Antibacterial peptides are secreted into the midgut lumen to provide antibacterial midgut defense in the soft tick, *Ornithodoros moubata* (Acari: Argasidae)

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Abstract
Antibacterial peptide defensin isoform A was previously isolated from the midgut contents of *Ornithodoros moubata* blood-fed females. However, not only defensin A, but also three other defensin isoforms showed gene expression in the midgut, suggesting the possibility that these antibacterial peptides are secreted into the midgut lumen. To further understand tick immune mechanisms, the involvement of antibacterial peptides in midgut defense was investigated. Three antibacterial peptides with molecular masses near defensin isoforms B, C and D were detected in the midgut contents of blood-fed females. Enzyme-linked immunosorbent assay analysis revealed that the antibacterial peptides in the midgut contents cross-reacted with defensin A antibodies and increased as a response to blood feeding. Simultaneously, the antibacterial activity of the midgut contents was enhanced by blood feeding. Secretion of antibacterial peptides into the midgut lumen and an increase in the peptide concentration following blood feeding was also confirmed. These findings further support the hypothesis that antibacterial peptides play an important role in the midgut defense of ticks.

Key words: *Ornithodoros moubata*; antibacterial peptides; midgut; blood feeding; antibacterial activity

INTRODUCTION

Ticks must defend themselves from infection without antibodies and lymphocytes normally involved in the adaptive immunity of vertebrates. An innate immune response is the only known way for ticks to combat microorganisms. Research on the immune mechanisms of ticks has progressed over the last few years showing that ticks are equipped with an immune system consisting of cellular and humoral responses similar to other arthropods. In cellular defense, the phagocytotic activity of *Ornithodoros moubata* sensu Walton hemocytes and protease activity in phagosomes of the hemocytes have been reported by Inoue et al. (2001). Encapsulation and nodulation were shown in *Dermacentor variabilis* (Say) (Ceraul et al., 2002). In humoral defense, borreliacidal activity has been demonstrated with hemolymph plasma from *D. variabilis* (Johns et al., 2000, 2001a). Production of a reactive oxygen species, a well-known mechanism for microorganism killing by vertebrate phagocytes, was shown in *Boophilus microplus* (Canestrini) hemocytes (Pereira et al., 2001). Antimicrobial proteins and peptides, major components of innate immune defense, have been characterized in ticks. The lysozyme of *O. moubata* was purified from the gut by Kopáček et al. (1999). Johns et al. (2001b) purified antibacterial peptide defensin from the hemolymph of *D. variabilis*. Isolation of defensin (van der Goes van Naters-Yasui et al., 2000) and an increase in defensin concentration after bacterial inoculation (Nakajima et al., 2003) were demonstrated in *O. moubata* hemolymph.

Antimicrobial peptides are generally induced by septic injury and released into the hemolymph. On the other hand, recent studies have revealed that
some hematophagous insect antimicrobial peptides appear to be involved in midgut defense. Antimicrobial peptide defensin has been isolated from the midgut of *Stomoxys calcitrans* (Lehane et al., 1997) and *Anopheles gambiae* (Vizioli et al., 2001b). Antimicrobial peptide stomoxyn with antiparasitic activity was also identified from the midgut of *S. calcitrans* (Boulanger et al., 2002). Gene expression of antimicrobial peptides in the midgut has been demonstrated in *Aedes aegypti* defensin (Lowenberger et al., 1999), *Rhodnius prolixus* defensin (Lopez et al., 2003) and gambicin (Vizioli et al., 2001a). The midgut of hematophagous arthropods is vulnerable to attack from microorganisms ingested with the blood meal. Therefore, hematophagous arthropods must possess an efficient defense mechanism within their midgut.

In *O. moubata*, gene upregulation of defensin (Nakajima et al., 2001, 2002) has been demonstrated in the midgut after blood feeding. However, little is known about the contribution of the mature antibacterial peptides to tick midgut defense. In order to clarify the importance of antibacterial peptides in midgut defense, secretion of the peptides into the midgut lumen and post blood-feeding regulation of the peptide levels were investigated.

