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An Arthropod Defensin Expressed by the Hemocytes of the American Dog Tick, Dermacentor variabilis (Acari: Ixodidae)

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Abstract.

Both soluble and cell-mediated components are involved in the innate immune response of arthropods. Injection of *Borrelia burgdorferi*, the Lyme disease agent, results in the secretion of defensin into the hemolymph of the ixodid tick, *D. variabilis*. The presence of the peptide is observed as early as 15 minutes post-challenge and remains present through 18hrs post-challenge. As is observed in insects and soft ticks the transcript for defensin is detected as early as 1 hour post-challenge in *D. variabilis*. RT-PCR resulted in an amplicon of 624bp with a 225bp region that translates to a 74 amino acid preprodefensin. The defensin encoding region was amplified, cloned and sequenced from the hemocytes. It appears as though defensin is stored in the granulocytes of the hemolymph and secreted into the hemolymph upon bacterial insult. The role of defensin as a contributing factor in determining vector competency is discussed.

Keywords: Defensin, hemocytes, ticks, innate immunity

Introduction.

The innate immune system is one of the most important factors in the ability of metazoan organisms to survive when challenged by microbes. The innate immune system is comprised of cell-mediated and soluble components and is initiated through recognition of pathogen-associated molecular patterns (PAMP's) (Girardin et al., 2002). In many invertebrates, the soluble component of the system includes antimicrobial peptides such as lectins (Chen et al., 2001),

attacins (Gillespie et al., 1997), lysozyme (Kopacek et al., 1999), defensin (Johns et al., 2001; Nakajima et al., 2002; Nakajima et al., 2001) and others.

In insects, the expression of defensin is induced in the fat body following bacterial injection (Gillespie et al., 1997). In *Drosophila*, septic injury alone, bacterial challenge with *Escherichia coli* or *Micrococcus luteus*, or challenge with the fungus *Beauveria bassiana* induced the expression of defensin between 0 and 3 hours post-challenge (Lemaitre et al., 1997). However, this pattern of rapid induction is not universal. In the Mediterranean mussel, *Mytilus galloprovincialis,* defensin is constitutively expressed and the mature peptide is secreted from the granulocytes into the plasma 24 hours post-injection of heat killed *Vibrio alginolyticus* (Mitta et al., 1999). In the soft tick *O. moubata*, defensin is expressed in the midgut as early as 1 hour post-feeding (Nakajima et al., 2001). A defensin (varisin) was observed in the hemolymph of the hard tick, *Dermacentor variabilis*, between 1 and 6 hours post-injection of the Lyme disease spirochete, *B. burgdorferi* (Johns et al., 2001). The sequence for the first 30 amino acids of the predicted 38 to 40 amino acids was determined by Edman degradation. A MALDI-TOF predicted a molecular weight of 4228.66 for the secreted peptide (Johns et al., 2001). However, neither the full amino acid sequence for the mature peptide nor the preprodefensin was determined. This paper reports the nucleotide sequence and the derived amino acid sequence of the preprodefensin from the hemocytes of *B. burgdorferi*-challenged

D. variabilis ticks. In addition, a mechanism of storage and release of defensin into the hemolymph by hemocytes is also discussed.

Materials and Methods.

D. variabilis ticks collected near Suffolk, VA were reared and maintained as described (Johns et al. 1998). Female ticks fed for 6 days were used in all experiments. All use of animals in this research was done in accordance with protocols approved by the Old Dominion University Institutional Animal Use and Care Committee protocols 01-006 and 01-007 approved on February 13, 2002. Three-day *B. burgdorferi* cultures were adjusted to approximately 1.0×10^4 cells/μl and three microliters injected into the hemocoel as described (Johns et al., 1998). Depending upon the experiment, hemolymph was collected at 1hr, 6hr, 9hr, 12hr, 15hr and 18hr post-injection and diluted in either RNA later (Ambion, Austin, TX) or modified tick saline (1mM EDTA, 0.1mM PMSF, pH 8.5) adjusted to approximately 300 mOsm using alkaline water (pH 11). Hemocytes were removed by centrifugation at 830 x g for 15 minutes at 4°C washed gently with tick saline and the cell pellet resuspended in 50μl of lysis buffer (20mM Tris-HCl, 137mM NaCl, 2mM EDTA, 0.1% Triton-X 100, 10% glycerol, pH 7.2) (Han and Ip, 1999). The hemocyte lysates were sonicated for 90s on ice and the lysate clarified by centrifugation at 16,000 x g for 15 min at 4°C. The hemolymph plasma and hemocyte lysate were stored in aliquots at –20°C until used.

