ORIGINAL ARTICLE

Induction of defensin response to dengue infection in Aedes aegypti

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Abstract

Innate immune-related defensin peptide expression, the major antimicrobial peptide (AMP) in the dengue vector mosquito Aedes aegypti, was analyzed following infection by dengue virus type 2 (strain 16681) (DENV-2). In vitro, the mosquito cell line C6/36 was exposed to dengue virus at different multiplicities of infection (MOI) in an hour; it was found that the expression level of defensin transcripts was dependent upon viral dose. In addition, using both polymerase chain reaction (PCR) and Western blotting assays to determine defensin transcription and translation at a series of times post-inoculation, we found that the time course of defensin transcripts correlated with the expression of defensin peptide post-infection. In vitro, female Ae. aegypti mosquitoes were fed different meals (DENV-2 infected blood, non-infectious blood and sugar) at 14 days post-feeding, to determine defensin transcription in response to dengue infection, by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) assay. The results showed that a peak of 4.25 kDa peptide, composed of defensin, was predominantly induced by DENV-2 infected blood-fed mosquitoes. We observed that DENV-2 could trigger the defensin peptide, defined molecularly as an innate immune response. Therefore, this peptide may be involved in dengue infection and/or transmission. Improved understanding of the mosquito’s responses to dengue virus should strengthen our understanding of this vector’s innate immune system.

Key words: antimicrobial peptides, Antivirus defense mechanisms, innate immune response.

INTRODUCTION

The Aedes aegypti (Diptera: Culicidae) mosquito is the major vector of dengue infection; it has a significant and increasing impact on human morbidity and mortality. The World Health Organization estimates 2.5 billion people are currently living in over 100 endemic DENV-transmission areas (Anonymous 2009). As yet, no proven antiviral treatment or vaccine is publicly available to prevent dengue infection, and these are much needed to fight and control the virus.

The mosquito’s immune system plays a key role in pathogen–vector interactions. Mosquito innate immunity is a first line of defense against pathogenic infection. Immune peptides are produced by the activation of hemocytes and/or the fat body, which in turn induce immune effectors and release antimicrobial peptides (AMPs) into the hemolymph. They are also delivered to other tissues (Hoffmann et al. 1999). Antimicrobial peptide activity is specific to different classes of pathogen and to killing mechanisms. The immune response of insects in general, especially mosquitoes, is not standardized. Although all insects are capable of producing immune peptides belonging to the same family, the expression and concentration of specific peptides differs among species, families and orders of insect. Specific AMP induction during bacterial and fungal infection in insects is relatively well defined; for instance, during bacterial infections, cecropin, defensin and diptericin are all induced (Engström 1999).
Defensins are the best characterized of the immunity proteins produced by adult female mosquitoes, including *Aedes aegypti* (Chalk et al. 1994; Lowenberger et al. 1995; Cho et al. 1996; Gao et al. 1999). Mosquito defensins are chiefly active against Gram-positive bacteria (Lowenberger et al. 1999a, 1999b), although some Gram-negative bacteria are also sensitive, and defensins reportedly combat some fungal and parasitic infections (Lowenberger et al. 1995, 1996; Cho et al. 1996; Dimopoulos et al. 1997; Tereza et al. 2008). Previous studies have shown that mosquitoes inoculated with bacteria or with purified defensin had a reduced prevalence and more limited microbial infection than control mosquitoes (Lowenberger et al. 1996, 1999a). However, during dengue viral infection in *Aedes* mosquitoes, the role and specificity of inducible peptides remain poorly understood.

For the present study, we characterized defensin production in the *Aedes aegypti* mosquito. *Aedes* defensin expression was induced in mosquitoes infected with dengue virus type 2. We observed dose-dependent defensin transcription and translation in vitro and obtained evidence of immune peptides for translational regulation, which have not previously been described in *Aedes aegypti* mosquitoes.

