

Reassessing the role of defensin in the innate immune response of the mosquito, *Aedes aegypti*

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Abstract

Defensin is the predominant inducible immune peptide in *Aedes aegypti*. In spite of its activity against Gram-positive bacteria *in vitro*, defensin expression is detected in mosquitoes inoculated with Gram-positive or negative bacteria, or with filarial worms. Defensin transcription and expression are dependent upon bacterial dose; however, translation is inconsistent with transcription because peptide is detectable only in mosquitoes inoculated with large doses. *In vitro* translation assays provide further evidence for post-transcriptional regulation of defensin. Clearance assays show that a majority of bacteria are cleared before defensin is detected. In gene silencing experiments, no significant difference in mortality was observed between defensin-deficient and control mosquitoes after bacteria inoculation. These studies suggest that defensin may have an alternative function in mosquito immunity.

Introduction

The inducible immune peptides that exhibit antimicrobial activity have long been considered a primary defence element in the innate immune response of insects. These antimicrobial peptides, such as defensin, have received a great deal of research attention, in part because of their potential to impede parasite development in insects that vector such

devastating diseases as malaria and lymphatic filariasis. There is ample evidence to support the occurrence of inducible insect immune peptide production in conjunction with the innate immune response to bacteria and fungi. For example, defensin A transcription is robustly induced in the fat body of *Aedes aegypti* mosquitoes following bacteria inoculation, and high levels (45 μM) of peptide are secreted into the fluid-filled open circulatory system (Lowenberger *et al.*, 1999b). *Anopheles gambiae* defensin production is also inducible, but less peptide is produced (3 μM) (Vizioli *et al.*, 2001). Promoter analyses of the defensin A sequence from both *Ae. aegypti* and *An. gambiae* defensins reveal binding sites for immune responsive transcriptional regulators that parallel those employed in vertebrate immunity, including NF- κ B (Cho *et al.*, 1997; Eggleston *et al.*, 2000). Mosquitoes inoculated with bacteria or purified defensin had a reduced prevalence and mean intensity of infection with parasites than did control mosquitoes (Albuquerque & Ham, 1996; Lowenberger *et al.*, 1996, 1999a; Shahabuddin *et al.*, 1998).

Despite these correlations, our understanding of the role of these peptides in insect innate immunity is limited to their *in vitro* antimicrobial activity – for example, *Ae. aegypti* defensin primarily has activity against Gram-positive bacteria (Lowenberger *et al.*, 1995). This property *in vitro* does not necessarily reveal the function of the defensin peptide *in vivo*. In fact, mosquitoes are well equipped to mount immune responses to Gram-positive bacteria many hours before the peak of defensin production. Recent studies demonstrate that the mosquitoes *Ae. aegypti*, *Armigeres subalbatus* and *An. albimanus* efficiently melanize Gram-positive bacteria within minutes of inoculation (Hernandez-Martinez *et al.*, 2002; Hillyer *et al.*, 2003b; Hillyer *et al.*, 2003a).

We have characterized defensin production in the mosquito *Ae. aegypti*. Like other immune peptides from insects, *Ae. aegypti* defensin was induced in, and isolated from, mosquitoes that were inoculated with a probe dipped into a slurry of Gram-negative and Gram-positive bacteria, resulting in infections with large, unmeasured and presumably unnatural numbers of bacteria. Using multiple experimental conditions to characterize the responsive nature of defensin in *Ae. aegypti*, we observed dose-dependent defensin transcription and translation, and obtained evidence for a type of post-transcriptional regulation not previously described for this immune peptide. Additionally, our data clearly

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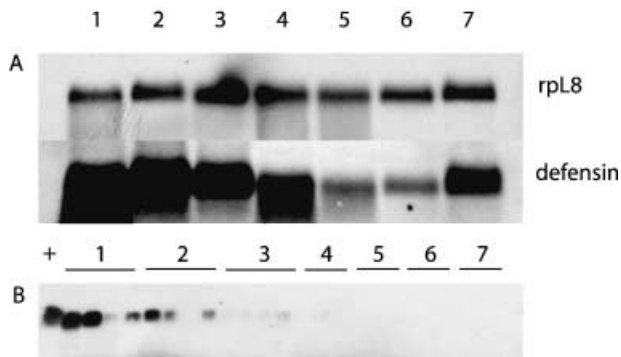


Figure 1. Dose-dependent defensin transcription in *Ae. aegypti* inoculated with serial dilutions of bacteria as measured by Northern analysis (A) and translation as measured by Western analysis of haemolymph (B). All materials were collected 24 h after bacteria inoculation. Five micrograms of RNA from whole body material was used for Northern analysis and rpl8 was used as a loading control. Columns represent material from mosquitoes inoculated with approximately: (1) 400 000 bacteria, (2) 40 000 bacteria, (3) 4000 bacteria, (4) 400 bacteria, (5) forty bacteria, (6) four bacteria or (7) saline. For Western analysis, columns 1–3 contain four lanes of haemolymph from individual mosquitoes, and columns 4–7 haemolymph from three individual mosquitoes. Purified defensin (0.1 ng) was used as a positive control (+). These results were seen in six separate experiments.

document that survival of bacteria-challenged *Ae. aegypti* is independent of the production of defensin peptide, which is detected only in mosquitoes inoculated with large numbers of bacteria, and only at a time when a majority of those bacteria have been cleared from the system, most likely by phagocytosis or melanization (Hillyer *et al.*, 2003b).

