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Innate immune response of *Aedes aegypti*

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Abstract

Insects are able to protect themselves from invasion by pathogens by a rapid and potent arsenal of inducible immune peptides. This fast, extremely effective response is part of the innate immunity exhibited by all insects and many invertebrates, and shows striking similarities with the innate immune response of vertebrates. In *Aedes aegypti* invasion of the hemocoel by bacteria elicits the production of defensins, cecropins, a peptide active only against Gram-negative bacteria, and several other peptides that we are now characterizing. However, not all insects utilize the same peptides in the same concentrations, which may reflect the pathogens to which they may have been exposed through evolutionary time. These protective measures we see in mosquitoes are the current state of the evolution of a rapid immune response that has contributed to the success of insects in inhabiting essentially every niche on earth. The molecules involved in the response of *Aedes aegypti* to pathogens, and the potential role of these peptides against eukaryotic parasites ingested and transmitted by mosquitoes are discussed.

Keywords: *Aedes aegypti*; Innate immunity; Defensin; Cecropin

1. Introduction

When we consider the diverse environments in which insects live, the pathogens to which insects are constantly exposed, and the success of insects in colonizing essentially every niche on the earth, part of their overall success must be attributed to their ability to withstand invasion by microorganisms. This self-protecting immune response, that has undoubtedly played a major role in the success of this class of organisms, is a component of what is now termed innate immunity.

Innate immunity in insects has been described as the germ line-encoded anti-infection response of the host organism (Boman, 1998). In this process immune peptides are produced de novo, usually in the fat body or hemocytes, or are activated from precursors and released into the hemolymph or delivered to other tissues. This response is innate, and generally lacks immunologic memory and the discrete specificity of the antigen:antibody response components of classical immunology (Boman, 1998). A keystone element of innate immunity is the speed with which these responses occur: transcripts can be found within minutes of stimulation, and proteins found within hours. The activity spectrum of the immune peptides is diverse: some peptides such as the cecropins are active against Gram-negative and Gram-positive bacteria, the defensins active mainly against Gram-positive bacteria, and other molecules active only against Gram-negative bacteria. In addition, specific anti-fungal peptides have been identified from *Drosophila* (Fehlbaum et al., 1994; Michaut et al., 1996; Bulet et al., 1999), and it is likely that they also exist in other insects.

Although transcription of most insect immune peptides has been reported to occur in the fat body of the insect, there is growing evidence that other tissues may also be involved in the immune response. The midgut is one site of transcription for defensins from *Aedes aegypti* (Lowenberger et al., 1999b), *Anopheles gambiae* (Richman et al., 1997), and *Stomoxys calcitrans* (Lehane et al., 1997). Transcripts for *An. gambiae* defensin also have been reported from the salivary glands of parasite-infected insects (Dimopoulos et al., 1998), and Brey et al. (1993) described the induction of cecropin transcripts in cuticular cells of *Bombyx mori* after abrading the cuticle, and also in fat body cells remote from the abraded area. Thus, as we explore the immune responses of insects, we find that several tissues and organs may be

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involved, and that structures such as the cuticle, formerly considered solely as a physical barrier to pathogen invasion, may be involved actively in the overall immune response.

Innate immunity is not limited to insects. It is apparent, as more data become available, that antimicrobial peptides are highly conserved members of the innate response of widely diverse taxa, including single celled organisms (Leippe, 1999), various classes of invertebrates (for a review see Hetru et al., 1998), plants (Broekaert et al., 1995) and vertebrates, including mammals, birds, amphibians, and fish (Lehrer et al., 1993; Lehrer and Ganz, 1996). The majority of studies over the last 10 years, however, have been carried out on insects. *Drosophila melanogaster* has become the most extensively studied immune system in insects (Hoffmann and Reichhart, 1997) because of the recent advances in developing mutant lines, and understanding the regulation of developmental processes. Studies on *Drosophila* immune peptide signal transduction and gene regulation have demonstrated startling similarities with the innate immune system of vertebrates (Hoffmann et al., 1999), and consequently we may be able to look at immune responses in insects as the primordial response and progenitor molecules from which have arisen the innate responses of higher organisms. If we can study similar molecules through evolutionarily diverse taxa, we may develop a model for the origins and evolution of the vertebrate innate response.

Our major interest lies in the response of mosquitoes to pathogens and parasites, especially in light of the increasing number of cases of mosquito-transmitted diseases: malaria infects approximately 500 million people, 3 million deaths/year (WHO, 1998), lymphatic filariasis causes morbidity in over 100 million people (WHO, 1999), and tens of thousands of people are affected by arboviruses such as Dengue fever and yellow fever. Several laboratories are vigorously investigating the antimicrobial response of the major disease vector species with the principal aim of these studies to characterize components of the immune response that may be evaluated in terms of anti-parasite activity, with the ultimate aim of generating transgenic mosquitoes that will kill ingested parasites. Several reports have suggested that these immune peptides have a negative effect on the development and transmission of mosquito-borne parasites (Chalk et al., 1995a,b; Lowenberger et al., 1996; Lowenberger et al., 1999b; Shahabuddin et al., 1998), however, the role of individual peptides, or the synergistic effects of several peptides, on these parasites has not been resolved.

