobial substances are peptides: squalamine, for instance, is a steroid. I expect there will be many more non-peptide antimicrobial substances discovered. It is interesting to discover new classes of antibiotics. Now we have peptides, we have steroids—what comes next? Animal antibiotics with new mechanisms of action or new targets are also clearly of interest.

And finally, new information concerning gene organization and peptide biosynthesis is certainly going to be very useful. To understand processing and control of expression are clearly very important goals.

Concerning practical applications, the use of antimicrobial peptides may be a transition phase in the history of medicine. I think the future pharmacology will include agents that can turn on the biosynthesis or the delivery of the peptides in the places where they are needed. This time may come when we understand the gene organization better and know more about tissue differentiation, physiology and so on. I probably won't be around in 20 years, but if the Ciba Foundation is still here then, perhaps it will be the right time and place for a symposium on this type of pharmacology.

Index of contributors

Non-participating co-authors are indicated by asterisks. Entries in bold type indicate papers; other entries refer to discussion contributions.

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