Results and discussion

The inducible insect immune peptides, including *Ae. aegypti* defensin, have been isolated and studied using a technique that involves inoculation with a probe dipped into a slurry of Gram-negative and Gram-positive bacteria (Lowenberger, 2001). The resulting infections with large numbers of bacteria undoubtedly do not reflect the numbers of bacteria that insects are exposed to in natural environments. We estimate that this method results in infections with more than 5×10^5 – 1×10^6 bacteria per individual mosquito. The transcriptional and translational profiles of defensin production in *Ae. aegypti* after inoculations with serial dilutions of bacteria reveal a dose-dependency not previously described. Defensin transcripts increase with increasing numbers of inoculated Gram-negative (*Escherichia coli*) and Gram-positive (*Micrococcus luteus*) bacteria, as detected by Northern blot analysis (Fig. 1A). It is interesting to note that defensin detection is more intense in RNA from saline inoculated mosquitoes in this Northern; this is attributed to inherent variation in defensin production per individual, an observation we came across frequently.

To confirm and quantify dose-dependency, cDNA synthesized from mosquitoes exposed to different doses of

bacteria was used in real-time PCR reactions. Analysis of real-time results from three independently generated sets of cDNA confirmed a continuous increase in transcript numbers with increasing numbers of inoculated bacteria. Mosquitoes inoculated with bacteria using a probe, or $0.5 \mu\text{l}$ of a thick slurry of concentrated bacteria (in excess of 1×10^6), show no apparent plateau in number of transcripts produced within the limits used in these experiments (data not shown).

In conjunction with transcriptional analyses, we examined the presence of peptide in haemolymph perfused from mosquitoes 24 h post exposure to different concentrations of bacteria. Western analysis was performed on either haemolymph from individuals or quantified from pooled haemolymph and, in six separate experiments, revealed a dose-dependent translational response. Importantly, despite the presence of defensin transcripts, peptide was not detected in haemolymph from mosquitoes inoculated with greater dilutions of bacteria (equating to approximately 1–100 bacteria in six different experiments), nor was peptide found in saline-inoculated mosquitoes. Additionally, peptide production does not appear to increase with dose in an approximately linear fashion as does transcription (Fig. 1B). A similar phenomenon has been observed in the *Ae. aegypti* cell line, Aag2, wherein defensin transcripts can be detected when cells are exposed to lipopolysaccharide (LPS), but defensin peptide is not detected by Western analysis (W. L. Cho, personal communication). These results suggest post-transcriptional regulation of the defensin gene.

The defensin A mRNA sequence, beginning with the transcription start site and ending with the last nucleotide in the second exon of the genomic sequence (Cho *et al.*, 1997), was submitted for MFold secondary structure analysis (Zuker, 1989). This sequence was chosen because defensin A/B is the isoform of defensin produced in the fat body, and defensin C is produced in the midgut (Lowenberger *et al.*, 1999a). In each of twenty-five predicted output structures, a large stem loop (thirty-three bases) followed by a small stem loop (eight bases) in the 5' untranslated region (UTR) immediately preceded the initiation codon, which was not predicted to be involved in any secondary structure, and extensive secondary structure was predicted in the 3' UTR (data not shown).

We designed constructs of the defensin A gene that were flanked by (1) both the 5' and the 3' UTR, (2) either the 5' or the 3' UTR or (3) just the coding region, and used these in *in vitro* translation assays. A peptide migrating at approximately 4 kDa was produced when the coding region alone was used in the assay (Fig. 2, lane 2), but not when the coding region was flanked by both the 5' and the 3' UTRs (Fig. 2, lane 1). Less peptide was produced in assays that used the coding region flanked by either the 5' or the 3' UTR (Fig. 2, lanes 3–5). These results were seen in four different experiments. It is important to note that the 75 °C

