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Investigations into the resistance of the tick, *Dermacentor variabilis* (Say) (Acari: Ixodidae)

following challenge with the bacterium, *Escherichia coli* (Enterobacteriales: Enterobacteriaceae)

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ABSTRACT In addition to a soluble response, many invertebrates control bacterial infections by means of phagocytosis or melanotic encapsulation. In some insects, *Escherichia coli* growth is reported to be inhibited by aggregation/encapsulation. Soluble and phagocytic responses to bacterial challenge have been reported in ticks, but evidence of an aggregation/encapsulation response was reported only for inanimate (araldite) implants. Ticks were challenged by direct inoculation of bacteria into the hemocoel cavity. By plating, no viable *E. coli* were detected 1 h post-inoculation. A direct fluorescence assay (DFA) revealed aggregated bacteria 1 h post-inoculation. Furthermore, DFA showed aggregated bacteria at 6, 24 and 48 h post-inoculation associated with masses of tissue, presumably of cellular origin, suggesting events similar to those described as nodulation. These findings suggest that encapsulation/nodulation may be an important component of the immune response in ticks.

Ticks manifest a nonspecific response towards bacterial challenge (Johns et al. 1998). The soluble response is comprised of antimicrobial proteins with lytic and/or agglutinating properties that are upregulated in response to microbial challenge (Gillespie et al. 1997). Antimicrobial proteins known to occur in ticks include lysozyme, a protein known for its antibacterial activity against gram-positive bacteria, purified from the midgut of *Ornithodoros moubata* (Kopacek et al. 1999) and defensin, a 4.2 kDa peptide, purified from the hemolymph of the hard tick, *Dermacentor variabilis* (Johns et al. 2001) and the soft tick, *O. moubata* (Nakajima et al. 2001). A lectin (Dorin M), isolated from the hemolymph of the soft tick *Ornithodoros moubata* (Kovar et al. 2000) induces agglutination and may aid lysozyme and defensin in preventing infection by invading microbes. Also, lectins may serve to aid in the recognition of foreign substances, thereby identifying them for destruction by phagocytic cells.

Cellular responses are also crucial in the control of microbes in ticks (Zhioua et al. 1996). Hemocyte populations both increase and migrate towards microbes upon exposure to microorganisms (Kryuchechnikov 1991, Johns et al. 1998) as well as clustering around inanimate objects (Eggenberger et al. 1990). However, little is known of the intimate interactions that take place between hemocytes and bacteria. Studies in insects show that, once contact with the microbe is made, granulocytes may lyse and release chemotactic factors. These factors in turn summon plasmatocytes, the phagocytic cells in insects, to the area, which exert their killing action against the bacterial targets (Gotz and Bowman 1985).

Once other hemocytes are summoned to the area, microaggregates comprised of hemocytes and microbes may form. The microaggregates may adhere to various organs within the hemocoel, a process known as nodulation. During nodulation, aggregates enlarge to approximately 0.1 mm in diameter and darken via the melanization process (Miller and Stanley

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1998). Nodulation is quite effective as thousands of bacteria may be cleared from the hemocoel within 1 h post-inoculation (Miller and Stanley 1998).

In a process similar to nodulation, encapsulation has been observed to surround inanimate araldite slivers implanted within ticks such as *D. variabilis* (Eggenberger et al. 1990). Soluble and cell-mediated responses are well documented in the tick, *D. variabilis*, in response to *B. subtilis*, a gram-positive bacterium, and *B. burgdorferi*, a spirochete (Johns et al. 1998, 2000). However, while the nodulation and encapsulation processes have been described in insects, little is known about these processes in ticks. Although known to occur in ticks in response to inanimate objects (Eggenberger et al. 1990), it is not clear whether these processes would also occur in response to bacterial challenge. Because *D. variabilis* is capable of encapsulation, it was of interest to investigate what occurs when microorganisms are used to stimulate the immune system in such arthropods. This study was carried out to determine whether nodulation and or encapsulation contribute to the control of *E. coli* infections in the tick, *D. variabilis*.

MATERIALS AND METHODS

Ticks. The American Dog tick, *Dermacentor variabilis,* was maintained in an AMINCO-AIRE incubator at 27°C $\pm$ 1°C with 92 $\pm$ 1% relative humidity. Immature stages were fed on rats (*Rattus norvegicus*) (larvae approx. 4 days, nymphs approx. 5 days) while adult ticks were fed and bred on New Zealand White Rabbits (*Oryctolagus cunniculus*) for 7 days. All animal procedures and tick incubations were performed as outlined by Sonenshine (1993).

