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**Contrasts in Tick Innate Immune Responses to *Borrelia burgdorferi* challenge: Immunotolerance in *Ixodes scapularis* versus Immunocompetence in *Dermacentor variabilis* (Acari: Ixodidae).**

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**Abstract** The Black legged tick *Ixodes scapularis* (Say) transmits the Lyme disease spirochete (*Borrelia burgdorferi*) whereas the American Dog tick (*Dermacentor variabilis* (Say)) is unable to transmit the bacterium. We compared the innate immune response of these ticks against spirochetes directly inoculated into the hemocoel cavity of ticks. In *I. scapularis*, some *Borrelia* were found associated with hemocytes, while numerous other spiral-shaped, intact bacteria remained free in the hemolymph. In contrast, in *D. variabilis* only remnants of the bacteria were evident in the hemolymph, indicating lysis; intact spirochetes were rare. Spirochetes were observed bound to or within the organs of both tick species, although many more spirochetes were found associated with the *I. scapularis* organs. The few spirochetes observed with the *D. variabilis* organs appeared to be dead since *D. variabilis* tissues rarely contained culturable bacteria, unlike *I. scapularis* tissues. When spirochetes were incubated with *I. scapularis* hemolymph plasma in vitro, bacterial survival and motility were not reduced. In contrast, incubation of spirochetes with *D. variabilis* hemolymph plasma resulted in greater than 50% of the spirochetes becoming non-motile by 45 min. The differences in the responses of the two different tick species indicate that *I. scapularis* is immunotolerant when challenged with *B. burgdorferi* and dependent upon a slow phagocytic response to clear *Borrelia* from the hemolymph. In contrast, *D. variabilis* is highly immunocompetent (i.e. innate immunity), utilizing plasma borreliacidal factors and a rapid increase in phagocytic cells to clear the infection and limit tissue invasion.

**Key words:** Immunotolerance, immunocompetence, *Borrelia burgdorferi*, *Ixodes scapularis*, *Dermacentor variabilis*.

Lyme borreliosis is caused by the spirochete, *Borrelia burgdorferi s.l.* (Johnson et al. 1984) transmitted by ticks of the genus *Ixodes*. Lyme disease, caused by these bacteria, is now recognized as the most important vector-borne disease in the United States (CDC 1999), with more than 15,000 cases per year, and many thousands of cases in Eurasia.

The passage of spirochetes from the midgut to the salivary glands and other internal organs during feeding of infected vector ticks has been documented in numerous studies (Lane et al. 1991). In nymphs, dispersal to the salivary glands occurs over a period of several days, with maximum numbers of spirochetes in the saliva within 72 h after the commencement of tick feeding (Piesman 1995, deSilva et al. 1995; Zung et al. 1989). However, little is known about the survival of these bacteria during hemolymph passage, wherein they are likely to encounter antimicrobial peptides and phagocytic hemocytes. Phagocytosis of invading *Borrelia* has been reported (Coleman et al. 1997) and the possible role of lysozyme in bacteriolysis has been suggested (Kühn and Haug 1994, Podboronov 1991). How the bacteria survive in the presence of these immune challenges, how long they survive, the percentage of surviving bacteria that invade nearby tick organs and other aspects of the dynamics of spirochete passage in the ticks have received little attention.

Evidence of spirocheticidal activity by antimicrobial hemolymph peptides was described in *Dermacentor variabilis* (Say) (Johns et al. 2000), a tick which does not transmit *B. burgdorferi*; it is not known whether similar peptides are active in *Ixodes scapularis* (Say). By following the fate of spirochetes inoculated into the hemocoel of ticks, it may be possible to gain

a better understanding of the role of the tick's immune system in determining vector competence. In this study, we used direct inoculation of cultured spirochetes into the hemocoel of ticks to follow the fate of the bacteria in the hemolymph of competent (*I. scapularis*) and incompetent (*D. variabilis*) vectors of *B. burgdorferi*. We report that spirochetes survived and invaded organs in *I. scapularis* whereas they were rapidly cleared from the hemolymph of *D. variabilis* ticks.