Be it through the use of one, several individual molecules, or the combined effects of several immune peptides, the overall goal of the innate response is a rapid and complete elimination of invading pathogens or their relegation to nonpathogenic members of the natural flora of the insect (Boman, 1998). One of our objectives is to isolate and characterize the induced immune peptides of *Ae. aegypti* and to evaluate them in terms of antimicrobial and anti-parasite activity. To this end this review will focus on the immune peptides we have isolated from this mosquito species, compare them with peptides from other species, and outline future directions of this research.

2. Methodologies

In the majority of studies on invertebrate immunity, the host organisms are injected with a combination of Gram-negative and Gram-positive bacteria, and at various time points, most commonly 24 h after inoculation, the tissues, hemolymph, or whole bodies are collected, ground up, the proteins extracted and the resulting products analyzed using standard HPLC techniques. This is the technique we used to isolate 3 isoforms of insect defensin (Lowenberger et al., 1995) and cecropin (Lowenberger et al., 1999c) from *Ae. aegypti*. These processes are labor intensive. During the initial experiment we inoculated 1500 mosquitoes with bacteria, 1500 with sterile saline, and 1500 naïve mosquitoes. After proteins are extracted, and processed through HPLC, individual peaks are collected and assayed for antimicrobial activity in standard assays (Bulet et al., 1993). Positive fractions are then subjected to HPLC purification, mass spectrometry, Edman degradation and sequencing to give a partial amino acid sequence. Degenerate primers then can be designed to amplify the sequence from cDNAs, or libraries to isolate the complete cDNA or gene. Recently, several techniques have been used to reduce the time consuming process of rearing and inoculating large numbers of insects. The laboratory of Ann Fallon in Minnesota has successfully developed a model system to induce the expression of mosquito immune peptides in mosquito cell lines by exposing them to bacteria (Hernandez et al., 1994; Gao et al., 1999; Sun et al., 1998, 1999). The supernatants can then be collected and processed. This technique provides much greater quantities of peptide for processing than does inoculating individual mosquitoes.

With advances in technology, we are able now to assess peptides/proteins using ever-decreasing amounts of raw material. Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF) allows us to look at the production of immune peptides in hemolymph from individual insects. This technique has been used successfully by Uttenweiler-Joseph et al. (1997, 1998) to identify peptides induced in *D. melanogaster* by the introduction of bacteria into the hemocoel. We have used this technique in *Ae. aegypti* (Fig. 1) to demonstrate the induction of several peptides in response to bacterial challenge. Although this technique

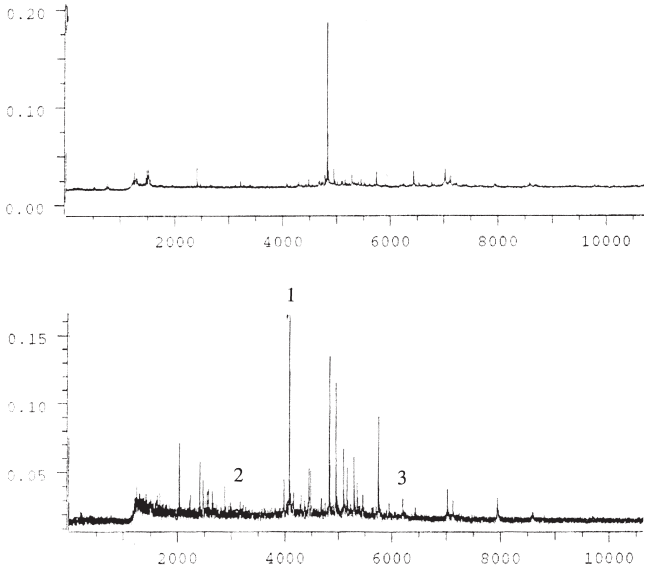

Fig. 1. Differential MALDI-TOF MS analysis of hemolymph from a control (top) and an immune activated mosquito (bottom) 24 h after inoculation with bacteria. Among the peaks induced by immune activation are defensin, cecropin, and a peptide active against Gram-negative bacteria, numbered 1–3 respectively.

is qualitative only, it does allow us to refine the HPLC processing step when we know the mass of the peptides being produced by the insects.

3. Immune peptides in *Ae. aegypti*

3.1. Defensins

Insect defensins were first described simultaneously and independently from *Sarcophaga peregrina* by Matsuyama and Natori (1988a,b) and *Phormia terranovae* by Lambert et al. (1989). Since then over 30 defensins have been reported from several insect orders including Diptera, Coleoptera, Hemiptera, Hymenoptera, Trichoptera, and Odonata (see reviews by Hetru et al., 1994, 1998). The peptides isolated from the Diptera (Fig. 2) share 68–98% similarity at the amino acid level and mosquito defensins have about 35% shared identity with those isolated from the dragonfly, *Aeshna cyanea*, which evolved about 100 million years before the emergence of the higher Diptera, but the defensin from *A. cyanea* shares about 75% identity with the defensin isolated from scorpions as determined by Clustal analysis (DNAStar). These comparisons, along with the similarities of insect defensins with amoebae (Leippe, 1999), nematodes (Kato and Komatsu, 1996), scorpions (Cociancich et al., 1993; Ehret-Sabatier et al., 1996), molluscs (Charlet et al., 1996; Hubert et al., 1996; Iwanaga et al., 1998), mammals (Ganz and Lehrer, 1994) and plants (Broekaert et al., 1995), suggest an ancient origin of this molecule that has been maintained in the innate immune response of these organisms through evolutionary divergence.