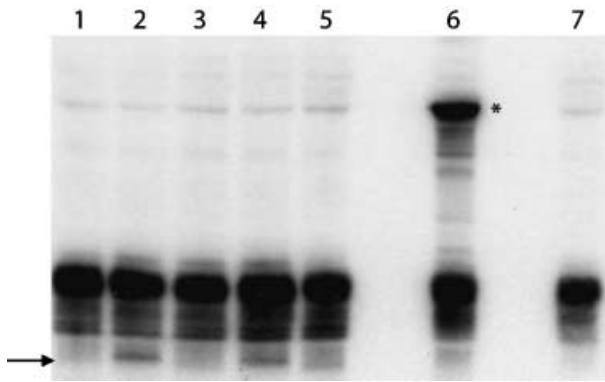


Figure 2. Post-transcriptional gene regulation as measured by *in vitro* translation assays. Biotinylated protein samples were produced from transcripts that contained: in lane (1) the coding region surrounded by both the 5' and 3' untranslated regions (UTRs); lane (2) the coding region alone; lane (3) the coding region with the 5' UTR only; lane (4) the coding region and 5' UTR with an extra CGGG at the 5' end; and lane (5) the coding region with only the 3' UTR. Lane (6) contains control RNA that produces a 50 kDa protein (*), and in (7), no RNA was added. Protein was detected using streptavidin–HRP followed by chemiluminescent detection. The arrow points to the defensin peptide that is approximately 4 kDa in size. Endogenous biotinylated proteins from rabbit reticulocyte lysate (evident in lanes 1–7) migrate above the defensin peptide. These results were seen in four different experiments.

pretreatment of RNA transcripts, which melts the secondary structure of the RNA, was necessary for peptide production with the constructs containing the 5' and 3' UTRs. Without this pretreatment, only the coding region construct produced peptide (data not shown). It is likely therefore that both the 5' and the 3' UTR are involved in the regulation of defensin peptide production; consequently, when one of the UTRs is removed, some peptide production occurs. The level of peptide production from the 5'b construct (Fig. 2, lane 4), which contains an additional CGGG sequence at the 5' end, is similar to that seen when the coding region alone was used in the assay. When subjected to MFold secondary structure analysis, the start codon of the 5'b construct is not embedded in stem loop structures; the opposite is true for the start codon of the 5'a construct.

The mechanism for post-transcriptional gene regulation of defensin may require a factor or physiological state that facilitates ribosome loading on to the mRNA. It is possible, for example, that defensin translation is controlled by a translation regulator such as the immune-responsive *Drosophila Thor* 4E-binding protein (Bernal & Kimbrell, 2000). Alternatively, translational efficiency may be dictated by the untranslated regions of the mRNA. At the initiation of translation, the 40S ribosomal complex binds to and migrates through the 5' UTR until it encounters the first AUG codon. The defensin 5' UTR is GC-rich and forms an extensive secondary structure (46–49% of the nucleotides are GC base-paired) between the cap and AUG codon. When this occurs translational efficiency is drastically reduced, as is seen in many house-keeping genes and genes that encode

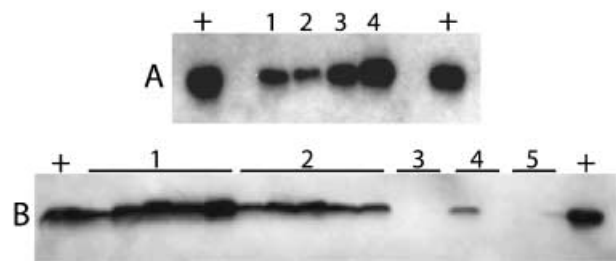


Figure 3. Defensin expression is detected by Western analysis in haemolymph from individual *Ae. aegypti* inoculated with various bacteria (A) and parasites (B). (A) +: defensin-positive control (0.1 ng) recombinant defensin, lane 1: *E. coli* inoculated, lane 2: *M. luteus* inoculated, lane 3: combined, heat-killed *E. coli* and *M. luteus* inoculated, lane 4: combined *E. coli* and *M. luteus* inoculated. (B) Defensin peptide in haemolymph from individual *Ae. aegypti* as a result of inoculation with: (1) cryopreserved *Dirofilaria immitis* (five lanes), (2) cryopreserved *Brugia malayi* (five lanes), (3) saline (two lanes), (4) supernatant from cryopreserved *B. malayi* (two lanes), (5) supernatant from cryopreserved *D. immitis* (two lanes), +: bacteria-inoculated positive control haemolymph.

growth factors, receptor proteins, transcription factors, signal transduction components and tumour-related proteins (Kozak, 1991; Kozak, 1999). Post-transcriptional regulation could also be facilitated at the 3' UTR because poly(A) binding protein (PABP) and certain translation initiation factors may interact on actively translated messages to deliver initiation complexes to the 5' end of the mRNA (Dever, 2002). In support of this, the defensin construct containing the coding region and 3' UTR produced only trace amounts of defensin peptide (Fig. 2, lane 5). Recently, another mechanism of post-transcriptional regulation that involves activation by a serine protease was described for a constitutively expressed defensin in the gut of the fly, *Stomoxys calcitrans* (Hamilton *et al.*, 2002). Experimental evidence therefore points to multiple levels of regulation of the defensin gene, from induction to peptide production.