**MATERIALS AND METHODS**

**Ticks.** Soft ticks, *O. moubata* sensu Walton (Acari: Argasidae) (Walton, 1962), were obtained in 1994 from Prof. Yasuo Chinzei, School of Medicine, Mie University and have since been maintained in our laboratory. The ticks were fed on rabbits (*Oryctotagus cuniculus*) and maintained at 30±1°C, 70±10% relative humidity and total darkness. Feedings and rearing of ticks were as described by Chinzei (1983).

**Peptides extraction.** The midgut was dissected from unfed and engorged adult females (1, 3, 5, 10 and 15 d after engorgement). The content of each midgut was separately diluted in phosphate-buffered saline (PBS) containing protease inhibitors (Complete, Roche Diagnostics, Tokyo, Japan), acidified with trifluoroacetic acid (TFA) to a final concentration of 0.1% and kept on ice for 1 h. The acid extract was heated at 100°C for 10 min and centrifuged at 10,000×*g* for 10 min. The supernatant was loaded onto a reverse-phase (RP) Sep-Pak C18 cartridge (Walters, Milford, MA, USA). After washing with acidified water (0.05% TFA), two successive elutions were performed with 20 and 30% acetonitrile in acidified water. Peptides eluted in 30% acetonitrile were lyophilized and stored at −20°C until further analysis.

**Separation of Sep-Pak 30% fraction.** The Sep-Pak 30% fraction of 15 midguts (10 d after engorgement) was reconstituted in distilled water and separated by RP-high-performance liquid chromatography (RP-HPLC) using a SMART system (Amersham Pharmacia Biotech, Uppsala, Sweden) on a μRPC2/C18 column (Amersham Pharmacia Biotech) with a linear acetonitrile gradient of 20–30% in 0.05% TFA. The antibacterial activity against the *Staphylococcus aureus* subsp. *aureus* (NBRC 12732) of each fraction was monitored by radial diffusion assay (Lehrer et al., 1991). The well diameter was 2 mm and a value of 2 mm indicates no formation of an inhibition zone. The peptides from fractions showing antibacterial activity were further eluted through a Sephasil C18 SC 2.1/10 column (Amersham Pharmacia Biotech) with a linear gradient of acetonitrile containing 0.05% TFA.

**Matrix-assisted laser desorption/ionization-time of flight mass (MALDI-TOF MS) spectrometry.** MALDI-TOF MS analysis was performed by Voyager RP Biospectrometry (Applied Biosystems, Foster City, CA, USA) in a positive linear mode. Samples were deposited on the target, followed by the addition of a saturated solution of the matrix (α-cyano-4-hydrocinnamic acid) in 50% acetonitrile.

**Antibody preparation.** Four milligrams of synthetic *Ornithodoros* defensin A was coupled to ovalbumin using glutaraldehyde. A male rabbit was immunized with a first intradermal injection of 1 mg antigen in Freund’s complete adjuvant followed by five additional intradermal injections of 0.5 mg antigen in Freund’s incomplete adjuvant at 2-wk intervals. The antiserum was purified from rabbit serum by a hitrap protein-G Sepharose column (Amersham Pharmacia Biotech) according to procedures described by the manufacturer.

**Detection of antibacterial peptides by enzyme-linked immunosorbent assay (ELISA).** The Sep-Pak 30% fraction of midgut contents was reconstituted with PBS (8 μg/ml). Microtiter plates were coated overnight at 4°C with 50 μl of the Sep-
Pak 30% fraction. After washing with PBS, the microtiter plates were incubated with blocking buffer (PBS containing 5% BSA) for 1 h, washed and incubated with anti-defensin IgG (30 μg/ml) for 1 h. After washing, goat anti-rabbit IgG conjugated to peroxidase (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA) was added and incubated for 1 h. Finally, the microplate wells were washed and assayed with 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) as a substrate. The assays were spectrophotometrically quantified by measuring absorbance at 405 nm. Negative control wells coated with only PBS were quantified and subtracted from each value.