Defensins are cationic peptides approximately 40 amino acids in length, and are characterized by 6 cysteine residues arranged in three intramolecular bridges, giving the 3-D structure consisting of an N-terminal loop, an α-helix, and two twisting antiparallel β-sheets (Hetru et al., 1998). The three dimensional structure of insect defensins has been described by Cornet et al. (1995) and is available on the NIH web page (http://www.ncbi.nlm.nih.gov/entrez/query). The activity spectrum of mosquito defensins is primarily against Gram-positive bacteria (Lowenberger et al., 1995), although some Gram-negative bacteria also are sensitive.

Insect defensins were the first immune peptides isolated from *Ae. aegypti* (Chalk et al., 1995a,b; Lowenberger et al., 1995). Subsequently, Cho et al. (1996) isolated full length cDNA clones and sequenced the gene from *Ae. aegypti*, including 1400 bp upstream of the coding region (Cho et al., 1997), and Gao et al. (1999) isolated defensins from cell lines of *Ae. aegypti* and *Ae. albopictus*. Defensins also have been isolated and characterized from *An. gambiae* (Richman et al., 1996; Dimopoulos et al., 1997, 1998).

Three isoforms of insect defensins have been reported from *Ae. aegypti*, and their transcription profiles characterized (Lowenberger et al., 1999a). In the samples obtained from whole bodies of immune-activated *Ae. aegypti*, isoforms A and B were found in equal amounts whereas isoform C was found at levels only one fifth of isoforms A and B. In addition, the signal peptide and pro-defensin regions of isoforms A and B are so similar that we believe that these are allelic variants of one gene. Isoform C, however, has a different length signal peptide, and modifications within the signal peptide and propeptide region and we believe it is encoded by a different gene. These genes also have a distinct transcription profile: only transcripts for defensin C were found in the midguts of naïve *Ae. aegypti* (Lowenberger et al., 1999b) whereas isoforms A/B were transcribed very strongly in the fat body after immune activation. These differences in hemolymph concentration and transcription site may reflect tissue specific expression patterns for the different genes. In addition to *Ae. aegypti*, midgut expression of defensin has been reported in *An. Gambiae* in response to parasite invasion (Richman et al., 1997) and in *S. calcitrans* (Lehane et al., 1997).

In immature stages of *Ae. aegypti* we find, by Northern analysis, transcripts for defensins only in callow pupae (Lowenberger et al., 1999a). These data suggest that the hemocoel from the larvae we examined did not contain bacteria, because when larvae are injected with bacteria, transcription occurs rapidly (Richman et al., 1996). Why then would a callow pupa, a transient stage in itself, up-regulate defensin only for the period before sclerotization and melanization of the cuticle, when 12