A previous study using *Drosophila melanogaster* described differential induction of immune peptides according to the type of bacteria or fungi infecting the flies (Lemaitre *et al.*, 1997); by contrast, the production of defensin in *Ae. aegypti* is not dependent on the organism inoculated. Defensin was detected in the perfusate of mosquitoes inoculated with various combinations of live or heat-killed *E. coli* and/or *M. luteus* (Fig. 3A). We also inoculated mosquitoes with the eukaryotic parasites *Brugia malayi* or *Dirofilaria immitis*. Microfilariae of these parasites are encapsulated by melanin when inoculated into *Ae. aegypti*; however, in mosquitoes that are exposed via blood feeding, the parasites develop normally and do not trigger up-regulation of defensin (Lowenberger *et al.*, 1996). Defensin peptide was detected in the haemolymph of mosquitoes inoculated with microfilariae of either species (Fig. 3B). It is unlikely that defensin production is triggered by bacterial symbionts within the filarial parasites because these parasites have a thick cuticular covering, and are rapidly encased in a capsule

of melanin and cross-linked proteins. The detection of an anti-Gram-positive peptide in these situations where it would seem to be biologically irrelevant is surprising, especially in light of the hierarchy of regulatory interventions involved in defensin expression, and raises questions concerning its function in innate immunity.

It is becoming increasingly clear that the mosquitoes employ a multifaceted response against bacteria. Within minutes of inoculation bacteria are subjected to phagocytosis and melanization (Da Silva *et al.*, 2000; Hernandez-Martinez *et al.*, 2002; Hillyer *et al.*, 2003a,b). This information, in conjunction with data from experiments described above, led us to hypothesize that defensin functions in the immune response in a capacity beyond its antibacterial activity. Mosquitoes were inoculated with tetracycline resistant XL-1 blue *E. coli* and 3–6 individuals were perfused on to tetracycline LB agar plates to monitor bacteria clearing. In four experiments, clearing of bacteria was measured over time and rapid clearing was observed. In one of these four experiments, colony forming units (cfu) were too numerous to count at the early time points. Data from the remaining three experiments were combined, and the number of colonies counted was used to calculate an average number of colony forming units at each time point. When mosquitoes were inoculated with bacteria from an overnight culture diluted ten-fold (approximately 10–15 000 per individual), 61% of bacteria were cleared between 1 and 9 h post inoculation, and an average of 73% of bacteria were cleared by 24 h. Mosquitoes that were exposed to the same cultures diluted 1000-fold (100–150 bacteria per individual) cleared 70% of bacteria by 9 h and 95% of bacteria by 24 h (Fig. 4). Analysis of peptide production in these mosquitoes shows that consistent, but low levels of defensin are detectable 6 h post inoculation, but only in mosquitoes inoculated with concentrated bacteria, and that defensin expression steadily increases such that the peptide is most

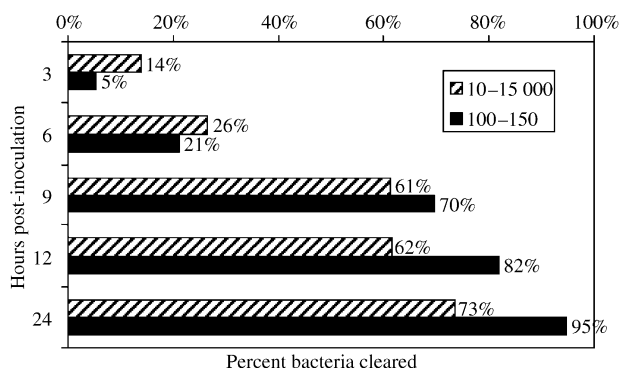


Figure 4. Rapid clearing of antibiotic-resistant bacteria in *Ae. aegypti*. Perfusate from mosquitoes was incubated on tetracycline containing LB agar plates. The resulting colony forming units were counted. Results shown are the averages from three experiments for a total of twelve individuals per time point. The percentage of bacteria cleared (*x*-axis) was calculated at various times post inoculation (*y*-axis).

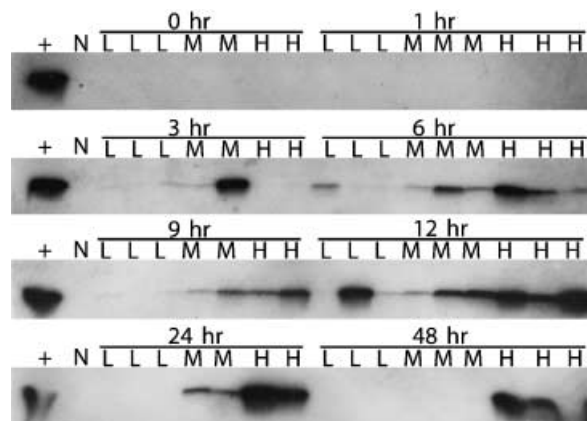


Figure 5. Western analysis of the presence of defensin peptide over time in haemolymph samples from individual *Ae. aegypti* inoculated with different concentrations of antibiotic-resistant bacteria. Bacteria-inoculated positive control haemolymph (+) is at left. Hours post inoculation are indicated by bars over lanes. Lanes are labelled for the dose of bacteria inoculated: (N) naïve (no bacteria), (L) low (c. 1 bacteria per individual), (M) medium (100–150 per individual), or (H) high (10–15 000 per individual).

abundant 24 h post inoculation – a time when the majority of the inoculated bacteria have been cleared (Fig. 5). Mosquitoes inoculated with the least concentrated numbers (1–2 per individual) cleared these bacteria immediately and defensin peptide was found only rarely in mosquitoes inoculated with this low dose of bacteria (5/44 were defensin positive in Fig. 5), which we attribute to inherent individual variability in defensin expression between individual mosquitoes.