Bacteria. *Escherichia coli* American Type Culture Collection (ATCC) #25922 was used in bacterial injection experiments. A stock culture of *E. coli* was maintained at -80°C from which aliquots were subcultured on tryptic soy agar (TSA) (Difco, Detroit, Michigan). Overnight broth cultures (tryptic soy broth, TSB), were made for each set of experiments (Difco). Plasmid pGFP (CLONTECH laboratories, Palo Alto, CA USA) was transformed into *E. coli* strain JM109 (Promega Corporation, Madison WI, USA) and maintained on 2YT + ampicillin agar media for use in the GFP inoculation studies.

Tick Inoculations and Hemolymph Collections. Bacteria were grown in TSB overnight and enumerated as described by Johns et al.(1998). The overnight culture of *E. coli* was adjusted to 1×10^4 cells/μl by direct count and suspended in Shen's saline solution (Oliver et al. 1974). Seven microliters of *E. coli* inoculum was administered to each tick as previously described (Johns et al. 1998). Hemolymph collections were performed immediately and at 15 min, 1, 6, 24, 48, 72 and 96 h post-inoculation. Hemocyte counts, protein assays, bacterial counts and hemolymph collection were done as described by Johns et al. (1998). For DFA's and electron microscopy preparations, which required larger amounts of hemolymph, the posterior cuticle was nicked and hemolymph was expressed by gentle pressure; hemolymph contaminated with midgut contents, guanine or other visible materials was discarded.

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Bacterial survival *in vivo*. All hemolymph collections for *in vivo* survival assays were performed aseptically. Briefly, at each designated time point through 72 h, the anterior portion of each tick was swabbed with 95% ethanol after which the ethanol was allowed to evaporate. The coxa-trochanter joint was severed using flame-sterilized micro-surgical scissors (Roboz Surgical Instruments Co., Washington, D.C. 20036) and hemolymph collected using sterile micropipette tips. Collections of hemolymph designated for bacterial survival studies were diluted in serial ten-fold dilutions through 10^{-5} in sterile Shen's saline pH 7.2. Twenty-five microliters of each dilution was spread-plated in triplicate onto TSA and incubated overnight at 37°C. The results for each time point were averaged from six individual ticks.

Protein Assays. Bradford protein assays were performed as described by the manufacturer (BioRAD, Richmond,CA).

Hemocyte counts. Hemolymph was diluted 2-fold in Shen's saline pH 7.2 and enumerated as described by (Johns et al.1998). The hemocyte concentrations in cells/μl were determined by averaging cell counts (n=16) from each time point.

Direct Fluorescence Assays (DFA). Hemolymph was collected at 1, 6, 24 and 48 h post-inoculation. Hemolymph was placed on silyated slides (CEL Associates, Inc., Houston, Texas 77099), allowed to airdry and stained with polyclonal antibody as described by Bisset and Hill (1987). Prior to antibody staining, each slide was blocked overnight at 4°C using 5% fetal bovine serum in PBS (0.2M Na_2HPO_4, 0.2M NaH_2PO_4, pH 7.2) (Bollag et al. 1996) containing 0.05% sodium azide. An anti-*E. coli* polyclonal rabbit antibody (DAKO Co., Carpinteria, CA 93013) at a final concentration of 1.3 mg/ml was used to probe for *E. coli*. Twenty microliters of rabbit anti-*E. coli* serum was incubated with both control and experimental hemolymph preparations for 90 min at 37°C. Following incubation, the slides were washed three times in PBS, pH 7.2

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before the second antibody was applied. Twenty microliters of a FITC-labeled anti-rabbit antibody (SIGMA, St Louis, MO) was incubated with the slide-fixed hemolymph samples for 90 min at 37°C. Each sample was then washed three times with PBS pH 7.2 buffer and mounted using slow-fade mounting medium (Molecular Probes Eugene, OR USA) to prevent fluorescence fading. The DFA preparations were viewed at 1000x using epi-fluorescence optics.

Electron Microscopy Hemolymph samples were collected aseptically at 1, 6, 24 and 48 h after inoculations and prepared for TEM by conventional methods (Eggenberger et al. 1990). Briefly, samples were centrifuged to pellet the cells at 300 x g for 3 min at 4°C (Brouwer et al. 1984). The cells were briefly resuspended in a 4% agar solution and centrifuged again for 30 s at 1600 rpm to form a pellet. Thin sections in the gray-silver spectrum were cut on an RMC Ultramicrotome MT2C using a Dupont Diatome 45° diamond knife. The sections were positively stained using uranyl acetate and lead citrate. The sections were viewed using a JOEL 100 CX II transmission electron microscope at an accelerating voltage of 60 kV.