**MATERIALS AND METHODS**

**Cell culture and dengue virus infection**

Mosquito cell line C6/36 cells were grown at 28°C in Minimal Essential Medium (MEM) supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% sodium bicarbonate, 1% nonessential amino acids, 50 U/mL of penicillin and 50 µg/mL of streptomycin. Dengue virus serotype 2 strain 16681 (DENV-2) was about 2.1 x 10⁴ PFU/mL for this study. The C6/36 cells were inoculated with viable DENV-2 at a multiplicity of infection (MOI) of 1, 5, 10 and 15 at 28°C for 1 h. Excess viruses were removed and the complete medium was added to the infected cells. The supernatants from each time were determined. The infected cells were incubated at 28°C for the duration of this experiment. The supernatants from each time were determined. DENV-2 was inactivated by treating the purified virus with formaldehyde (Eckels & Putnak 2003). Mock infected cells were used as the normal physiological control. The experiment was conducted in triplicate.

**Mosquito infection**

Five- to seven-day-old adult female mosquitoes were orally infected with dengue virus via an artificial membrane feeding apparatus. Mosquitoes were divided into three treatment groups: (i) those fed on 10% sugar solution; (ii) those fed on defibrinated human blood; and (iii) those fed on defibrinated human blood with dengue virus. Engorged mosquitoes were collected at 14 days post-infection (dpi) from each group.

**Dengue viral detection by semi-quantitative reverse transcription-PCR**

Reverse transcription-polymerase chain reaction (RT-PCR) was used to detect dengue viral type 2-RNA (DENV-2-RNA) in *Ae. aegypti* mosquitoes post-oral infection (data not shown). The DENV-2-RNA was extracted from the samples using a QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA, USA), following the manufacturer’s instructions. The presence of DENV-2-RNA was determined using RT-PCR as described by Lanciotti et al. (1992). RT-PCR was carried out on 0.5 µg of RNA using a OneStep RT-PCR Kit (Invitrogen, Carlsbad, CA, USA), following the manufacturer’s protocol. The following primers were used:

- DENV-2_F: 5' - CAAATGCTGAAACGGCGAGAAGCC - 3'
- DENV-2_R: 5' - CCACAAGGGCCATGAACAG - 3'

Ribosomal protein S7 (*rpS7*) was used as an internal control. Primers for *rpS7* were:

- rpS7_F: 5'-TCAGTGTACAGAGCGCGAGAAACC′-3'
- rpS7_R: 5'-AACGATGCCAGCAAAAGATG-3′

RT-PCR products were detected by gel electrophoresis with ethidium bromide staining. The gel was digitized using the Gene Genius Gel documentation system, with Gene Snap software (Syngene Laboratories, Cambridge, UK) for ultraviolet visualization.

**Defensin A transcript detection by RT-PCR**

This method was used as a convenient, sensitive and semi-quantitative way to analyze changes in gene expression. For the RT-PCR analysis, cDNA was synthesized from 10 µg of RNA using an oligo-dT primer from three separate experiments. Gibco Platinum Taq (Invitrogen) and related reagents were used for PCR reactions, as per the manufacturer’s instructions. The following specific primers were used. Primer sequences for *defensin A* were:

- defensin A_F: 5’-CCGCACCTGATCTGCTG-3’
- defensin A_R: 5’-TCAATTTCGACAGACGCAGACCTT-3’

Normalization was performed using a set of internal control genes. Primers for *actin* were:
Defensin A peptide detection by Western blot assay

Supernatants of the infected- and mock-infected C6/36 cells were collected and protein concentrations were measured using a 2-D Quant Kit (GE Healthcare, Piscataway, NJ, USA).

Defensin A peptide expression was determined in the DENV-exposed cells by using Western blot analysis. The supernatant was mixed with loading buffer (25% 4× Tris-Cl/SDS pH 8, 20% glycerol, 1% SDS, 0.5% 2-mercaptoethanol) and boiled for 2 min. Using this assay, about 40 μg of purified protein was separated on precast 12% SDS-PAGE gels (Bio-Rad, Hercules, CA, USA) and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blotted with a polyclonal anti-defensin antibody raised against defensin in rabbits (diluted 1:45 000) (Perkin-Elmer, Foster, CA, USA), followed by horseradish peroxidase (HRP) conjugated secondary antibody (diluted 1:3000), and exposed to Lumilight Western Blotting Substrate (Roche, Indianapolis, IA, USA), as per the manufacturer’s instructions.