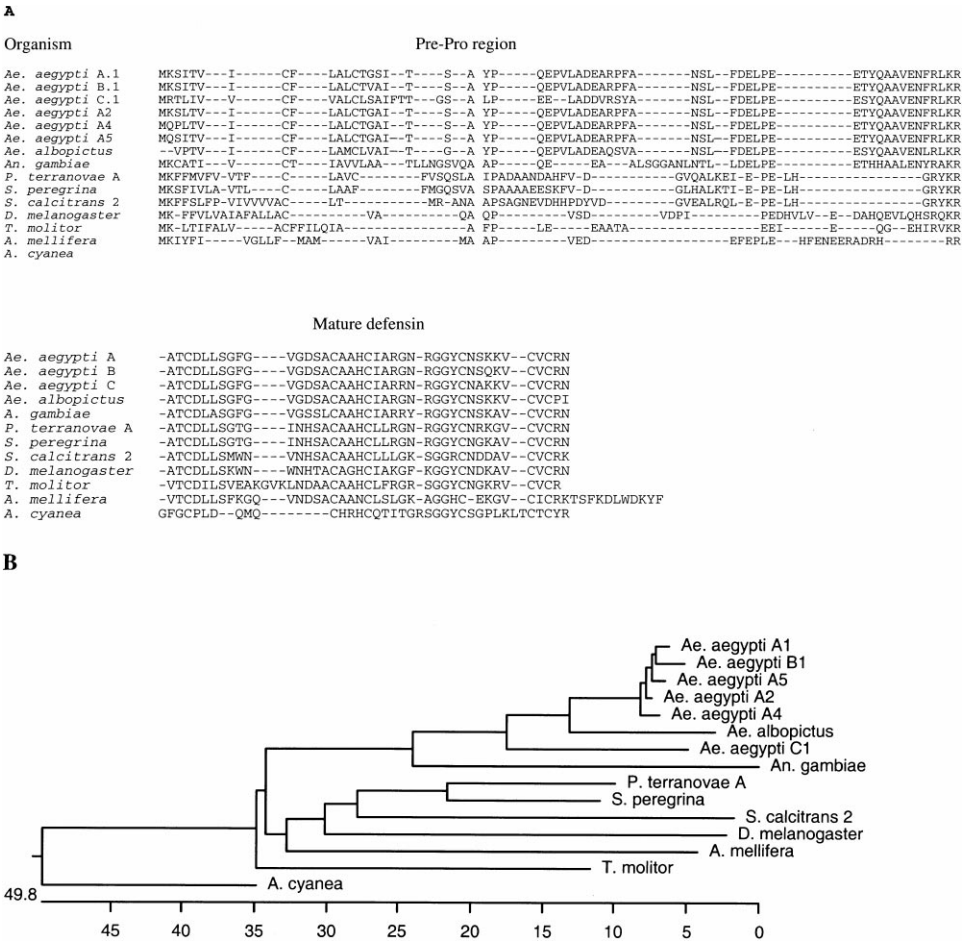

Fig. 2. Comparison of cDNA sequences from insect defensins. (A) Alignment of deduced sequences, and (B) phylogenetic relationship of the defensin cDNAs. Alignments and phylogenetic relationships were done using DNASTAR Megalign module using the Jotun–Hein algorithm with PAM250 weight table. The length of each pair of branches represents the distance between sequence pairs and the scale beneath the phylogenetic indicates the number of substitution events.

h after emerging as pupae, we can find essentially no defensin transcripts in the black pupae? We hypothesized (Lowenberger et al., 1999a) that there were two distinct possibilities: (1) that the cuticle on the white pupae is so tender that small tears in the soft tissue may allow the entry of pathogens from the bacteria-laden medium in which the larvae and pupae live, or alternatively, (2) during the transformation from larva to pupa to adult, there is a histolysis of the larval gut that is replaced by an adult gut, and during the breakdown of these tissues, bacteria may be released from the larval gut into the hemocoel, activating the innate immune response of the pupae. With defensin as our only immune gene marker it was difficult to determine which, if either, of these two hypotheses was correct. With the identification of other immune peptides, especially cecropin (Lowenberger et al., 1999c) we then were able to compare and contrast the transcription of these two immune peptides (see below).

3.2. Cecropins

Cecropins were the first immune peptides fully characterized from insects by Steiner et al. (1981). Since then at least 20 cecropin molecules have been identified from insects and two from mammals (see review by Hetru et al., 1998), and more recently three cecropin like molecules, styelins, have been isolated from the tunicate *Styela clava* by Zhao et al. (1997) (Fig. 3). Cecropins, like defensins, are inducible peptides with a mass of ~4 kDa. However, in contrast to defensins, cecropins lack cysteine residues, and are made up of two alpha helices linked by a short hinge. Characteristically the N-terminal region of insect cecropins is an amphipathic α-helix comprising hydrophobic and hydrophilic regions, whereas the C-terminal region is more hydrophobic. The helical structure of cecropins was first demonstrated by Steiner (1982) using circular dichroism spectroscopy of cecropin A from *H. cecropia*. Cecropins tend to have

(A)

Organism	Pre-proregion	Mature peptide
DIPTERA		
Ae. aegypti	MNFTKLFLLIAVAV-LLLTGQSEA	GGLKKLGKKLEGAGKRVFNAAEKALPVVA--G-AKALRK
Ae. albopictus A	MNFTKLFAIVLLAA-LVLLGQTEA	GGLKKLGKKLEGVGKRVFKASEKALPVAV--G-IKALGK
Ae. albopictus B	MNFNKLFALVLLIGLVLLTGQTEA	GGLKKLGKKLEGVGKRVFKASEKALPVLT--G-YKAIGK
Ae. albopictus C	MNFTKIFVLIAMAA-LLLVGQSEA	GGLKKLGKKLEGAGKRVFNAAEKALPVVA--G-AKALGK
An. gambiae	MNFSKIFIFVVLAV-LLLCSQTEA	GRLKKLGKKIEGAGKRVFKAAEKALPVVA--G-VKAL*
D. melanogaster A	MNFYNIFVFVALIL-AITIGQSEA	GWLKKIGKKIERVGQHTRDATIQ-GLGIP--QQAANVAATARG*
D. melanogaster B	MNFNKIFVFVALIL-AISLGNSEA	GWLRKLGKKIERIGQHTRDASIQ-VLGIA--QQAANVAATARG*
S. peregrina IA	MNFQNIFIFVALIL-AVFAGQSEA	GWLKKIGKKIERVGQHTRDATIQ-GLGIA--QQAANVAATARG*
C. capitata A	MNFNKVFILVAIVI-AIFAGQTEA	GWLKKIGKKIERVGQHTRDATIQ-TIAVA--QQAANVAATARG*
LEPIDOPTERA		
H. cecropia A	MNFSRIFFFVFACLTALAMVNAAPEP	KW--KLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAKG*
H. cecropia B	MNFSRIFFFVFALVLALSTVSAAPEP	KW--KVFKKIEKMGRNIRNGIVKAGPAIAVLGEAKAL*
A. pernyi B		KW--KIFKKIEKVGRNIRNGIIKAGPAVAVLGEAKAL*
M. sexta B-5P	MNFSRVLFFVFACLSAFAMASAAP	-W--NPFKELERAGQRVRDAVISAA-AVATVGQAAAIARGG*
B. mori A	MNFVRILSFVFALVLALGAVSAAPEP	RW--KLFKKIEKVGRNVRDGLIKAGPAIAVIGQAKSLGK*
B. mori D	MKFSKIFVFVFAIVFATASVSAAP	G---NFFKDLEKMGQRVRDAVISAAPAVDTLAKAKALGQG*
T. ni A	MNLVKILFCVFACLVFT--VTAVPEP	RW--KFFKKIEKVGQNIRDGIIKAGPAVAVVGQAASITGK*
H. cunea A	MNFSRIIFLVFACFVALASVSAAPEP	RW--KIFKKIERVGQNVRDGIIKAGPAIQVLGTAKALGK*
H. cunea A3	MNFSRILFFMFACFVALASVSAVPEP	RW--KVFKKIEKVGRHIRDGVIKAGPAITVVGQATALGK*
TUNICATE		
S. clava C	MQMKATILIVLVAL-FMI-QQSEA	GWFGKAFRSVSNFYKKHKTYIHAGLSAATLLGDMTDEEFQEFMQDIEQAREEELLSRQ
S. clava D	MQMKATILIVLVAL-FMI-QQSEA	GWLRKAAKSVGKFYYKHKYYIKAAWQIGKHALGDMTDEEFQDFMKEVEQAREEELQSRQ