A synthetic defensin peptide representing the first 20 amino acids of the mature peptide was prepared and conjugated to keyhole limpet hemocyanin (KLH) (Genemed Synthesis, Inc., San Francisco, CA). A New Zealand White rabbit, *Oryctolagus cunniculus*, was immunized subcutaneously with 0.6 mg of synthetic defensin-KLH conjugate mixed 1:1 with Freund's complete adjuvant (Sigma, St. Louis, MO) followed by two-booster immunizations of 0.25 mg mixed 1:1 in Freund's incomplete adjuvant (Sigma) spaced at 2 to 3 week intervals. Immune serum was collected 2 weeks after the final immunization and antigen-affinity purified. The antigen-affinity column was made by linking the synthetic defensin peptide to an activated 4% agarose Aminolink Plus Immobilization affinity column according to the manufacturers instructions (Pierce Biotechnology Inc., Rockford, IL). Immune rabbit serum was passed twice over the affinity column, eluted with 0.2 M glycine buffer pH 2.5, the eluate neutralized with 50μl of 1M Tris-HCL, pH 9.0 and concentrated in a 5000 MWCO Microcon filter (Millipore, Billerica, MA) according to the manufacturer's instructions. The rabbit anti-defensin antibody was resuspended in PBS (0.1M NaH_2PO_4, 0.1M Na_2HPO_4, 0.15 NaCl, pH 7.2), supplemented with 0.02% NaN_3 and 10% glycerol, and stored in aliquots at –20°C until used.

Hemocyte lysate, 60ug, was run on 4%-12% NuPAGE Tris-Bis SDS gels (Invitrogen) with Mark XII molecular weight markers (Invitrogen) and silver stained using Silver Express staining kit (Invitrogen). For Western blots, gels were transferred to 0.45μm pore-size nitrocellulose for 1hr 30 min at 25V

constant voltage in an EC140 Miniblot System (ThermoEC, Holbrookn, NY). Tick hemocyte protein bands were detected using primary antibodies (1:75) incubated overnight at 4°C followed by goat anti-rabbit IgG (H+L) conjugated to horseradish peroxidase (1:1000) and the bands visualized with Protein Detector TMB Western Blotting kit (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md). Western blot controls included testing the conjugate with and without pre-immune rabbit sera as the primary antibody where we observed bands > 14 kDa consistent with the presence of imbibed IgG from ticks fed on rabbits (Jasinskas et al., 2000) or IgG-binding proteins (Wang and Nuttall, 1995). However, only purified rabbit anti-defensin antibody reacted with the 4.2 kDa defensin protein.

Poly A RNA was extracted from the hemocytes on the day of collection using QuickPrep™ Micro mRNA Purification Kit (Amersham Biosciences, Piscataway, NJ). First-strand synthesis was performed using 25ng of mRNA in Promega's ImProm-II Reverse Transcription System (Promega, Madison, WI). Five hundred nanograms of cDNA cloning primer (5'GAAGAATTCTCGA GCGGCCGCTTTTTTTTTTTTTTTTTTV 3') (Integrated DNA Technologies, Coralville, IA) were used to prime the mRNA during first strand synthesis. PCR using 200nM of Vsn F2-I (5'GGITTYGGITGYCCICTIA AYCAR 3') and the cDNA cloning primers were used to amplify the cDNA. PCR cycling was as follows: 95°C for 5 min followed by 30 cycles of 95°C for 30 sec, 54°C for 1 min, 68°C for 1 min followed by a final extension at 68°C for 10 min. The resultant amplicon