### MATERIALS AND METHODS

**Ticks.** *Dermacentor variabilis* ticks were colonized as described by Sonenshine (1993) using rats (*Rattus norvegicus*) and rabbits (*Oryctolagus cuniculus*).<sup>1</sup> *I. scapularis* was colonized from ticks collected near Armonk, New York, fed on rabbits and the spirochete-free progeny reared to adults. All life stages were fed on rabbits and the fed ticks were incubated at 26°C ± 1°C and 92 ± 1% RH. All *I. scapularis* used in the experiments were from the F<sub>1</sub> generation.

**Bacteria.** The *B. burgdorferi* used in this study were the low passage B31 strain from Center for Disease Control, Fort Collins, Co. The spirochetes were cultured and prepared for inoculation as described by Johns et al. (2000).

**Tick Inoculations and Tick Tissue Collections.** All tick inoculations with *B. burgdorferi* were done as described by Johns et al. (2000). Briefly, bacteria were suspended in phosphate-buffered saline (PBS) (pH 7.4). Next, 5 µl aliquots containing 35,000 spirochetes were injected into the hemocoel cavity of partially fed virgin female *D. variabilis* or *I. scapularis* via the foramen

between the capitulum and the anterior end of the scutum. Ticks were also sham inoculated with PBS alone. A 50  $\mu$ l Hamilton syringe (Hamilton, Reno, NV) with a 30-gauge hypodermic needle was used for the bacterial inoculations. Hemolymph collections were made at 1 and 24 h following the inoculations. Hemolymph collected as described previously (Johns et al. 1998) was diluted 1:1 in Shen's tick saline (Oliver et al. 1974) with 10 mM Phenylmethyl-sulfonyl fluoride) (PMSF) (Sigma); samples contaminated with midgut, malpighian tubules or other tissues were discarded. Following hemolymph collection, 3 – 5  $\mu$ l aliquots were smeared onto CSS-100 silylated slides (CEL Associates, Houston, TX). Immediately following hemolymph collection, the same tick specimens were dissected at the same time intervals with the aid of a stereoscopic microscope and samples of salivary glands and ovary were transferred to microscope slides. The same procedures were also repeated with inoculations of 3,500 spirochetes per tick so as to assess the effects of low dose inoculations on spirochete survival within ticks.

**Direct Immunofluorescence Assay.** A direct immunofluorescence assay (DFA) was used to detect spirochetes in tick hemolymph, salivary glands and ovaries. The collected hemolymph was spotted directly on CSS slides and air-dried. The tissue samples were dissected out in a drop of Shen's tick saline on a regular glass slide, transferred to a CSS slide and air-dried. The slides were fixed in acetone for 20 min, then blocked with 5% FCS, PBS and 0.05% sodium azide for 30 min at room temperature. Next, the slides were incubated with fluorescein

isothiocyanate (FITC) conjugated goat anti-*Borrelia* antibody (KPL, Inc., Gaithersburg, MD), diluted 1:50 with blocking buffer and incubated at room temperature for 1 h. After incubation, the slides were washed 3 times with PBS for 5 min each, then mounted with SlowFade Light Antifade kit (Molecular Probes, Inc., Eugene, OR).

**Digital Imaging and Confocal Microscopy.** The slides were observed by epifluorescence with an ECLIPS E 600 microscope (Nikon, Tokyo). Both bright field and fluorescent images were captured with a SPOT II digital camera and processed using SPOT software V2.2 (Diagnostic Instruments Inc., MI). Slides were also observed with a Leica TCS-NT confocal microscope system (Leica Microsystems, Germany). To determine whether the FITC-labeled spirochetes were inside of cells or merely bound to their membranes, serial optical sections were observed at 1  $\mu\text{m}$  intervals. Images of the cells at the same optical section thickness were captured using Normarski differential interference contrast (DIC).

**Bacterial Survival in Tissues of Infected *I. scapularis*.** To assess the ability of *B. burgdorferi* to survive in salivary glands and ovaries after hemocoelic inoculation, samples of these organs were dissected at 1 and 24 h from *B. burgdorferi*-inoculated ticks, washed 3 x in PBS buffer, pH 7.5, and cultured individually in BSK-H media that contained an antibiotic mixture for *Borrelia* (Sigma, St Louis, MO) at 33<sup>o</sup>C. The culture tubes were examined by dark field microscopy and DFA for evidence of spirochete growth at 9-16 d after inoculation.