(B)

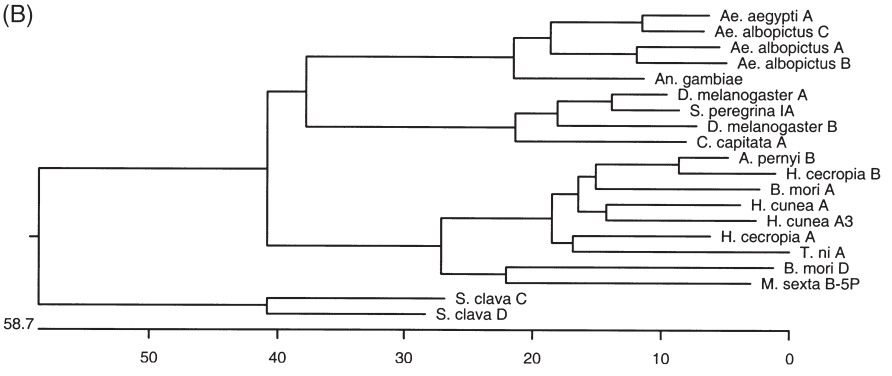

Fig. 3. Comparison of cDNA sequences from insect and tunicate cecropins. (A) Alignment of deduced sequences, and (B) phylogenetic relationship of the cecropin cDNAs. Alignments and phylogenetic relationships were done using DNASTAR Megalign module using the Jotun–Hein algorithm with PAM250 weight table. The length of each pair of branches represents the distance between sequence pairs and the scale beneath the phylogenetic indicates the number of substitution events.

unordered structures in solutions but adopt the helical structure in hydrophobic conditions (Shai, 1998). Subsequent studies using NMR demonstrated that the hinge region, that separates the two helices of the cecropin molecule isolated from *H. cecropia*, consists of an alanine–glycine–proline sequence. The structure and composition of the α-helix is an important determinant of the activity of the peptide because amino acid substitutions that alter the helical structure may cause a reduction in the potency of the peptide (Gazit et al., 1995; Steiner et al., 1988).

In mosquitoes there were reports of cecropin like sequences (Knapp and Crampton, 1990), and a peptide isolated from an immune-challenged *Ae. albopictus* cell line that demonstrated cecropin-like characteristics (Hernandez et al., 1994). Sun et al. (1998, 1999) provided the first detailed description of a mosquito cecropin from an *Ae. albopictus* cell line, and subsequently we reported the isolation of a cecropin molecule from the hemolymph of immune-activated *Ae. aegypti* (Lowenberger et al., 1999c) and *An. gambiae* (Vizioli et al., 2000). We since have sequenced another cecropin molecule from a cDNA made from the whole bodies of immune-activated *Ae. aegypti*. This cDNA sequence differs minimally from the published sequence (Lowenberger et al., 1999c). We were unable to design primer pairs to distinguish between these two sequences, but could do so using restriction enzyme analysis (Fig. 4). DNA from individual mosquitoes was extracted (Severson and Kassner, 1995) and used as template in a PCR reaction using primers that amplify the entire

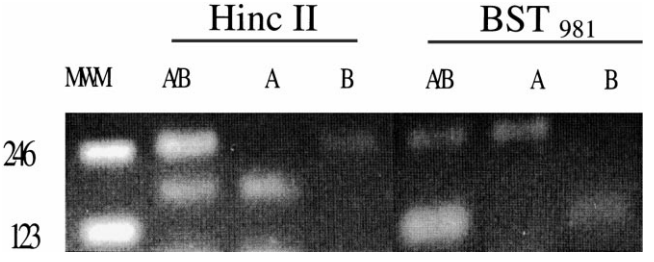

Fig. 4. Picture of a gel demonstrating differential digestion of two *Ae. aegypti* cecropin sequences amplified from genomic DNA from individual mosquitoes. The 250 bp fragment was purified and digested with HincII or BST_{981}. Lane A/B contains a sequence that is cut by both enzymes, indicating the presence of each sequence of cecropin while lanes A or B contain sequences that are cut completely or not at all by HincII or BST_{981}, indicating the presence, in a single mosquito, of cecropin forms A, B, or both A and B.