was cloned into pCR4 TOPO vector (Invitrogen, Carlsbad, CA) and sequenced on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). Two hundred nanomoles of the nested gene specific primers based on the sequence obtained following RT-PCR above (GSP) (GSP 1, 5'GTCTGCTTG ATGATGCCAGAGC AGTA 3'; GSP 2, 5'GAGCAGTAGCCGCCTCGGCG CCGAAT 3') was used with the GeneRacer RACE kit (Invitrogen) to obtain the 5' region. The initial PCR cycling parameters were: denaturation at 95°C for 5 min followed by 30 cycles of 95°C for 30 sec, 58°C (GSP 1) for 1 min, 68°C for 1 min followed by a final extension at 68°C for 10 min. One microliter from the initial PCR was used in nested PCR and was cycled under the following conditions: denaturation at 95°C for 5 min followed by 30 cycles of 95°C for 30 sec, 65°C (GSP 2) for 1 min, 68°C for 1 min followed by a final extension at 68°C for 10 min. Amplification of the 624bp cDNA fragment for defensin from hemocyte cDNA was done using primers, Vsn F (5'GACTGCGCTTTGAGACGACAAA 3') and Vsn R (5'AGAAAGCATAA CCATTTTTAATATGCATTT 3') that were designed from the sequence for the defensin from hemocytes. The cycling parameters were as follows: denaturation at 95°C for 5 min followed by 30 cycles of 95°C for 30 sec, 56°C for 1 min, 68°C for 1 min followed by a final extension at 68°C for 10 min. All sequencing was performed using ABI Prism BigDye Terminator V3.0 Ready Reaction Kit as described above. The defensin sequence designated vsnA1 and associated region was submitted to GenBank under accession number **AY181027**.

Results and Discussion.

A previous study using silver-stained, SDS-PAGE gels of plasma from *D. variabilis* injected with *B. burgdorferi* showed the 4.2 kDa defensin band as early as 15 minutes through 6 hr (Johns et al., 2001) and we have subsequently detected defensin in plasma through 18 hours (data not shown). This finding suggested that defensin is available immediately after bacterial challenge, as is found in many insects (Bulet et al., 1999; Gillespie et al., 1997).

In *M. galloprovincialis*, defensins are secreted from the hemocytes within 24hrs post-injection, and remain in the hemolymph plasma through 48 hours following challenge. The data suggests that expression continues during this period and then is down regulated (Mitta et al., 1999). In *M. galloprovincialis* defensin is transcribed as a 550 bp fragment, translated, and then stored in the granulocytes until needed; no defensin was present in the hemolymph plasma of unstimulated animals (Mitta et al., 1999). Interestingly, in *D. variabilis*, a Western blot confirmed the presence of defensin in hemocyte lysates collected from unstimulated ticks (Fig. 1). Similar to the situation in the mussel, this suggests that defensin is stored in the hemocytes of the tick and is released upon bacterial stimulation.

In the soft tick *O. moubata*, two defensin isoforms were amplified at 1 hour post-injection of *Escherichia coli* JM109 and *Stahylococcus aureus* (Nakajima et al., 2001). More recently an additional two isoforms were reported from this species

(Nakajima et al., 2002). Similarly, we amplified a defensin gene from the hemocytes as early as 1hr post-borrelial challenge. However, in contrast to *O. moubata*, only one isoform of defensin has been found in the hemocytes of *D. variabilis*. Sequencing the 624 bp cDNA fragment revealed a 225 bp ORF that translated to a 74 amino acid preprodefensin (Fig. 2). This was found to be identical to the defensin peptide that was previously purified from *D. variabilis* plasma with the exception of one amino acid at residue 27 (Johns et al., 2001). Examination of the Edman degradation results suggest the possibility for two residues at position 27, either glutamine or glycine; however, glutamine predominated in the amino acid sequence obtained. If confirmed this data would suggest two isoforms; however only the gene encoding the peptide with a glycine at position 27 has been isolated. Therefore, at this time, it appears as though the defensin present in *D. variabilis* plasma is similar to the defensin produced by the hemocytes. The presence of defensin in the hemolymph of *D. variabilis* early after infection and the detection of transcript as early as 1hr post-challenge suggest a rapid response similar to that observed in the soft tick *O. moubata* and insects.