Using double stranded RNA, *Ae. aegypti* defensin A was silenced as evidenced by Northern and Western analyses (Fig. 6). Mosquitoes were first inoculated with double-stranded RNA (dsRNA) or diethyl pyrocarbonate (DEPC) water, and then inoculated with bacteria 24, 48 or 72 h later. Optimal defensin silencing occurs in the fat body of mosquitoes 48 h post inoculation with dsRNA. To assess the importance of defensin in survivability, mosquitoes were inoculated with either high (c. 250 000 bacteria) or lower concentrations of combined Gram-positive and Gram-negative bacteria (c. 250 bacteria), or with saline (to control for the inoculation process) 48 h after treatment with dsRNA or DEPC water. Fat body and haemolymph samples were collected from a cohort of mosquitoes 24 h after bacteria inoculation. Defensin silencing is evident at the level of both transcription and translation in dsRNA-inoculated mosquitoes compared with water-inoculated mosquitoes (Fig. 6). Mosquito mortality was observed for 21 days and the proportion of surviving mosquitoes (combined from all three experiments) was plotted against time (Fig. 7). Data were combined for all three experiments ($n = 122$ –157 per group). The rate of mortality (as measured by the slope of the calculated linear regression) for mosquitoes inoculated with the same dose of bacteria or saline was compared using a

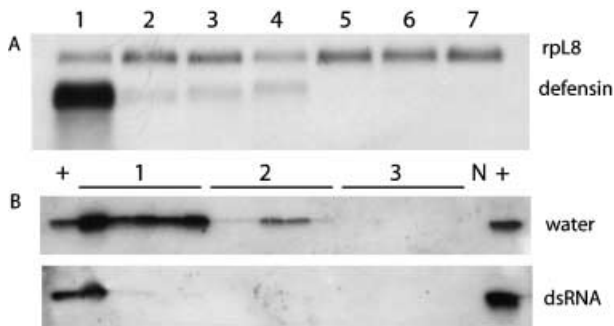


Figure 6. Representative results of defensin silencing in *Ae. aegypti* after inoculation with defensin A dsRNA. (A) Northern analysis of 5 µg of fat body material from mosquitoes inoculated with DEPC water (lanes 1–3) or dsRNA (lanes 4–6), then combined Gram-positive and Gram-negative bacteria, or saline 48 h later. Mosquitoes were inoculated with DEPC water, then with (1) undiluted bacteria (c. 250 000 bacteria), (2) diluted bacteria (c. 250 bacteria) or (3) saline, or dsRNA followed by inoculation with (4) undiluted bacteria, (5) diluted bacteria or (6) saline. Lane 7 contains material from naive mosquitoes. (B) Western analysis of defensin silencing in mosquitoes from the same experiment. DEPC water-inoculated *Ae. aegypti* haemolymph is shown in the top gel and dsRNA-inoculated haemolymph is on the bottom. Haemolymph of individual mosquitoes inoculated with: (N) no bacteria/naïve, (1) undiluted bacteria-inoculated (five lanes), (2) diluted bacteria-inoculated (five lanes) and (3) saline-inoculated (five lanes). Bacteria-inoculated positive control haemolymph (+) is on either side.

Student's *t*-test according to a previous mosquito mortality study (Christensen, 1978). No significant difference was observed between groups exposed to saline or bacteria at high or lower concentrations, whether or not defensin was silenced (Fig. 7). This demonstrates that although defensin is produced (Fig. 6), sometimes in abundance (depending on the dose), it does not contribute to the survival of *Ae. aegypti* exposed to these bacteria. These results contrast with those of a previous study performed with *An. gambiae*, wherein defensin silencing resulted in increased susceptibility to Gram-positive bacteria (Blandin *et al.*, 2002). In support of our findings, a recent study of a transgenic line of *Ae. aegypti*, deficient in defensin expression, did not show an increased susceptibility to Gram-positive bacteria (Shin *et al.*, 2003). It is difficult to compare these studies in relation to our results. In the work with *An. gambiae*, there was no control for mortality attributed to the inoculation process alone, and in the *Aedes* studies involving the transgenic line, the inoculation procedure alone caused 50% mortality at 24 h.

The innate immune response of mosquitoes and other insects employs humoral elements, such as the immune peptides, and cellular elements to manage invading pathogens. The cellular and cell-mediated components of the innate immune response involve phagocytosis and melanization of foreign substances. Several recent studies demonstrate that *Culex*, *Anopheles*, *Armigeres* and *Aedes* – four genera of mosquito vectors – are capable of rapid and robust cellular responses to bacteria and fungi (Da Silva *et al.*, 2000; Hernandez-Martinez *et al.*, 2002; Hillyer *et al.*,

2003a,b). Specifically, an individual granulocyte in *Ae. aegypti* is capable of phagocytosing at least 100 bacteria, and phagocytosis is initiated within minutes of inoculation (Hillyer *et al.*, 2003b). Therefore, this mosquito is probably capable of handling reasonable loads of bacteria by phagocytosis or melanization alone.