coding region of our published cecropin sequence (Lowenberger et al., 1999c). These PCR bands were excised from a low melting agarose gel, purified, and subjected to restriction analysis. Two enzymes, A and B, were used. Enzyme A cuts in the published sequence and not in the novel sequence, and enzyme B cuts in the novel sequence but not in the published sequence. An individual mosquito may have cecropin A, cecropin B, or both sequences, suggesting that these sequences are alleles of one gene. In addition, we have only found one transcript in our Northern analysis, but RT–PCR using RNA from immune activated mosquitoes generates both sequences, suggesting both sequences are transcribed.

A characteristic of insect cecropins has been the presence of a tryptophan residue at position 1 or 2, and amidation of the C-terminus. However, none of the cecropin molecules reported from mosquitoes has a tryptophan residue in this location, and only some are reported to have C-terminal amidation. The activity spectrum of the *Ae. aegypti* cecropin was similar to the cecropin isolated from *D. melanogaster*, however, the mosquito molecule was generally less active than the fruit fly cecropin that contains the tryptophan residue at position 2, and is amidated (Lowenberger et al., 1999c). Whether the difference in potency in the molecules from these Diptera is due to these different characteristics is not known. The replacement of tryptophan by a non-aromatic residue in a synthetic cecropin analog strongly reduced its activity (Andreu et al., 1983, 1985), and amidated cecropins isolated from *S. peregrina* had a 3–4 fold higher activity against bacteria than did non-amidated molecules (Li et al., 1988). Similar data from *H. cecropia* report a lowered activity in non-amidated cecropins (Andreu et al., 1983; Merrifield et al., 1982), suggesting that these factors may contribute to stability and activity of the molecule, be they purified or synthesized (Callaway et al., 1993).

Northern analysis of cecropin in *Ae. aegypti* provided us with another marker to look at levels of constitutive transcription for immune peptides and those that occur after immune activation. As was the case with defensins, there is a strong and rapid transcription of cecropin in adult *Ae. aegypti* after injection with bacteria (Lowenberger et al., 1999c). However, whereas transcription for defensin lasts in excess of 21 days, that for cecropin is only minimally detectable 10 days after immune challenge (Lowenberger et al., 1999c). No transcripts are detected by Northern analysis in any developmental stage (Lowenberger et al., 1999c), in contrast to the defensin transcription detected in callow pupae. These data add credibility to the suggestion that defensin transcription in callow pupae may be a developmentally regulated process.

In comparing the cecropin sequences, those from mosquitoes share >80% identity, whereas the level of shared identity between *Ae. aegypti* and *D. melanogaster* is 35%. If we analyze different regions of the molecule, the cecropin from *Ae. aegypti* shares 87% identity with molecules from other, non-mosquito Diptera over the initial 13 amino acids, but shares only 2 amino acids in the C-terminal region (Fig. 3).

3.3. Transferrins

Transferrins are a family of proteins usually considered as iron transport and storage molecules in insects. Transferrins have been identified from *M. sexta* (Bartfeld and Law, 1990), *S. peregrina* (Kurama et al., 1995), *D. melanogaster* (Yoshiga et al., 1999) and *Ae. aegypti* (Yoshiga et al., 1997). In *D. melanogaster* and *Ae. aegypti*, there is an increase in transcription for transferrin after inoculation with bacteria (Yoshiga et al., 1997, 1999) and it has been hypothesized that insects use transferrin to sequester iron from pathogens, thus reducing their ability to establish an infection (Yoshiga et al., 1997). This hypothesis is strengthened by the discovery that insect transferrins contain the characteristic iron binding domain in the N-terminal region but typically lack the iron-binding domain in the C-terminal region of the molecule, a modification that have may arisen to prevent iron piracy by microorganisms (Yoshiga et al., 1997). In *Ae. aegypti* there is an increase in transferrin transcription after inoculation with bacteria, by sterile wounding, and after filarial worms were injected into the hemocoel (Beerntsen et al., 1994). However, the processes of injection, by which the bacteria or pathogens were introduced into the insects, also can result in increased transcription. In another mosquito, *Armigeres subalbatus*, a similar response occurs when bacteria are injected into the hemocoel. However, *Ar. subalbatus* kills microfilariae of the filarioid nematode *Brugia malayi* acquired in a bloodmeal by a melanotic encapsulation response. We have found (Falk, Lowenberger, Kamal, Neumann and Christensen, unpublished data) that concomitant with the melanotic encapsulation response is a significant increase in transcription for transferrin, suggesting once again a role for this molecule in the immune response of mosquitoes to metazoan parasites as well as microorganisms.

4. A mosquito is a mosquito is a mosquito?

The immune response of insects in general, and specifically mosquitoes, is not a standardized response. Although all insects are capable of producing immune peptides belonging to the same families, the expression of specific peptides, or the concentration of the peptides differs among species, families, and orders of insects.