During feeding ticks are exposed to the normal flora or pathogens infecting the host. Hence, the ability to respond to microbial challenge is presumed to be present in the midgut. Defensin has been observed in the midgut of several blood-feeding insects, e.g., the biting fly *Stomoxys calcitrans* (Munks et al., 2001), the malaria mosquito *Anopholes gambiae* (Richman et al., 1996), the

tsetse fly, *Glossina morsitans* (Boulanger et al., 2002) as well as the soft tick *O. moubata* ; Nakajima et al., 2001). Whether or not defensin is produced by the midgut of *D. variabilis* is currently unknown.

The insect fat body, believed to be functionally analogous to the vertebrate liver, detects microbial cell wall components known as PAMP's through the Toll or *imd* pathways (Belvin and Anderson, 1996). The fat body is one of the tissues responsible for defensin production (Lowenberger et al., 1999). Protein analysis of fat body lysates from unchallenged *D. variabilis* ticks suggests that defensin is stored in this tissue as well (Ceraul and Sonenshine, unpublished). With regard to the defensin peptide purified from the hemolymph of *D. variabilis,* secretion may take place from the fat body, the hemocytes or both.

Defensin is present in all types of organisms from humans to plants. A common function of defensins from all organisms is to lyse bacterial cells; however, the amino acids sequences can vary. A database (BLAST) search shows that the mature defensin peptide from *D. variabilis* has 89% identity to the defensin from the scorpion, *Leiurus quinquestriatus*, also an arachnid but in a different subclass (Subclass Scorpionomorphae) than the ticks (Subclass Acari). Less similarity is observed between the preprodefensins of *D. variabilis* and *O. moubata* (Fig. 2). Comparisons like these are interesting from a phylogenetic standpoint where it is hoped that the evolution of defensins can be mapped.

The function of each tissue involved in the innate response may be to complement one another thereby protecting the organism from infection. Organisms such as the Rocky Mountain spotted fever agent *Rickettsia rickettsii* in the case of *D. variabilis* or the Lyme disease agent *B. burgdorferi* in *I. scapularis* are imbibed with a blood meal. These organisms then interact with antimicrobial peptides in the midgut that contribute to their destruction. Transmigration of surviving microbes to the hemocoel again exposes them to secreted antimicrobial peptides as well as to a cell-mediated response.

The efficiency of the innate immune response in different tick species may contribute to a better understanding of vector efficiency for the diverse microbes they transmit. However, the question of why some ticks are competent vectors of pathogens and others are not is more complicated than the elucidation of the presence or absence of specific peptides as constituents of the antimicrobial defenses. Other considerations, such as the rate and sustainability of expression may contribute to the efficacy of the host's ability to destroy invading microbes. Thus, the robustness and rapidity of the immune response may be as important as the peptides themselves. Future work will include amplifying the defensin gene cDNA from the fat body and midgut, investigating the rate of expression for defensin within the fat body and midgut, as well as expression of defensin at different life stages of *D. variabilis* and in other tick species.