The studies presented here demonstrate another level in the hierarchy of defensin production and simultaneously de-emphasize the importance of defensin as a cytotoxic molecule in the immune response. We propose that the peptide plays an important role, perhaps in another aspect of the immune response, or as a stress response element when pathogens in the haemolymph exceed the phagocytic capacity of the haemocytes, and that cytotoxicity is an ancillary property. In support of this contention, recent studies in vertebrates have revealed chemotactic properties of defensins and, in turn, chemokines exhibit antimicrobial activity *in vitro* (Lehrer & Ganz, 2002; Salzet, 2002; Yang *et al.*, 2002). Thus far, studies of immune peptide production have employed very large doses of bacteria that do not reflect the type, nor the dose of bacteria that a mosquito would encounter naturally. Future studies with more appropriate bacterial infections are necessary truly to elucidate the function of molecules, such as defensin, in the innate immune response of mosquitoes and other insects.

Experimental procedures

Mosquito maintenance

The *Aedes aegypti* Liverpool strain was originally obtained from a colony from the University of London in 1977 and was reared as previously described (Christensen & Sutherland, 1984). Adult female mosquitoes were used for experimentation within 3 days of eclosion.

Bacteria, parasite and saline inoculations

Bacteria used for inoculations were *Escherichia coli* K12 and *Micrococcus luteus* (University of Wisconsin-Madison). The procedure described previously was used for inoculations of large amounts of bacteria with a stainless-steel probe (Lowenberger *et al.*, 1999b). Preparation of inocula to contain quantified bacteria involved mixing equal amounts of the cultures together and performing ten-fold serial dilutions in sterile *Aedes* saline (Hayes, 1953). The number of bacteria in each dilution was approximated by microscopical analysis of Gram-stained slides, and from plates (LB agarose plates, 37 °C overnight) containing 0.5 µl aliquots of each dilution. A 0.5 µl inoculum was dispensed on to a Parafilm™-wrapped slide, drawn into pulled capillary needles and then inoculated into mosquitoes as described previously (Beerntsen & Christensen, 1990). An equal volume of *Aedes* saline was injected into mosquitoes as a control. Using this technique, we routinely see greater than 90% survivorship after 24 h. To monitor the rate that mosquitoes could clear bacterial infections, XL1-Blue tet-R *E. coli* (Clontech) were serially diluted and injected as described above. Haemolymph then was perfused (three drops in ddH₂O to displace as many bacteria as possible) from individual mosquitoes