GFP (Green Fluorescent Protein) Bacterial Inoculations. A colony of JM109 was resuspended in 200 μl of sterile Shen's saline and bacterial numbers adjusted as described above. Hemolymph samples were collected by nicking the cuticle 1, 6 and 48 h post-inoculation. The hemolymph was spread onto silyated slides, mounted under slow-fade mounting media (Molecular Probes, Eugene, OR) and viewed by epi-fluorescence microscopy using an excitation wavelength of 365nm.

RESULTS

Bacterial survival in vivo. Groups of ticks were inoculated with 1 x 10^4 *E. coli* cells and the hemolymph extracted at selected time points. At time zero, 7,053 ± 797 CFU were obtained from the hemolymph. At 15 min post-inoculation 1675 ± 535 CFU were detected in the removed hemolymph. At 1 h post-inoculation there were 850 ± 272 CFU, i.e. virtually unchanged (Fig. 1). However, no bacteria were detected at 6 h or any subsequent time thereafter through the 72 h observation period (Fig. 1).

Protein concentration The protein concentration of hemolymph from sham inoculated ticks were assayed at selected time points (Fig. 2). At 15 min post-inoculation, the protein concentration was 69 ± 20 mg/ml in the sham-inoculated controls. Thereafter, the protein concentrations declined to 37 ± 7 mg/ml at 1 h. Protein levels gradually rose reaching 64 mg/ml by 72 h post-inoculation. In contrast, the protein concentration in the bacteria-challenged ticks was 48 mg/ml at 15 min post-inoculation and remained virtually unchanged through 48 h post-inoculation. Both the 24 h (t = 1.88, d.f. = 8, P < 0.05) and 48 h (t = 2.23, d.f. = 8, P < 0.05) time periods show significant difference in hemolymph protein concentration between the experimental and control populations. The protein levels in the challenged ticks rose to a level not significantly different from the control levels at 72 h post-inoculation (t = 0.736, d.f. = 4, NS).

Hemocyte counts Following *E. coli* inoculation, lower hemocyte concentrations were observed in the experimental group, compared to the control, as early as 15 min post-inoculation (t = 1.8921, d.f. = 31, P < 0.05) (Fig. 3). The experimental group remained significantly lower at 1 h post-inoculation (t = 3.4309, d.f. = 31, P < 0.05). At 1 h post-inoculation hemolymph from sham inoculated ticks had an elevated hemocyte population of 8194 ± 1647 cells/μl. Thereafter, cell

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numbers from the control population declined, and remained relatively level between 6 and 96 h post-inoculation (Fig. 3). The hemocyte count in the experimental ticks increased gradually, reaching a peak at 48 h post-inoculation (t = -5.1988, d.f. = 35, P < 0.05). Hemocyte population values differed significantly between the *E. coli* challenged and control ticks at all time points sampled.

DFA Hemolymph was examined by DFA to look for aggregations of *E. coli* cells. Auto-fluorescence from hemocytes in the control samples was minimal (Fig. 4a). The earliest evidence of aggregation/nodulation by DFA was observed at 1 h point (Fig. 4b). The fluorescence image revealed an aggregated mass of bacteria readily distinguishable from dispersed bacteria. By 6 h, in addition to the clumps of bacteria, hemocytes were observed aggregated around the bacterial cluster (Fig. 4c and 4d). Note also that, in addition to the hemocytes aggregated around the bacterial cluster, several hemocytes appear free. When viewed with epi-flourescence alone, the free hemocytes are no longer evident (Fig. 4c, white arrow). At 24 h, the mass of hemocytes surrounding the bacterial cluster appears larger and individual hemocytes are no longer evident (Figs. 4e and 4f).

GFP bacterial inoculations. Aggregations of *E. coli* were evident at all time points in the experimental samples and appear associated with hemocytes. The fluorescence emitted by the GFP allowing excitation was not as intense as that emitted by the FITC-labeled antibodies. Again, as in the previous experiment there was virtually no auto-fluorescence from tick hemocytes (Fig. 5a) ensuring that fluorescence was attributed to the *E. coli* producing GFP. The aggregations took the form of compact nodules that were surrounded by disintegrating or disintegrated hemocytes, indicative of nodulation (1 h post-inoculation, 5c and 5d; 6 h post-inoculation, 5e and 5f).