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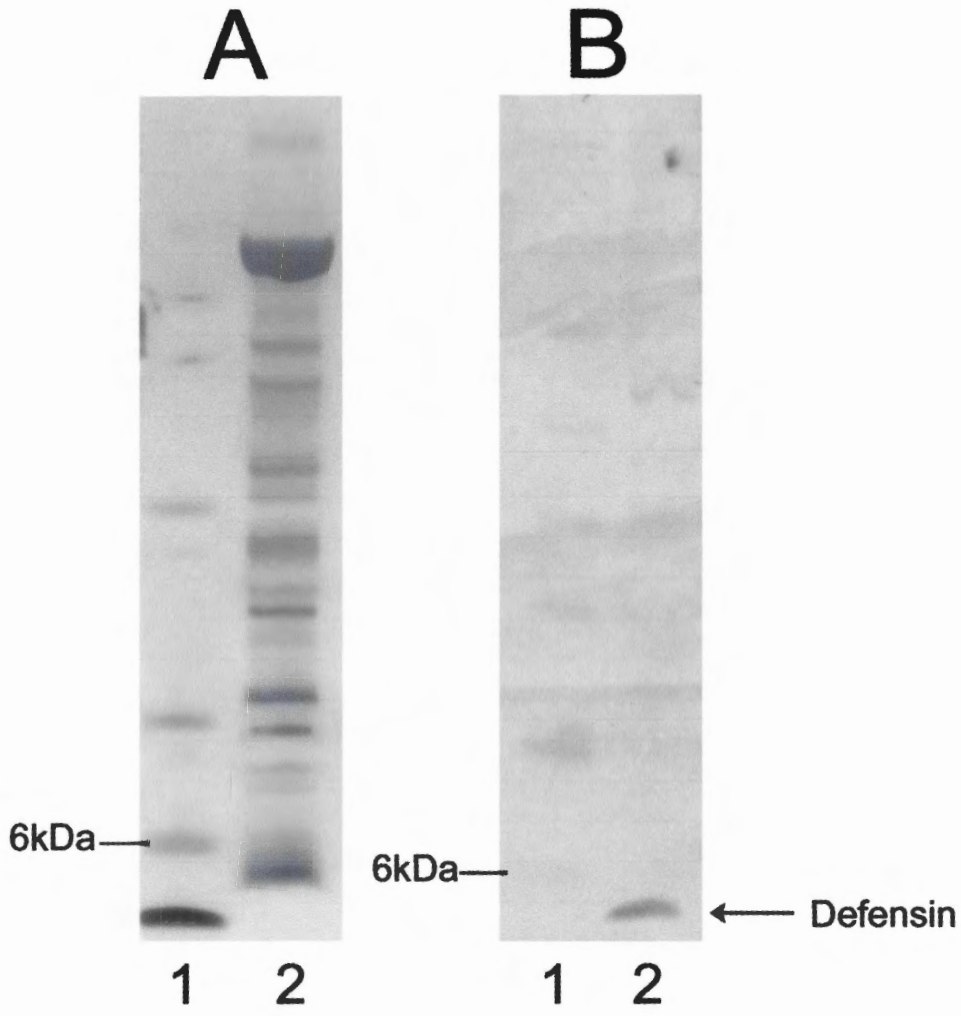