**Antibacterial activity assay.** *Escherichia coli* JM109 and *Micrococcus luteus* (NBRC 12708) were grown in Mueller Hinton Broth (MHB). Exponential phase bacteria were diluted in MHB and 90 μl (2×10⁵ cells) dispensed to sterile 96-well polypropylene microtiter plates. The Sep-Pak 30% fraction of the midgut contents was reconstituted with distilled water (40 μg/ml). Ten microliters of diluted Sep-Pak fraction was added to the bacterial suspension in the wells. The plates were incubated at 37°C for 24 h and bacterial growth was quantified by measurement of optical density at 600 nm.

**RESULTS AND DISCUSSION**

Previously, we isolated *Ornithodoros* defensin A from the midgut of blood-fed ticks (Nakajima et al., 2002). However, not only defensin A, but three other defensin isoforms showed gene expression in the midgut, suggesting the possibility that these antibacterial peptides are secreted into the midgut lumen. In addition, although the upregulated gene expression of defensin in response to blood feeding has been demonstrated in *O. moubata* (Nakajima et al., 2001, 2002), little is known about changes in the peptide levels of antibacterial peptides. To further understand tick immune mechanisms, we investigated the involvement of defensin in midgut defense.

First, we focused on the detection of antibacterial peptides in the midgut. Acid extracts of midgut contents from blood-fed females were purified by Sep-Pak C₁₈ cartridges. The Sep-Pak 30% acetonitrile fraction was submitted to RP-HPLC and eluted with a gradient of 20–30% acetonitrile, yielding the chromatogram shown in Fig. 1. Four zones showed activity against *S. aureus* (Fig. 1, zone “a” to “d”). A previous study revealed the active zone eluted between 18–19 min (Fig. 1, zone “b”) contained *Ornithodoros* defensin A (Nakajima et al., 2002). Therefore, the other three active zones (Fig. 1, zone “a”, “c” and “d”) were submitted to the second purification step. After the second purification step, the three active zones were separated into single peaks with antibacterial activity (Figs. 2–4). Although mass measurement by MALDI-TOF MS showed the presence of multiple molecules in these three peaks (Figs. 2–4), all three peaks contained a molecule with a molecular mass

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**Fig. 1.** First RP-HPLC separation of an acidic extract obtained from *O. moubata* midgut contents. The Sep-Pak 30% acetonitrile fraction of the midgut contents was analyzed on a μRPC C₂/C₁₈ column with a linear gradient of acetonitrile (dotted line) and absorbance was monitored at 220 nm (solid line). Shaded bars indicate anti-*S. aureus* activity measured by radial diffusion assay. The four zones showed anti-*S. aureus* activity (zone “a”–“d”).
of about 4 kDa. An antibacterial peak (eluted around 17.5 min) derived from zone “a” in Fig. 1 contained a molecule with a molecular mass at 4266 MH⁺, which is near to the molecular mass of *Ornithodoros* defensin B (theoretical molecular mass at 4265.8 Da) (Fig. 2). Zones “c” and “d” also contained molecules with masses of 4163.6 MH⁺ and 4060.5 MH⁺, which are close to *Ornithodoros* defensin C (theoretical molecular mass at 4166.6 Da) (Fig. 3) and *Ornithodoros* defensin D (theoretical molecular mass at 4062.5 Da) (Fig. 4). Consequently, the three antibacterial peptides were newly detected as mature peptides in the midgut contents of blood-fed females. The previous and present studies strongly support the hypothesis that antibacterial peptides are secreted into the midgut lumen in *O. moubata*. Antibacterial activity against Gram-positive bacteria in the Sep-Pak 30% fraction of the midgut contents may be derived from defensin-type antibacterial peptides. However, direct evidence is necessary to prove this hypothesis.