Digital images of the Defensin A (Def A) gene/peptide bands were captured at 600 dpi using a color scanner (SPEC; GE Healthcare). The density of band expression for each level was determined using GeneTools v4.0 (Syngene).

SELDI-TOF-MS

Surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF-MS) ProteinChip Arrays (Bio-Rad) were used, as previously described (Misse et al. 2007). Mosquito extracts (from three groups fed on different meals: sugar, non-infectious blood and DENV-2-infected blood) were diluted in a binding buffer (100 mM ammonium acetate, pH 4) and applied to a cation exchanger (CM10) chip. Chip-captured proteins were air-dried and covered with a matrix (3,5-dimethoxy-4-hydroxycinnapynic acid (SPA) in 99.9% acetonitrile and 0.1% trifluoroacetic acid) to absorb the laser energy. Ionized and desorbed proteins were detected and their molecular masses displayed on a proteogram. Peaks were determined by using SELDI-TOF-MS analysis with Protein-Chip Biology System 3.5 software (Luplertlop et al. 2011).

Data analysis

Differences among samples collected at various time points were compared under each experimental condition, using a standard Student’s t-test to determine statistical significance. P ≤ 0.05 was considered statistically significant. These assays were performed using SPSS statistical software v13.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Defensin detection on C6/36 cells at different MOI

The mosquito C6/36 cell line was used to investigate the expression of defensin A gene (Def A) after dengue viral infection. The expression of Def A on C6/36 cells infected with DENV-2 with different MOI of 1, 5, 10 and 15 were determined using RT-PCR coupled with densitometry.

The presence of Def A showed that expressions increased with MOI of 1, 5, 10 and 15, in a dose-dependent manner (Fig. 1). These results indicate that the expression Def A could be enhanced by increasing the level of DENV-2 challenge.

Expression of defensin A transcript and peptide on C6/36 infection

In this study, Def A gene expression was determined in C6/36 cells infected with viable DENV-2 and inactivated DENV-2 at a MOI of 1, for 30 min to 48 h post-inoculation, using RT-PCR coupled with densitometry.

The results showed that Def A transcript expression was induced in response to both viable DENV-2 and inactivated DENV-2 challenge. Both viable and inactivated DENV-2 could be highly expressed by intensity level at 30 min post-infection, continually increasing to the highest level at 6 h post-infection (hpi), before decreasing to a low level at 12 to 48 hpi (Fig. 2). Moreover, Def A transcript expression on C6/36 cells infected with viable DENV-2 at 6 hpi was higher than cells infected with inactivated DENV-2 (2.8 times).
addition, the expression of Def A peptide was determined, to confirm and quantify the induction of Def A transcript that continued to be processed to produce the Def A peptide, using Western blot assay coupled with densitometry. The Def A peptide was produced in response to the viable DENV-2 and inactivated DENV-2 challenge (Fig. 3). The time for Def A peptide expression was consistent with the time for expressed Def A transcripts on the C6/36 cells infected with viable DENV-2 and inactivated DENV-2.

Defensin A peptide expression in female *Aedes aegypti* mosquito infections

The expression of the defensin family belonging to the antimicrobial peptides (AMPs) was determined at 14 days post-feeding (dpf) in female *Aedes aegypti* mosquitoes fed on three different meals: DENV-2 infected blood, non-infectious blood and sugar. We used SELDI-TOF-MS to analyze components of the whole body, including the hemolymph and fat body. The mass spectrum from 3000 to 5000 m/z was used to determine defensin peptides in mosquitoes fed on DENV-2-infected blood, non-infectious blood and sugar meals.

The results of this analysis showed that DENV-infected blood-fed mosquitoes expressed a mass value peak 4.254 kDa higher than normal blood- and sugar-fed mosquitoes (Fig. 4). The 4.254 kDa peptide consists of the identified peptide sequencing data for mosquito defensin A (Lowenberger et al. 1995; Bartholomay et al. 2004). This result indicated that DENV-2 infection...
strongly induced Def A peptide expression at 14 dpi, supporting that the Def A peptide response may be due to the dengue virus. Our results suggest that the mosquito’s humoral immune response against DENV is activated early, through intracellular signaling pathways that induce expression of this defensin.