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Electron microscopy Examination of hemolymph samples, each comprised of hemolymph from 6 *E. coli* challenged ticks, revealed two nodule-like masses (Figs. 6a and 6b). In both cases, the center of the nodule is an elongated, ellipsoidal area comprising numerous irregularly-shaped electron-dense particles, without a distinct membrane. Surrounding the area are numerous circular or subcircular electron dense granules typical of granules found in Type-I granulocytes and fragments of membranes. No evidence of nodules or nodule-like masses similar to those described above were observed in samples from the sham-inoculated controls. Granulocytes and plasmatocytes found in the hemolymph samples from bacteria-challenged ticks showed no evidence of phagocytosis. It is also of importance to note that no evidence of poor fixation or artifacts were observed. Intact hemocytes were evident in all samples and no scattered, free granules that might arise from random cellular disruption were found (data not shown). Thus, the clustering of the circular granules reflects degranulation presumably induced by the developing nodule.

DISCUSSION

This is the first report of nodulation in a tick. Nodulation appears to be an important mode of control used by *D. variabilis* to control *E. coli*. No FITC-labeled particles, perhaps indicative of lysis, were found floating free in the hemolymph nor were any found attached to or within hemocytes. As of yet, we see no evidence to support the role of either bacteriolysis or phagocytosis in the control of *E. coli* infections in these ticks.

At 15 min post-inoculation the number of culturable bacteria decreased from the number of CFU inoculated at time zero (Fig. 1). Furthermore, the inability to culture bacteria 6 h post-inoculation, suggests that the ticks can mount a rapid response following *E. coli* challenge (Fig.1). The efficacy of this response is evident when one considers that tick hemolymph, when removed from the tick's body, is an excellent growth medium for *E. coli* (data not shown). Thus, some factor(s) produced by the living animal effectively stops *E. coli* growth. Similar findings have been reported following hemocoel inoculation with other bacteria (Johns et al 1998, Kryuchnekov 1991).

The findings reported in this paper suggest that *D. variabilis* controls gram-negative bacteria by the process of aggregation, followed by hemocytic encapsulation, i.e., similar to the nodulation process described in insects (Miller and Stanley, 1998). In this study, the resultant aggregates were not extracted due to the small diameter created at the coxa-trochanteral joint (< 50 μm) during the bleeding process. Therefore, the rapid entrapment of *E. coli* cells in numerous aggregates may account for the inability to culture bacteria 6 h post-inoculation. However, the possible role of other immune mechanisms, e.g., bacteriolysis and phagocytosis, cannot be excluded.

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Previous studies, summarized elsewhere (Kovar et al. 2000, Gillespie et al. 1997, Miller and Stanley 1998), allude to several soluble components of the innate response in arthropods that function in a concerted manner to eliminate invading bacteria. Lectins, proteins that bind specific subunits in the lipopolysaccharides (LPS) layer and cell wall of bacteria and induce aggregation, have been isolated from a soft tick, *Ornithodoros moubata* (Kovar et al. 2000) and a horseshoe crab, *Tachypleus tridentatus* (Chen et al. 2001). Antimicrobial peptides isolated from ticks include lysozyme, from the gut of *O. moubata* (Kopacek et al. 1999), defensin from the hemolymph of *D. variabilis* (Johns et al. 2001) and from the whole body of the soft tick *O. moubata* (Nakajima et al. 2001). Total hemolymph protein concentrations (Fig 2) suggest an early utilization of protein as reflected in the control group. The bacteria-challenged group indicated a continual use of protein for both antimicrobial peptide production, and expansion of the hemocyte population, facilitating the cell-mediated response.

The cell-mediated response and therefore the hemocyte load, is an important factor in the ability of various *Drosphilia melanogaster* subgroups to encapsulate parasitoid eggs (Eslin et al. 1996, 1998). Hemocytes are also important in the resistance of the tick *D. variabilis* to challenge with *Bacillus subtilis* (Johns et al. 1998). The initial increase in sham-inoculated hemocyte concentrations, up to 1 h post-inoculation (Fig. 3) probably reflect a wounding response. In the absence of a bacterial challenge, hemocyte numbers then decline rapidly. An increase, from constitutive levels (not inoculated) as reported by Johns et al. (1998) (approx. 1000 to 2000 cells/µl), in hemocyte numbers in *E. coli* challenged ticks ($\geq$ 2000 cells/µl), suggest the *E. coli* challenged ticks also exhibit a wounding response. The response does not appear as robust as the sham-inoculated response, perhaps due to the encounter between the hemocytes and *E. coli*, which proceeds to form aggregates and are unable to be extracted. Hemocyte numbers begin to

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increase 6 h post-inoculation, presumably in response to bacterial aggregates, thereby facilitating the encapsulation phase of nodule formation.