Next, the relative concentration of antibacterial peptides in the midgut contents was analyzed by
ELISA using rabbit anti-*Ornithodoros* defensin A polyclonal antibodies. Sep-Pak fractions (30%) of midgut contents were prepared from unfed and blood-fed females (1, 3, 5, 10 and 15 d after feeding). The relative concentration of antibacterial peptides in the midgut contents cross-reacting with *Ornithodoros* defensin A antibodies increased after blood feeding as indicated by ELISA absorbance values (Fig. 5). A low level of antibacterial peptides was detected in the midgut contents of unfed females. After feeding, the level of the peptides gradually increased and reached a plateau on day 3. There was a 100-fold increase in the peptides 10 d after feeding as compared to unfed females. The high level of the peptides remained almost constant through 15 d after feeding. Similar post blood-feeding kinetics of defensin mRNA were obtained in our earlier studies (Nakajima et al., 2001, 2002). Defensin has been identified in several other hematophagous arthropods, i.e. *A. aegypti* (Chalk et al., 1994; Lowenberger et al., 1995), *A. gambiae* (Richman et al., 1996), *Glossina morsitans* (Hao et al., 2001; Boulanger et al., 2002) and *R. prolixus* (Lopez et al., 2003). However, defensin upregulation at the peptide level in response to a blood-meal has only been confirmed in *S. calcitrans* and *O. moubata*. Therefore, identification of the antibacterial peptides from midgut lumen is important for better understanding the immune system of hematophagous arthropods.

In order to verify whether the relative concentrations of the antibacterial peptides reflect the antibacterial activity of the midgut contents, the antibacterial activity of midgut contents was analyzed by a liquid growth inhibition assay. Growth inhibition of both Gram-positive *M. luteus* and Gram-negative *E. coli* were not observed in unfed tick midgut contents (Fig. 6). However, the midgut contents of fed ticks showed a significant reduction in *M. luteus* growth, less than 5% growth as compared to 100% growth in the control. Midgut contents days 3, 5 and 10 after feeding also inhibited *M. luteus* growth to less than 2%. Midgut contents retained strong antibacterial activity against *M. luteus* (less than 2% growth) even up to 15 d after feeding. On the other hand, *E. coli* was not greatly affected by midgut contents and showed only 20–30% growth inhibition (Fig. 6). Generally, invertebrate defensins are mainly active against Gram-positive bacteria, while Gram-negative bacteria are not affected (Bulet et al., 1999). Therefore, the antibacterial activity of midgut contents (30% Sep-Pak fraction) appears to be greatly affected by the concentration of antibacterial peptides such as defensins. These observations suggest that antibacte-
rial peptides are secreted into the midgut lumen and play a major role in the midgut defense of *O. moubata*.

The midgut is the largest organ in the tick body and serves as a major organ for storage of the blood-meal. The virtual absence of extracellular digestive enzymes in the midgut lumen makes this organ a highly favorable environment for survival of ingested microbes (Sonenshine, 1991). Therefore, it is necessary for ticks to be equipped with antimicrobial substances in the midgut. Secrecion of antibacterial peptides into the midgut lumen and an increase in concentration of the peptides following blood feeding were confirmed in this study. Moreover, gene upregulation of lysozyme in the gut after a blood-meal has been demonstrated (Grunclová et al., 2003). In the hard tick *D. variabilis*, a synergistic effect between defensin and lysozyme against the Lyme disease spirochete, *Borrelia burgdorferi*, was demonstrated (Johns et al., 2001b). In *O. moubata*, there is a possibility that antibacterial peptides including defensins and lysozyme synergistically act against not only Gram-positive bacteria, but also Gram-negative bacteria and other microorganisms such as spirochetes, protozoan, fungi and viruses. Further research on the midgut defense of *O. moubata* will greatly contribute to our understanding of vector competency in ticks.

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REFERENCES