In *Ae. aegypti*, defensins are found at concentrations of about 45 μM (Lowenberger et al., 1999a), whereas cecropins are found at approximately 1–5 μM

(Lowenberger et al., 1999c). In *An. gambiae*, defensins and cecropins are both found in the 1–5 μM range (Richman et al., 1996; Vizioli et al., 2000). In *D. melanogaster*, the concentration of cecropin, defensin, and drosomycin are 20 μM, 1 μM and 100 μM respectively (P. Bulet, personal communication). Whether this differential response is due to the same molecule (i.e. defensin) being more potent in one species than in another, or whether defensin is the major antimicrobial peptide in *Ae. aegypti* while a yet to be identified peptide is the major antimicrobial factor in *An. gambiae* is not clear.

Why do different species preferentially use one immune peptide over another, or produce the same peptide in different concentrations in the hemolymph? Do these differences reflect differences in the environments in which the insects live, the microorganisms to which they normally are exposed, or to which they have been exposed through evolutionary time? The specific niche and pathogen to which these different organisms have been exposed routinely may have played a role in the evolution of the immune response we see today. In the case of defensins (Fig. 2) and cecropins (Fig. 3) we have demonstrated a different phylogenetic origin of these peptides from terrestrial and aquatic insects. Ideally we could test the effects of the pathogens normally found in one environment on the organisms that exist in the other, but these studies may prove exhaustive.

It is clear that several peptides are up-regulated in *Ae. aegypti* in response to the introduction of bacteria or wounding (Fig. 1). We currently are completing the sequencing the cDNAs of two novel immune peptides that are active only against Gram-negative bacteria. One of these molecules is constitutively expressed at low levels in *Ae. aegypti* whereas the other is only found after immune activation. Future studies will involve characterizing these peptides in terms of antimicrobial spectrum of activity. One of our limitations in determining why certain species rely on different immune peptides is our limited knowledge of the mode of action of these molecules. The precise manner in which some of the insect antimicrobial peptides act is not fully understood (Boman, 1995), but the interaction between the peptides and lipid membranes is paramount to success, rather than receptor-mediated specific recognition processes. The amphipathic nature of the helices allows permeation of the bacterial cell wall, based on the ability of cecropins to form channels in planar liquid membranes (Cruciani et al., 1992), and many studies have demonstrated the ability of these peptides to permeate model phospholipid membranes (Saberwal and Nagaraj, 1994) either by the formation of pores directly, or by a disruption of the lipid in a detergent-like manner (Shai, 1995, 1998).

5. Effects of immune peptides on eukaryotic parasites

Recently several research groups have reported the effects of insect immune peptides, or the general immune activation of mosquitoes, on the subsequent response of these insects to parasites. Gwadz et al. (1989) injected cecropins and magainins into mosquitoes and reported a significant reduction in *Plasmodium* development. Since that report several papers have reported similar results: Jaynes et al. (1989), Rodriguez et al. (1995), Possani et al. (1998), and Boisbouvier et al. (1998), Rodriguez et al. (1995), Possani et al. (1998) and Boisbouvier et al. (1998) injected cecropins or a modified synthetic cecropin molecule, Shiva, into mosquitoes and reported reduced development of malaria parasites in mosquitoes. Similarly the co-injection of cecropins (Chalk et al., 1995a,b) or defensins (Albuquerque and Ham, 1996) with *Brugia pahangi* into mosquitoes was reported to reduce parasite establishment. The injection of defensin at specific time points after bloodfeeding was reported to reduce the mean intensity of infection, but not the prevalence, of *P. gallinaceum* in *Ae. aegypti* (Shahabuddin et al., 1998). We previously reported that immune activation of mosquitoes, via bacterial inoculation 24 h prior to parasite exposure, reduced both the prevalence and mean intensity of infection with *B. malayi* and *P. gallinaceum* in *Ae. aegypti* (Lowenberger et al., 1996, 1999b) and *P. berghei* in *An. gambiae* (Lowenberger et al., 1999b). In the studies with filarial worms, the prevalence was reduced from 92–97% to 50–57%, and the mean intensity of infection from 8–16 worms to 2.5 worms/infected mosquito. Because we found dead, although not melanized, microfilariae in the hemocoel of immune-activated mosquitoes, we assumed that a hemolymph factor killed the parasites after they penetrated the midgut and entered the hemocoel.

In the studies with *Plasmodium*, the timing of events was crucial. Only mosquitoes inoculated with bacteria before, or immediately after exposure to an infectious bloodmeal, showed a reduction in parasites as measured by the number of oocysts on the midgut. Mosquitoes inoculated 1–5 days after ingesting parasites showed no significant reduction in prevalence or mean intensity of infection. These results suggested that a pre-oocyst stage, probably the ookinete, was affected by a product of immune activation, and that once the oocyst was formed, that this factor had no effect. These data seem to contradict those of Shahabuddin et al. (1998) who reported a reduction in viable oocyst numbers when the mosquitoes were injected with a defensin peptide purified from the dragon fly, *Aeshna cyanea* or the flesh fly, *P. terranovae.* In this study, there were no effects of the purified defensins on ookinetes or early oocysts but later oocysts (day 4 after bloodfeeding) and sporozoites were

susceptible to the purified peptides (Shahabuddin et al., 1998). The possibility exists that in our study, using a generalized immune activation, that several peptides were turned on, one or more of which combined to kill the pre-oocyst stage, whereas the injected purified peptide was sufficient to affect late stage oocysts.