Nodulation appears to be an efficient alternative for bacterial control, possibly as efficient for control of some organisms as the better-known lysis, phagocytosis and encapsualtion mechanisms in arthropods. Large numbers of bacteria, like those used in this study may overwhelm lytic and phagocytic responses (Wiesner et al. 1998). In the insect encapsulation response, hemocytes trap and gradually surround large foreign objects with consecutive layers of dead and degranulating cells, followed by melanization (Gillespie et al. 1997). Nodulation is similar but without the circularization of hemocyte layers. Encapsulation was demonstrated in *D. variabilis* in response to inanimate araldite sliver surgically implanted in the hemocoel of these ticks (Eggenberger et al. 1990). Presently, in *D. variabilis*, although hemocytes eventually surround the bacterial aggregates, they did not form circular layers, a pattern characteristic of nodule formation rather than encapsulation. Nodulation is well characterized in insects (Miller and Stanley 1998), where it is also accompanied by melanization. In contrast, the nodulation we observed in *D. variabilis* lacked any evidence of melanin formations. However, nodulation is difficult to assess in the hemocoel of a tick, as compared to the work presented by Miller and Stanley (1998). Breakdown products of hemoglobin, i.e. hematin, and midgut particulates make it difficult to distinguish any melanized nodules that may be attached to various organs from hematin granules.

The use of GFP-expressing *E. coli* revealed another response. Initially, the microaggregates formed by the GFP-expressing *E. coli* cells were similar to that seen with *E. coli* strain 25922, i.e., they were comprised solely of bacteria. However, instead of free floating, the GFP-expressing cells appear to bind to the tick's tissues, i.e, hemocytes. This trend became

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prominent after 6 and 24 h post-inoculation, a pattern similar to that seen in the nodulation responses in some insects (Miller and Stanley, 1998).

Innate resistance in ticks is comprised of both cell-mediated and soluble peptides that work in concert to prevent microbial infection. Nodulation, a cell-mediated event, has been reported in insects and appears to be vital to the immune response in the tick *D. variabilis*. The response is both fast and efficient as a dose of 26,800 *E. coli* CFU /μl are aggregated lowering the number of viable bacteria as early as Time 0 and unable to be cultured 6 h post-inoculation. The failure to demonstrate lysis or phagocytosis in this study does not exclude those antimicrobial mechanisms in *D. variabilis* towards challenge with *E. coli*.

Acknowledgments

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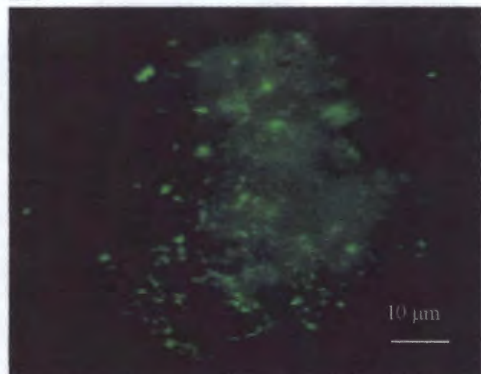
Fig. 1

Fig 2.

Fig 3.

Fig. 4.

A.

B.

10μm

C.

10 μm

D.

10 μm

E.

10 μm

F.

10 μm

Fig. 5

A.

B.

C.

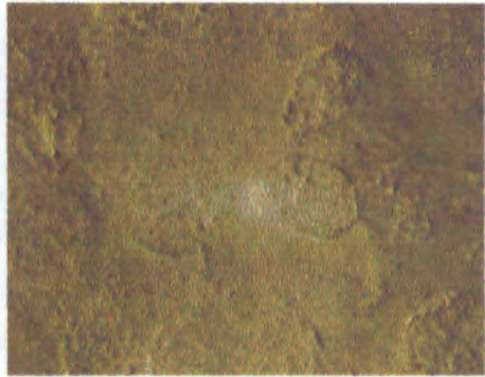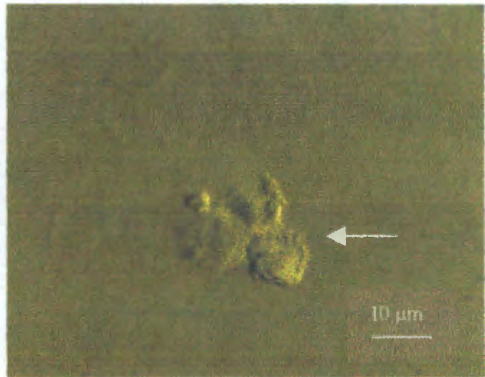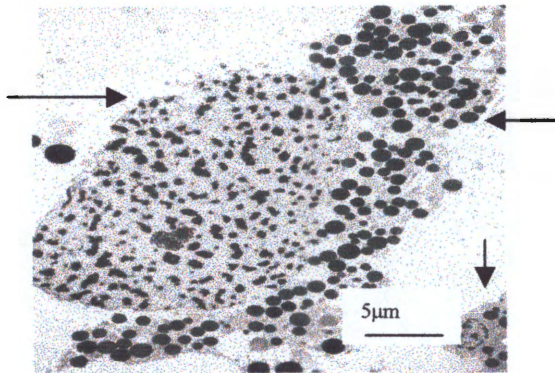10 μm

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F.