**DISCUSSION**

**Defensin A transcript and peptide expression in C6/36 infection**

Most studies that have focused on mosquito immune responses against pathogens have employed the lineage C6/36 (Igarashi 1978; Miller & Brown 1992; Adelman et al. 2002; Sanchez-Vargas et al. 2004). The C6/36 cell line has been used to demonstrate regulation of the synthesis and secretion of several important proteins, such as defensin (Cheng et al. 2001) and cecropin (Moon et al. 2011). Mostly, however, this cell line has been used to study aspects of vector–virus relationships (Sanchez-Vargas et al. 2004). This is because one of the main features of C6/36 is its capacity to grow a very broad spectrum of viruses and to produce higher virus titers than any other cell. It is now known that this property is, at least partially, due to the lack of a functional RNAi response in these cell lineages (Brackney et al. 2010; Barletta et al. 2012).

The present study, to investigate Def A transcript abundance in C6/36 cells challenged by dengue virus type 2 (DENV-2), revealed that the level of Def A expression could be increased in correlation with an increasing number of DENV-2 challenges to the mosquito cell line, in a dose-dependent manner. This result was consistent with a previous study on the transcriptional and translational profiles of defensin production was a dose-dependency with serial dilutions of bacteria in *Ae. aegypti* after inoculations (Bartholomay et al. 2004).

The expression of Def A transcripts and peptide in C6/36 cells challenged with live dengue virus and with inactivated dengue virus for 30 min to 48 h post-inoculation revealed the maximum levels of Def A transcript and peptide presented at 6 hpi, after which the signal decreased in band intensity. These results show that AMPs can be synthesized at relative levels according to microorganism (Shai 1998). Therefore, the increase and/or decrease in levels of Def A expression at different times may be in response to the external stimulator quite early, and reverts to normal very rapidly during the later stages of DENV infection. The length of the DENV replication cycle is estimated to be 30 h (Helt & Harris 2005; Sim & Dimopoulos 2010). In addition, the detectable Def A peptide was particularly strong 6 h after induction, which was consistent with the time of Def A transcripts, suggesting that the expression corresponds to the induction of Def A transcripts, which occurs relatively early in DENV infection, and replicates actively. However, a stimulated translation to produce both immature and mature forms of these peptides were found to be active against DENV in the time course of early DENV infection (Luplertlop et al. 2011). It has been demonstrated that AMPs in *Ae. aegypti* show that protein translation follows transcription. There seems to be a threshold of transcription leading to translation, but when transcription is very high, translation also occurred quite rapidly (Bartholomay et al. 2004). The result strongly suggests that the expression of the Def A transcripts and encoding Def A peptide, which are involved in the innate immune response, were induced by DENV infection.

Studying DENV infection in the mosquito cell line and in the mosquito carcass, resulting in regulation and induction of antimicrobial peptides in the immune response mechanisms, may vary due to certain factors (Xi et al. 2008), including: (i) the different time points, which are relatively late in the infection process; the C6/36 cell line transcription was determined at 30 min to 48 hpi, whereas mosquito transcription was characterized at 14 dpi; and (ii) the mosquito carcass comprises many different tissue types and organs. Thus, transcription may reflect an average across these heterogeneities, as well as the sum total of transcript abundance in the different tissues and cell types.

**Defensin A peptide expressions in *Aedes aegypti* mosquito infection**

The aim of this study was to determine expression of the defensin family on female *Ae. aegypti* mosquitoes fed on DENV-2-infected blood, non-infectious blood and sugar at 14 days post-feeding. Our results revealed that DENV-infected blood-fed mosquitoes predominantly expressed a peptide with a molecular weight of 4.254 kDa. This peptide with 4 kDa has been identified to have peptide sequencing as defensin-like peptide (Chalk et al. 1994, 1995; Lowenberger et al. 1995; Bartholomay et al. 2004). Defensins are cysteine-rich cationic peptides of about 40 amino acids, found in a variety of invertebrate and vertebrate organisms (Lehrer et al. 1993; Dassanayake et al. 2007). They are the major class of AMPs that have been found in *Ae. aegypti* (Lowenberger 2001).