Figure 1

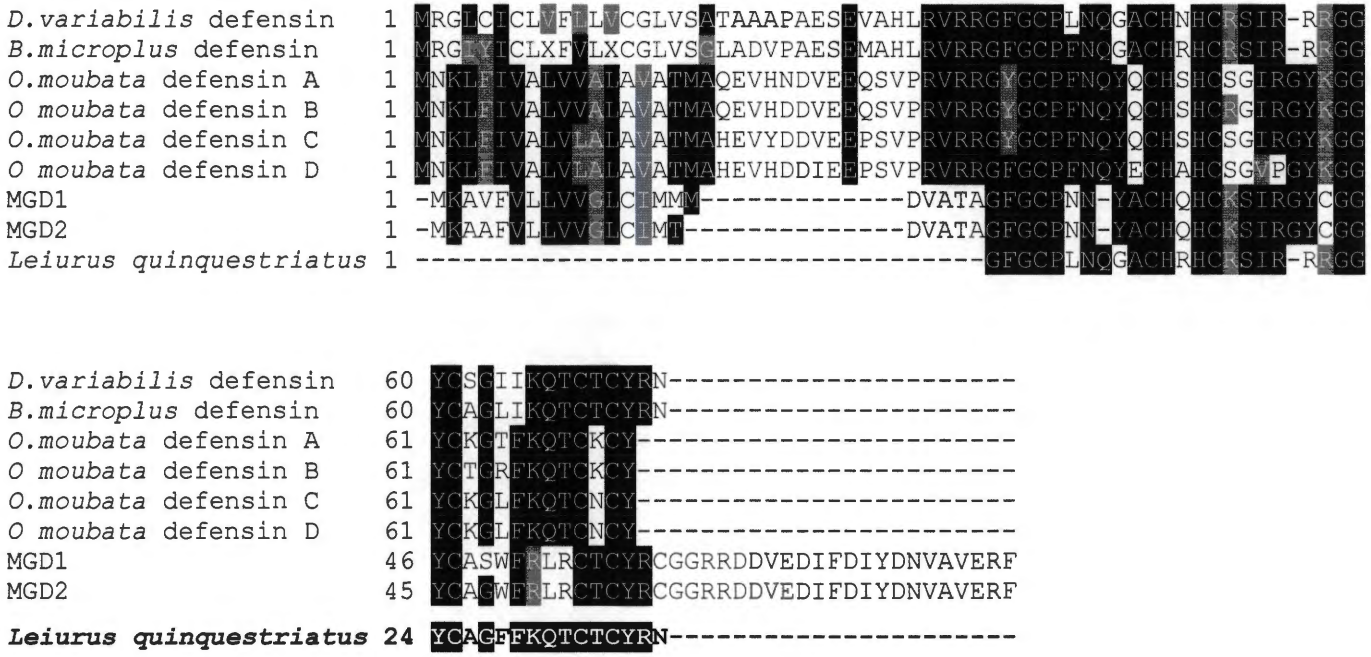
D.variabilis defensin 1 MRGLCICLVFLLVCGLVSATAAAPAESEVAHLRVRRGFGCPLNQGACHNHCRSIR-RRGG
B.microplus defensin 1 MRGLYICLXFVLXCGLVSCLADVPAESEMAHLRVRRGFGCPFNQGACHRHCRSIR-RRGG
O.moubata defensin A 1 MNKLEIVALVVALAVATMAQEVHNDVEEQSVPRVRRGYGCPFNQYQCHSHCSGIRGYKGG
O moubata defensin B 1 MNKLEIVALVVALAVATMAQEVHDDVEEQSVPRVRRGYGCPFNQYQCHSHCRGIRGYKGG
O.moubata defensin C 1 MNKLEIVALVLALAVATMAHEVYDDVEEPSVPRVRRGYGCPFNQYQCHSHCSGIRGYKGG
O moubata defensin D 1 MNKLEIVALVLALAVATMAHEVHDDIEEPSVPRVRRGFGCPFNQYECHAHCSGVPGYKGG
MGD1 1 -MKAVFVLLVVGLCIMMM----------DVATAGFGCPNN-YACHQHCKSIRGYCGG
MGD2 1 -MKAAFVLLVVGLCIMT----------DVATAGFGCPNN-YACHQHCKSIRGYCGG
Leiurus quinquestriatus 1 ----------GFGCPLNQGACHRHCRSIR-RRGG
D.variabilis defensin 60 YCSGIIKQTCTCYRN----------
B.microplus defensin 60 YCAGLIKQTCTCYRN----------
O.moubata defensin A 61 YCKGTFKQTCKCY----------
O moubata defensin B 61 YCTGRFKQTCKCY----------
O.moubata defensin C 61 YCKGLFKQTCNCY----------
O moubata defensin D 61 YCKGLFKQTCNCY----------
MGD1 46 YCASWFRLRCTCYRCGGRRDDVEDIFDIYDNVAVERF
MGD2 45 YCAGWFRLRCTCYRCGGRRDDVEDIFDIYDNVAVERF
Leiurus quinquestriatus 24 YCAGFFKQTCTCYRN----------

Figure 2.

Figure Legends

Figure 1. A. Silver-stained 4%-12% NuPAGE SDS gel and B. corresponding Western blot. Lane 1, Molecular weight markers; Lane 2, *D. variabilis* hemocyte lysate.

Figure 2. *D. variabilis* defensin peptide alignment. The defensin peptide sequence derived from the preprodefensin in these studies is identical to the peptide previously isolated from the hemolymph plasma (Johns et al., 2001) with the exception of residue 27 (**GenBank AY 181027**). The defensin from *D. variabilis* shares 68% identity with the putative defensin isolated from the ixodid tick *Boophilus microplus* (**GenBank AAO48943**) and 65%, 68%, 65% and 63% identity to the argasid tick *O. moubata* defensin A (**GenBank BAB41028**), B (**GenBank BAB41027**), C (**GenBank BAC10303)** and D (**GenBank BAC10304**), respectively (Nakajima et al. 2002). The mature defensin peptide from *D. variabilis* has 60% identity to MGD1 (**GenBank P80571)** and MGD2 (**GenBank AAD45118**) both defensins from the Mediterranean mussel, *Mytilus galloprovincialis*. The mature defensin peptide from *D.* variabilis shares 89% identity to the mature peptide from the scorpion *Leiurus quinquestriatus* (**GenBank P41965**). Black highlights indicate identical residues, gray highlights indicate similar residues.