10 μm
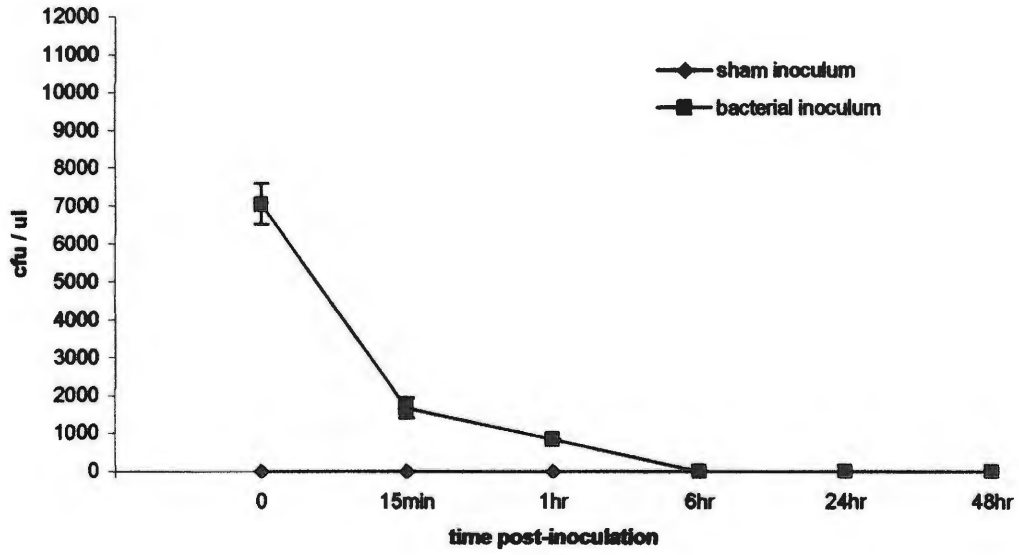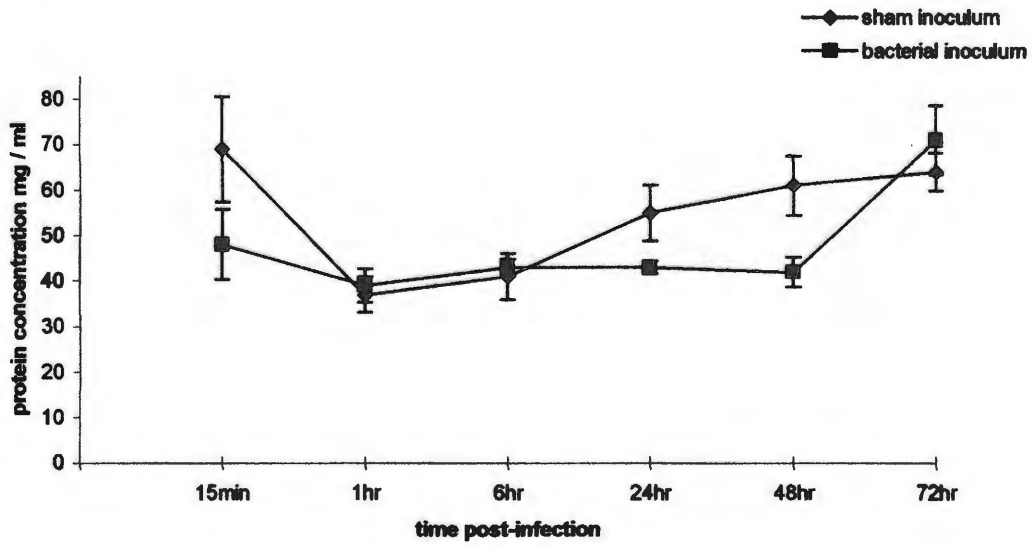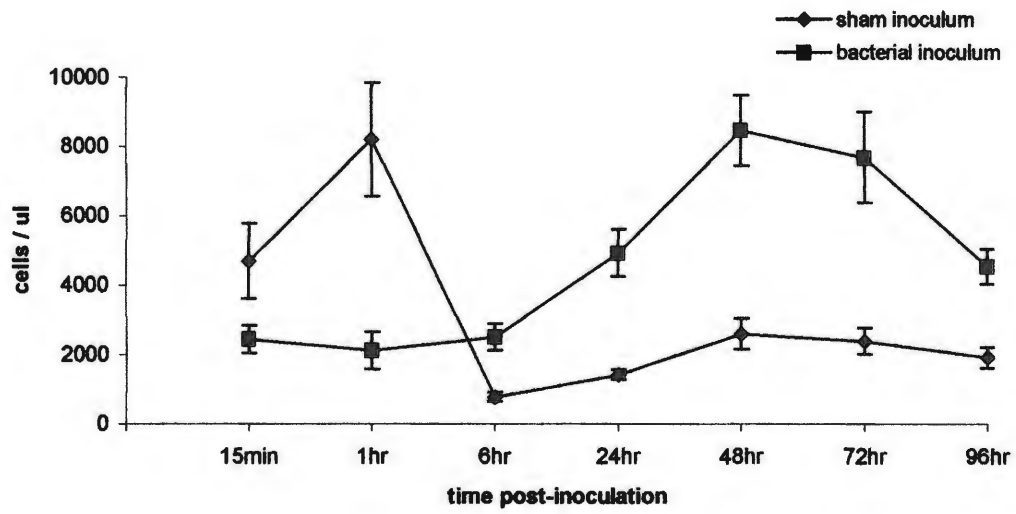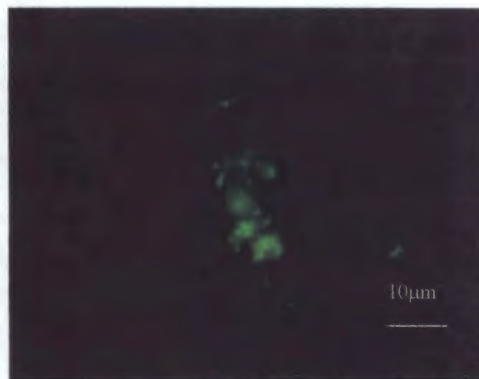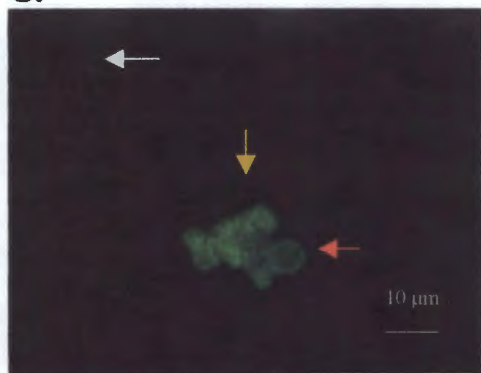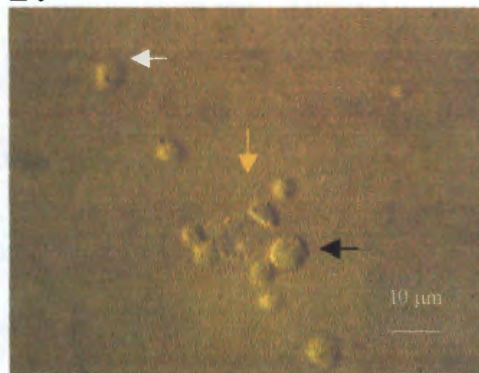