***Borrelia* motility inhibition assays.** To determine antimicrobial activity of *I. scapularis* hemolymph plasma against *B. burgdorferi*, hemolymph was collected at 1 h post-inoculation



from *B. burgdorferi* infected ticks or sham inoculated (non-infected) ticks and saved overnight at 4°C. On the next day, the hemolymph was centrifuged at 12,000 x g for 10 min to remove hemocytes and non-cellular particulates and the plasma were collected. Antimicrobial activity was assessed by adding 10 µl of tick hemolymph plasma sample to 50 µl of a 3 - 5 d *B. burgdorferi* culture in BSK-H (adjusted to  $4.5 \times 10^3$  cells per µl). Subsequently, at defined intervals samples were transferred to a hemocytometer and evaluated for bacterial numbers and motility by dark field microscopy.

**Hemocytote counts.** The hemocyte counts were done as previously described ( Johns et al. 1998) using a Brightline hemocytometer and viewed with a Nikon Optiphot compound microscope. Hemolymph samples were assayed at 1, 6, 18, 24, 48, and 72 h post-inoculation.

## RESULTS

**Observation of *B. burgdorferi* inoculated into the hemocoel of ticks.** One hour after spirochetes were inoculated into *I. scapularis*, examination of the hemolymph by DFA revealed numerous spirochetes. Most appeared as free, intact, elongated immunofluorescent organisms in the plasma but some could be seen adjacent to the surface of hemocytes and, occasionally, *several even appeared to be within* the hemocytes (Fig. 1a) [Compare the location of the organisms with the location of the cells in the bright field images.] In *other* instances, individual *Borrelia* appeared to be attached at their tips to a single hemocyte. Many spirochetes were also evident in *or on the surfaces* of the salivary gland acini (Fig. 3a) and in the ovarian tissues (data not shown).

At 24 h after *B. burgdorferi* inoculation into *I. scapularis*, examination of tissues revealed a different picture. In the hemolymph, DFA revealed few intact spirochetes in the plasma but numerous fluorescent fragments *presumably* from spirochetes were evident adjacent to or within the hemocytes (Fig. 1b). However, intact spirochetes were still evident in or on the surfaces of the salivary gland acini (Fig. 3b) and in the ovarian tissue samples (data not shown).

At 1 h after *B. burgdorferi* inoculation into *D. variabilis* ticks, few intact bacteria were evident in the hemolymph. Most organisms appeared to consist of small, cell-associated fragments (Fig. 1c). Confocal microscopy confirmed that these spirochetes were inside the hemocytes, not merely on the surfaces (Fig. 2). Only one intact spiral form characteristic of live spirochetes was found in the hemolymph plasma, among the many fields examined in contrast to the numerous apparently intact spiral forms seen in the hemolymph plasma of *I. scapularis*. Examination of the salivary glands at 1 h revealed several spirochetes apparently within a single acinus surrounded by other acini without *Borrelia* (Fig. 3c). Similarly, a few spirochetes were found in the ovarian tissue samples (data not shown).

At 24 h following inoculation, no spirochetes were observed in the hemolymph of *D. variabilis* (Fig. 1d). Spirochetes were still evident in several local regions of the salivary gland after 24 h, but most fields examined were free of *Borrelia* (Fig. 3d). A few spirochetes were also observed in the ovarian tissue at 24 h (data not shown).

**Survival of *Borrelia burgdorferi* in Tick Tissues.** Experiments were performed to determine if the spirochetes observed with DFA in tick tissues were viable. *I. scapularis* and *D.*

*variabilis* ticks were inoculated with high (35,000 per tick) and low (3500 per tick) doses of bacteria and then tick tissues were removed at 1 and 24 hr after inoculation, washed 3 x in PBS, and incubated with BSK-H media in an attempt to culture bacteria from the tissues. When tissues from *I. scapularis* treated with the higher dose of spirochetes for 1 h were cultured in BSK II media, spirochetes were found in all 3 salivary glands tested and 2 of the 3 ovary samples (Table 1A). Spirochetes were also cultured from 2 of the 3 salivary glands and 2 of the 3 ovary samples collected from *I. scapularis* at 24 h post-inoculation (Table 1A). When the studies were repeated with tissues from the ticks inoculated with the lower dose of *Borrelia*, the results were similar. Spirochetes survived in salivary gland and ovary samples for at least 24 h. Furthermore, bacteria were also readily cultured from hemolymph collected from *I. scapularis* treated with the low dose of bacteria for 1 h.