Our results showed expression of the peptide 4.254 kDa as the likely Def A peptide (Chalk et al. 1994, 1995; Lowenberger et al. 1995; Bartholomay et al. 2004), which is clearly presented in the DENV-infected blood-fed mosquitoes at 14 dpi, corresponding...
to the period of the governing infection, and to dissemi-
nation and transmission. In addition, the growth curve
of DENV infection in Aedes mosquitoes demonstrated
DENV titers could increase until 14 days post-infection,
relating to the extrinsic incubation period, which is
about 7–14 days (Black et al. 2002; Salazar et al. 2007).
The immune-response effect of DENV in the mosquito
during the early stages of infection could aid the estab-
lishment of viral infection, until dengue transmission by
mosquitoes, since infected mosquitoes can transmit
DENV for life (Kyle & Harris 2008). The immune path-
ways and effectors activated in response to pathogens in
Ae. aegypti still remain unclear.

Recent studies have provided important insights into
Ae. aegypti immune responses to DENV-2 infection,
showing that the Toll and the Janus kinase/signal trans-
duction and activators of transcription (JAK-STAT)
pathways, including RNAi machinery, are important for
the mosquito’s defense against DENV infection (Xi et al.
2008). These pathways have been reported to play an
important role in controlling DENV replication in the
midgut (Xi et al. 2008; Souza-Neto et al. 2009; Ramirez
& Dimopoulos 2010). The recent sequencing and anno-
tation of the Ae. aegypti genome is of importance for
identifying new effectors in the immune system of
dengue vector (Nene et al. 2007).

The mosquito salivary gland compartment harbors a
potent cellular response against DENV. The salivary
gland’s transcriptome profile has been reported to carry
many genes that are up-regulated after DENV infection,
and encode proteins that are involved in the innate
immune response and which participate in the IMD and
Toll-like signaling pathways for modulating DENV
infection. In addition, it also could induce the expression
of the gene, encoding a putative cecropin-like peptide,
which exhibits antimicrobial activity as a primary
defense in the innate immune response of insects
(Lupertlop et al. 2011). It has been reported that both
cecropin A and defensin A were co-overexpressed in a
cooperative antibacterial and anti-plasmodial action in
transgenic Ae. aegypti (Kokoza et al. 2010). The present
study suggests that the expression of Def A peptide may
thus be induced by dengue infection, rather than normal
blood meal or sugar feeding.

In Ae. aegypti, defensin is the most important AMP
(Lowenberger 2001). This molecule has a known activ-
ity against Gram-positive bacteria but is up-regulated in
mosquitoes on infection with a variety of pathogens,
including different types of bacteria, fungus, malaria
parasites and filarial worms (Lowenberger et al. 1995,
1996; Cho et al. 1996; Dimopoulos et al. 1997;
Bartholomay et al. 2004; Tereza et al. 2008). Several
pieces of evidence support the synthesis of immune
peptides, including defensin of mosquito responding to
limit development of pathogens in insect vectors
(Lowenberger et al. 1996, 1999b; Bartholomay et al.
2004). For example, promoter analyses of the defensin
A sequence from both Ae. aegypti and Anophe-
les gambiae defensins reveal binding sites for immune
responsive transcriptional regulators (Cho et al. 1997;
Eggleston et al. 2000). The mosquitoes inoculated with
bacteria or purified defensin had a lower prevalence
and intensity of infection than did control mosquitoes
(Albuquerque & Ham 1996; Lowenberger et al. 1996,
1999a; Shahabuddin et al. 1998). Our study showed
that DENV-2 infection strongly induced Def A expres-
sion, indicating that the response of this peptide is due to
potent inducible anti-viral activity in the mosquito.

In future studies, it would be interesting to know
whether the expression of defensin peptide in these situ-
ations is stimulated by the presence of the pathogens, by
a potential stress caused by the virus, or by mechanisms
to control or eliminate the pathogen. Our study may be
helpful in the discovery of new synthetic peptides, which
may be useful in the control of dengue disease.

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