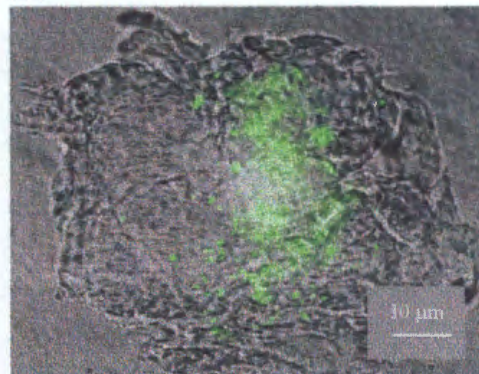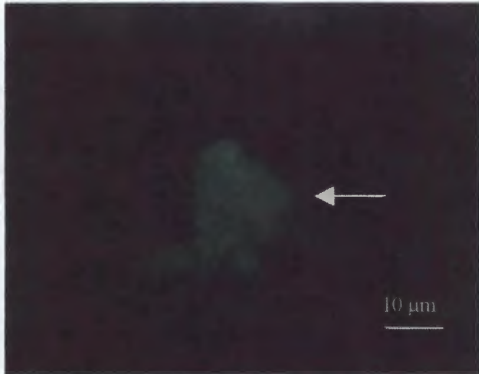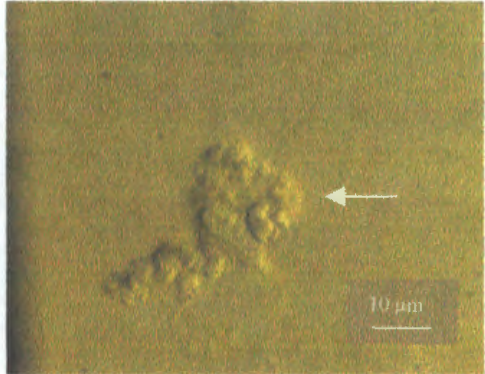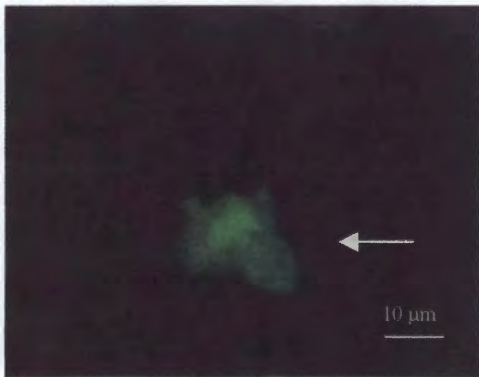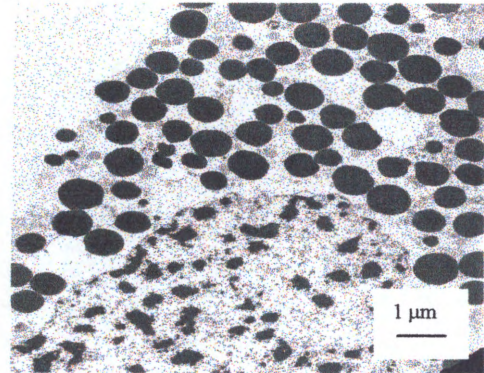
Fig 6.