Inherent with the experimental design of all of these studies was the need to inject the parasites, bacteria, or peptide into the insect and our interpretation of the results is confounded by our inability to distinguish all the events that occur solely due to the injection process alone, regardless of the compounds being injected. In our previous studies we immune-activated mosquitoes by injecting bacteria, and then only determined the transcriptional profile of defensins or cecropins, because at the time these were the only peptides for which we had sequence. We need a system that will allow us to determine in vivo what peptides, acting alone or in concert, are affecting the development of parasites as described in the cited papers. The development of Sindbis virus as a transient expression system in *Ae. aegypti* by the Arthropod and Infectious Disease Laboratories (AIDL) at Colorado State University has provided us with the ability to infect mosquitoes with a Sindbis virus designed to express one peptide, and determine the effects of this peptide on parasite development. This system has been used successfully to engineer resistance to dengue -2 virus (Olson et al., 1996), and yellow fever (Higgs et al., 1998) and to express the reporter gene, GFP, in *Ae. aegypti* larvae and adults (Higgs et al., 1996, 1999). The development of an orally infectious double subgenomic construct now allows the virus to enter the insect via ingestion instead of injection (Seabaugh et al., 1998).

We have used this technique, modified after Higgs et al. (1999), to infect mosquito larvae with the viral construct expressing *Ae. aegypti* defensin isoforms A and C, and confirmed the presence of mature peptide in the midgut and hemolymph by Western analysis after adult emergence, and after bloodfeeding (Cheng, Beaty, Higgs, Olson, Lowenberger, Myles, Vizioli, Beaty, and Christensen, unpublished data). The results demonstrate that the hemolymph of most virus infected mosquitoes contains significant amounts of defensin, and we are in the process of determining precise concentration, activity spectrum, and potency of the peptide produced in this manner. Because infecting mosquitoes with Sindbis virus in this manner does not induce transcription for cecropin, this technique will allow us to determine the effects of individual peptides on parasite development within the mosquito without the need to activate all components of the immune arsenal.

6. Defence budget

What is the cost to an insect of mounting an immune response? There is no doubt that the production of immune peptides is costly in terms of energy and resources. Ferdig et al. (1993) demonstrated that mounting a melanotic encapsulating response to microfilariae of *B. malayi* was extremely costly to *Ar. subalbatus* with the time required for egg development and the time to oviposit being increased. Yan et al. (1997) demonstrated that being refractory to a parasite had a cost in terms of size, volume of bloodmeal taken, and number of eggs layed/gonotrophic cycle.

Few studies have analyzed the cost of mounting a response to bacterial invasion; we assume that the insect must eliminate the infection, or succumb to it. Shai (1998) suggests that the main advantage of the innate system employing immune peptides is that this system functions without high specificity or memory requirement, the peptides are easily stored and readily available after an infection, and are rapidly synthesized at low metabolic cost to the host. But to a small insect how is low metabolic cost defined? If one assumes that the cost of forming a peptide bond is constant, and that immune peptides such as the defensins are good examples (~40 residues) that are synthesized as pre-pro proteins (~100 residues), then one can estimate the energy requirements to synthesize a single peptide (Boman, 1998). This must also encompass the 2–10 genes required for processing, activation etc. This cost is likely paltry compared to establishing the mechanisms to synthesize immunoglobulins, and all the genes required for IgM and IgG production (Boman, 1998). It has been estimated (Boman, 1998) that the cost to produce the innate immune response in insects requires at least 100 times less DNA than is the case in mammals. In addition, the pre-propeptides can be made at rates 100 times faster than IgM, and small peptides can diffuse more quickly than can antibodies (Boman, 1991).

Insects utilize this system that is rapid and amazingly effective against a wide range of microbial infections, and this is the ultimate goal of the innate response: eliminate the pathogen. And for this response to be successful, it must be rapid enough to produce peptides at speeds that exceed the rate of multiplication of the pathogen (Boman, 1991). Alternatively some organisms may have incorporated invading bacteria into integral components of their normal symbiotic flora. However, these symbionts do not normally inhabit the hemocoel. Should these or other microorganisms enter the hemocoel they elicit the immune response comprising phagocytosis by hemocytes, and the production of potent antimicrobial peptides. The evolution of this response has been tried and tested over the millenia. The responses we are now analyzing in insects, and particularly in mosquitoes, is the cost-effective mechanism by which these insects have maintained their existence and proliferated in environments laden with potential pathogens.

In our studies questions arise as to the evolution of the parasite–mosquito relationship, and the response of

mosquitoes to parasites ingested in a bloodmeal. Are there specific anti-parasite molecules involved that determine whether a species, strain, or individual mosquito will serve as a vector for a specific parasite? Or is there simply a physiological incompatibility between the majority of mosquitoes and parasites that cause disease in humans? Is there a manner to determine, emulate, and copy an immune response that will allow us to understand more deeply the innate response of mosquitoes, the mechanisms that trigger this response, and the signaling molecules involved in the production of an anti-parasite response? These questions will form the basis of concentrated research over the next decades.

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