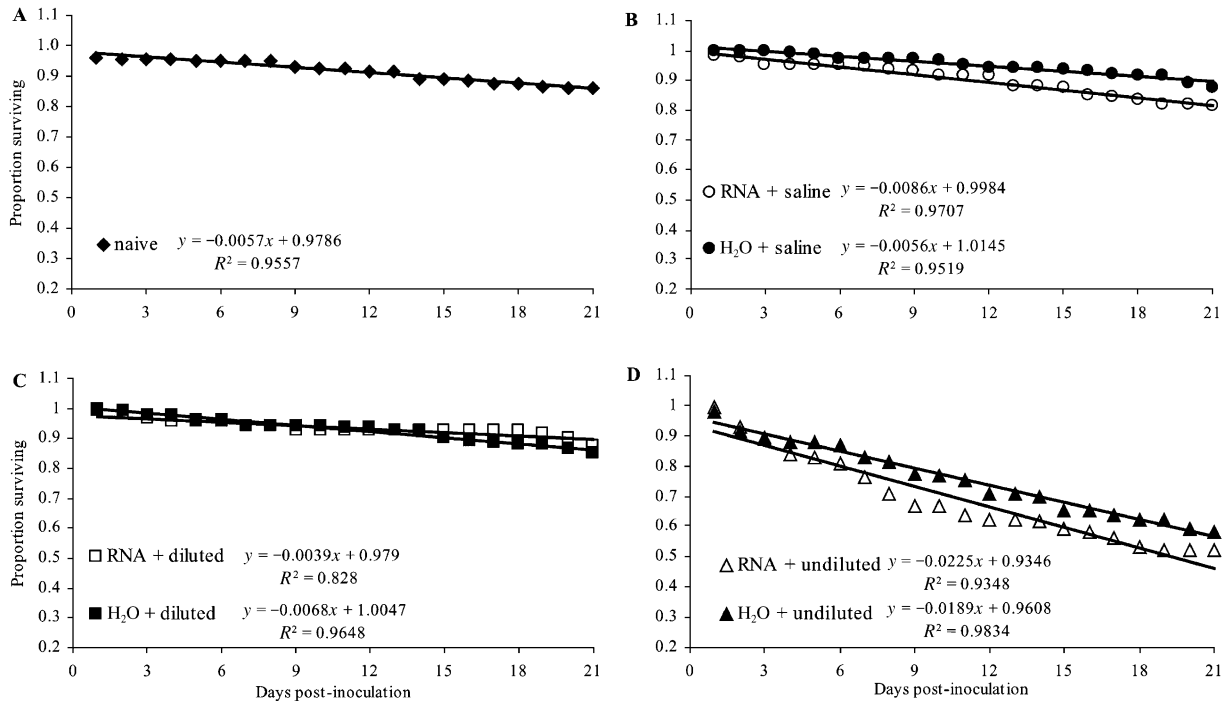


Figure 7. Survivorship of *Ae. aegypti* deficient in defensin expression. The number of days (after the second inoculation, x-axis) is shown vs. the proportion of surviving mosquitoes (y-axis). Baseline mortality is shown for naive mosquitoes, handled as per experimental mosquitoes, in A. Experimental mosquitoes were inoculated with 500 ng double-stranded defensin RNA or DEPC water, then with (B) saline, (C) a diluted culture of Gram-positive and Gram-negative bacteria, or (D) undiluted bacteria, 48 h later. Results shown are combined from three experiments totaling 122–157 mosquitoes per group. Double-stranded RNA inoculated mosquitoes are shown with open symbols, and DEPC water inoculated mosquitoes with closed symbols. Equations calculated for the line of best fit for each experimental group are shown. Using a Student's *t*-test (d.f. = 38) to compare the slope of regression lines, no statistical significance was observed between the rates of death in any of the corresponding groups inoculated first with DEPC water or dsRNA, then with: undiluted bacteria ($t = 0.191$, $P = 0.85$), diluted bacteria ($t = 0.120$, $P = 0.90$) or saline ($t = 0.124$, $P = 0.90$).

directly on to LB-tet (15 µg/ml) agar plates at 0 and or 1, 3, 6, 9, 12 and 24 h. Plates were allowed to dry, incubated overnight at 37 °C, and colony forming units counted.

To assess insults that elicit defensin expression, mosquitoes were inoculated with live or heat-killed *M. luteus* and *E. coli* either separately or combined, or with eukaryotic filarial worm parasites. *Dirofilaria immitis* and *Brugia malayi* microfilariae inoculated into mosquitoes had been cryopreserved and were thawed using the procedures of Bartholomay *et al.* (2001). Approximately thirty worms in *Aedes* saline were inoculated into each mosquito. The supernatant from processing cryopreserved parasites and *Aedes* saline were used as controls for parasite inoculation experiments.

Haemolymph and fat body collection

Haemolymph for Western analysis was perfused from the haemocoel of mosquitoes using the methods of Beerntsen & Christensen (1990). After perfusion, guts were dissected out and abdomens (containing the fat body) removed and stored at –80 °C for RNA extraction, in cases where both fat body and haemolymph were collected, as was done for gene silencing experiments.

Protein analysis

The presence of defensin peptide was detected by Western analysis of quantified protein from pooled haemolymph, or from individual mosquitoes. These results were similar, and individual

haemolymph was used in the figures presented herein. Protein was separated on precast 18% SDS-PAGE gels (Bio-Rad) and transferred to polyvinylidene fluoride (PVDF) membranes. For Fig. 1, detection was identical to that described previously using a Roche detection kit (Cheng *et al.*, 2001). For Figs 3, 5 and 6, detection was done with the Western Lightning detection kit (PerkinElmer) using the antidefensin antibody at a dilution of 1 : 30 000 with all other conditions as previously described. Using this assay, as little as 0.025 ng of purified defensin can be detected (data not shown). Loading controls are indicated in figure legends and were either measured quantities of recombinant defensin peptide or positive controls of haemolymph from bacteria-inoculated *Ae. aegypti* from a separate experiment.

RNA extraction and analysis

Unless otherwise indicated, mosquito tissues were collected 24 h post inoculation, a time at which defensin transcripts are abundant (Lowenberger *et al.*, 1999b). Carcasses and tissues were stored at –80 °C prior to RNA extraction and Northern analysis. RNA extractions were performed with five carcasses or ten abdomens (for Northern analysis), or one or five carcasses (for real-time PCR), using the single-step acid guanidinium thiocyanate–phenol–chloroform extraction method (Chomczynski & Sacchi, 1987). Nonradioactive detection was used for Northern analysis as described previously using 5 µg of total RNA (Johnson *et al.*, 2003). Blots were probed with defensin and rpl8 (Durbin *et al.*,