In *D. variabilis*, spirochetes were cultured from ticks at 1 h post-inoculation (high dose), including 3 of 6 hemolymph samples, but only 1 of 6 salivary gland or ovary samples (Table 1B). No spirochetes could be cultured from tick tissues collected at 24 h post-inoculation. When the study was repeated with the low dose of *Borrelia*, at 1 h post inoculation spirochetes were cultured from only 1 of 3 salivary glands while all the other samples were negative (Table 1B). No spirochetes were cultured from tissue samples collected at 24 h post-inoculation (Table 1B). In summary, culturing spirochetes from tissues taken from *B. burgdorferi*-inoculated ticks was successful in only 3 out of 36 attempts, and then only when taken at 1 h after inoculation. In contrast, cultures from *I. scapularis* were successful in 21 out of 24 attempts.

**Anti-Borrelia activity in hemolymph plasma.** *B. burgdorferi* were incubated with hemolymph plasma *in vitro* to directly test the effect of tick plasma on spirochetes. When *I. scapularis* plasma was incubated with cultured spirochetes *in vitro*, no significant differences in spirochete numbers or motility were found (Fig. 4). However, when the same experiment was done with plasma from *D. variabilis*, spirochete survival (assessed by motility) declined rapidly (Fig. 4).

**Hemocyte counts.** Hemocytes in the hemolymph of *B. burgdorferi*-challenged *I. scapularis* females were significantly elevated at 1 h after bacterial inoculation ( $t = 5.24$ ,  $p < 0.01$ ), but declined thereafter to normal levels (Fig. 5A). Similarly, Johns et al. (2000) showed hemocyte counts in the hemolymph of *B. burgdorferi*-challenged *D. variabilis* females increased 8-fold within 1 h and remained significantly greater than the controls for 6 h post-inoculation (Fig. 5B) (data from Johns et al. 2000, with permission of the publisher).

## DISCUSSION

*B. burgdorferi* infect the gut and then disseminate into diverse tissues of *I. scapularis* and other members of the *I. ricinus* complex of ticks (Burgdorfer et al. 1989, Zhu, 1998). *D. variabilis* is often sympatric with *I. scapularis* (Sonenshine 1993). Yet, although *B. burgdorferi* have been observed in *D. variabilis* guts, both laboratory and field studies indicate that *D. variabilis* is not a competent vector of *B. burgdorferi* (Mather et al. 1996, Mather and Mather 1990, Piesman and Sinsky 1988). The results we report here provide a basis for understanding the differences in vector competence between *I. scapularis* and *D. variabilis*.

The *I. scapularis* hemocoel was a relatively benign environment for *B. burgdorferi* in

comparison to the *D. variabilis* hemocoel. Within *I. scapularis* numerous spirochetes remained free and intact in the hemolymph plasma 1 h after direct inoculation into the hemocoel. Moreover, this period proved sufficient for viable spirochetes to penetrate the surrounding tissues, including the salivary glands. In contrast, the hemolymph of *D. variabilis*, rapidly destroyed *B. burgdorferi* and viable spirochetes were rarely recovered from other tissues (Johns et al. 2000 and Figs 1 and 4).

The *I. scapularis* hemocoel was not a completely benign environment for spirochetes because we observed a decline in the number of intact spirochetes 24 h after intra-hemocoelic inoculation. The gradual clearance of spirochetes from the *I. scapularis* hemocoel may explain why *B. burgdorferi* infection was usually confined to the gut of *I. scapularis* ticks. Spirochetes only disseminate beyond the gut to the hemocoel and other tick organs for a brief period during transmission (de Silva and Fikrig 1995, Piesman, 1995). Others have also reported that spirochetes do not persist in the hemolymph of *I. scapularis* ticks, suggesting that the hemolymph represents a hostile environment for these bacteria (Coleman et al. 1997, Munderloh and Kurtti 1995).