A.

B.

FIGURE LEGENDS

Fig. 1. Colony forming units per microliter (CFU/µl) of hemolymph detected post-inoculation into *D. variabilis* hemocoel. Hemolymph was extracted as described in the materials and methods at each time point. The hemolymph was serially diluted in Shen's saline and plated on TSA plates to obtain CFU's. Hemolymph for Time 0 was extracted immediately, serially diluted and plated for CFU assays. Each time point is the average of 5 replicates in the control and 7 replicates in the experimental.

Fig. 2. Total hemolymph protein concentration expressed as mg/ml. Hemolymph from 6 ticks was pooled (1 sample), centrifuged to remove hemocytes and the total protein concentration determined using the BioRAD Bradford protein assay with BSA to create a standard curve. Each time point represents an average $\pm$ S.E. (n = 3).

Fig. 3. Total hemocyte counts expressed as cells/µl. Hemolymph was collected at the designated time points and the hemocytes counted with a Brightline hemocytometer. Data for each time point represents the mean $\pm$ S.E. (n = 16).

Fig. 4. Images showing aggregation in tick hemolymph following bacterial inoculation. **A.** Hemolymph sample from a sham-inoculated (no bacteria) tick demonstrating the absence of non-specific fluorescence. **B.** Hemolymph sample collected at 1 h post-inoculation, DFA showing the earliest evidence of bacterial clumping. **C** (fluorescence) and **D** (DIC). Hemolymph assayed at 6 h post-inoculation showing aggregations (yellow arrow) associated with hemocytes (red and black arrow). **E** (fluorescence) and **F** (combined brightfield and fluorescence image). Aggregates of *E. coli* were enlarged at 24 h post-inoculation and were associated with larger masses presumably of cellular origin.

Fig. 5. Images showing aggregation of GFP-expressing *E. coli* following hemocoel inoculation of bacteria into *D. variabilis*. **A** (fluorescence) and **B** (DIC). Images from hemolymph showing hemocytes from sham-inoculated ticks. No auto-fluorescence was detected. **C** (fluorescence) and **D** (DIC). Images from hemolymph showing aggregation of GFP-expressing *E.coli*. Samples were collected at 1 h post-inoculation **E** (fluorescence) and **F** (DIC). Images from hemolymph showing aggregations of GFP expressing-*E.coli*. Samples were collected at 6 h post-inoculation.

Fig 6. Hemolymph samples were processed for electron microscopy as described in the materials and methods for each time point assayed. **A.** 48 h post-inoculation hemolymph sample showing a mass of granules surrounding some unknown mass. Right arrow: granules from degranulated hemocytes. Left arrow: electron dense granule presumably remnants of aggregated bacteria. Vertical arrow: Free hemocyte (2840x). **B.** Enlargement of mass observed in C (10,800x).