1988) probes (2 ng/ml hybridization solution) generated from cDNA clones. For real-time PCR analysis, cDNA was synthesized from 5 µg of RNA using an oligo-dT primer from three separate experiments. Gibco platinum *Taq* (Invitrogen Corporation) and related reagents were used for PCR reactions according to the manufacturer's instructions. Primer sequences for defensin A are: 5'-GCGACCTGCGATCTGCTG-3' (forward) and 5'-TCAATTCGACAGACGCAGACCTT-3' (reverse). Product was detected using the Bio-Rad i-cycler (Bio-Rad) (PCR program: denature xC, thirty-five cycles of 95 °C for 1 min, 66.8 °C for 30 s, 72 °C for 1 min) using SYBR green I (Sigma Aldrich) to quantify dsDNA produced. In parallel tubes, we amplified a 117 bp fragment of *Ae. aegypti* actin to control for similar starting quantities of cDNA. Primers for actin are: 5'-ATTAAGGAGAAGCTGTGCTACGTC-3' (forward) and 5'-GGCTGGAAGAGAGCTTCTGG-3' (reverse). The relative concentration (fold increase) in defensin transcript in bacteria-injected mosquitoes over saline-injected mosquitoes was calculated using the Comparative C_T Method (Applied Biosystems).

Post-transcriptional regulation of defensin mRNA

A defensin A clone, including the 3' and 5' UTR, was excised from the pTriplEx2 vector of a bacteria-inoculated *Ae. aegypti* cDNA library (Clontech Smart™ cDNA library), according to the manufacturer's instructions, and transformed into XL1-Blue cells (Clontech). Digestion with *EcoRI* and *NheI* removed the vector sequence between the SP6 promoter and the 5' end of defensin. Defensin fragments were generated using PCR to exclude either the 5' or the 3' UTR or both, ligated into the modified vector, and transformed into XL1-Blue cells. Two constructs containing the 5' UTR and coding region were made because it was not clear whether a CGGG sequence at the 5' most end was from the cloning vector or the actual sequence. We designated construct 5'b as the sequence containing the extra CGGG bases. Primers were designed for the coding region only: 5'-ATAAGCTAGCCCAAC-CATGAAGTCGATCAC-3' (forward) and 5'-ATAACTCGAGTCAAT-TCCGACAGAGGCAC-3' (reverse), the coding region without the 5' end: 5'-ATAAGCTAGCCCAACCATGAAGTCGATCAC-3' (forward) and 5'-ATAAGCTCGAGTCTAGAGTCGAC-3' (reverse), and the coding region without the 3' end: (5'a) 5'-ATAAGCTAGCGACAT-CAT TCAAT TCCACAAG-3' (forward) or (5'b) 5'-ATAAGCTAGCC-GGGGACATCATTCAATTCC-3' (forward) and 5'-ATAACTCGAGT-CAAT TCCGACAGAGGCAC-3' (reverse).

Defensin RNA was generated from linearized DNA templates using the mMESSAGING MACHINES™ kit (Ambion). RNA transcripts were purified by acid phenol/chloroform extraction, followed by isopropanol precipitation, and resuspended in nuclease-free ddH₂O. Transcript concentration was measured (A260) and the integrity verified on 1% formaldehyde agarose gels. Transcripts were denatured at 75 °C and immediately added to *in vitro* translation assays. The translation was performed using Retic Lysate IVT™ (Ambion) and Transcend™ Non-Radioactive Translation Detection System (Promega) according to the manufacturer's instructions. Biotinylated protein was isolated from the reaction using SoftLink™ Soft Release Avidin Resin (Promega) according to the manufacturer's instructions. Proteins were separated by SDS-PAGE and transferred to PVDF membranes, then blocked for 1 h and incubated with streptavidin-horseradish peroxidase (HRP) (diluted 1 : 10 000) overnight at 4 °C. After washing, membranes were incubated with the chemiluminescent substrate at room temperature for 10 min and exposed to X-ray film.

Defensin silencing

Templates for *Ae. aegypti* defensin A were generated by PCR amplification of template cDNA generated from haemocytes of bacteria-inoculated *Ae. aegypti*. A 359 bp DNA fragment, PCR amplified from the 5' end of a defensin cDNA clone, was subsequently cloned into pGEM T Easy (Promega). Primers used were: 5'-TTC AAT TCC ACA AGC TCG TTCAAG-3' (forward) and 5'-AT TCC GAC AGC GAC ACCT TCT TG-3' (reverse). Templates were linearized with either *NcoI* (SP6) or *SalI* (T7). The Ampliscribe High Yield Transcription Kit (Epicentre) was used to generate and purify RNA according to the manufacturer's instructions. RNA was resuspended in 40 µl DEPC-treated ddH₂O, quantified (A260), and the size and integrity checked on a 1% agarose gel stained with ethidium bromide. RNA concentrations were adjusted to 1 µg/µl. To anneal the strands, equal amounts of RNA were combined and incubated at 95 °C for 5 min. The samples then were allowed to cool to room temperature over several hours. The dsRNA was placed on ice briefly, spun down and stored at -80 °C. Silencing experiments were performed using 500 ng dsRNA per mosquito in a 0.5 µl volume injected into the haemocoel as described above. Mosquitoes were inoculated 48 h later with quantified numbers of bacteria as described above. For each experiment, a second group was inoculated first with DEPC water, and subsequently with bacteria or saline (as were experimental mosquitoes) to control for the potential detrimental effects on survivability induced by inoculation.

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