The mechanism of *Borrelia* clearance in *I. scapularis* appears to be primarily cell-mediated because hemolymph plasma from both *B. burgdorferi* challenged and unchallenged *I. scapularis* ticks was not borreliacidal in vitro. Phagocytosis appeared to be the primary means of *B. burgdorferi* control in *I. scapularis* because at 1 h after inoculation some spirochetes were cell-associated and by 24 h the bacteria were present mostly as cell-associated fragments. Under natural conditions, phagocytosis may proceed more slowly, perhaps, because the bacterial

challenge itself is more gradual as spirochetes emerge from the infected midgut over several days, rather than in a single burst (Ribeiro et al. 1987). In the Coleman et al. (1997) study, hemocytes with bound or incorporated spirochetes increased gradually, from only 2% of the hemocyte population on feeding day 3 to 13% by feeding day 5. Hemocyte abundance was distinctly higher in the *B. burgdorferi*-challenged *I. scapularis* ticks for at least 1 h after spirochete inoculation indicating that an increase in the number of hemocytes may contribute to the eventual clearance of spirochetes. Spirochetes introduced into the hemocoel of *D. variabilis* experienced a different fate than the bacteria within *I. scapularis*. Virtually all of these bacteria were destroyed within 1 h. The presence of innumerable fluorescent fragments scattered throughout the hemolymph plasma or attached to hemocytes suggests a dual mechanism of spirochete control, namely, bacteriolysis by plasma factors and phagocytosis. This is consistent with previous findings by Johns et al. (2000), who showed that hemolymph plasma from *B. burgdorferi*-challenged ticks rapidly cleared most of the microorganisms when added to cultures of the spirochetes growing in BSK-H and that hemocyte populations increased greatly during bacterial challenge.

We recognize that the unusually large number of spirochetes inoculated into the tick's hemolymph, perhaps hundreds of times the numbers reported in naturally acquired infections, was artificial. However, the results were similar with the lower dose, which is more representative of natural conditions. The ability of most *Borrelia* to survive in the hemolymph of *I. scapularis* for at least 1 h was long enough for them to migrate to the surrounding tissues and survive. Thus, tissue invasion can proceed very rapidly in *I. scapularis*. As noted by Kurtti et al.

(1993), *B. burgdorferi* adhere readily to actively transporting cells such as those of the salivary glands that have numerous coated and uncoated pits. In contrast, in the more the hostile environment of the *D. variabilis* hemolymph, most spirochetes are lysed within a few minutes by antimicrobial peptides (Johns et al. 2000). A few spirochetes did reach organs in *D. variabilis* also but these were mostly non-viable since spirochetes were rarely cultured from *D. variabilis* organs. Thus, in view of the robust immune system of *D. variabilis*, it is unlikely that naturally acquired spirochetes would survive hemolymph passage and tissue penetration, even in those rare occasions where they might survive in the midgut of ticks (Burgdorfer et al. 1989). We propose that the vector competence of tick species for *B. burgdorferi* is influenced strongly by the innate immune response of the tick vector.

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**FOOTNOTES**

1. Robert Johns and Jun Ohnishi contributed equally to the performance of these studies.
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**Table 1. *In vivo* survival of *Borrelia burgdorferi* in salivary glands and ovaries of *I. scapularis* and *D. variabilis* females following hemocoelic inoculation of cultured spirochetes**

**A. *I. scapularis***

Percent tissue or organs sampled with live spirochetes						
Treatment	Hemolymph 1 hr	Hemolymph 24 h	Sal glands 1 h	Sal glands 24 h	Ovaries 1 h	Ovaries 24 h
High dose <sup>a</sup>	N.D. <sup>3</sup>	N.D. <sup>c</sup>	3/3	2/3	2/3	2/3
Low dose <sup>b</sup>	3/3	N.D. <sup>c</sup>	3/3	3/3	3/3	3/3

**B. *D. variabilis***

Percent tissues or organs sampled with live spirochetes						
Treatment	Hemolymph 1 h	Hemolymph 24 h	Sal glands 1 h	Sal glands 24 h	Ovaries 1 h	Ovaries 24 h
High dose <sup>a</sup>	3/6	0/6	1/6	0/6	1/6	0/6
Low dose <sup>b</sup>	0/3	0/3	1/3	0/3	0/3	0/3

<sup>a</sup> Fed females were inoculated with  $3.5 \times 10^4$  spirochetes (high dose) from a laboratory culture by intra-hemocoelic injection, dissected at 1 and 24 h post-inoculation and organs removed. Samples of hemolymph or organs were incubated in BSK-H at 33° C for 9 – 16 d and examined for spirochetes using dark field microscopy and DFA.

<sup>b</sup> Females inoculated with  $3.5 \times 10^3$  spirochetes (low dose).

<sup>c</sup> N.D. = not done.

**Figure Legends.**

**Figure 1.** Images of tick hemolymph from fed female ticks following hemocoelic inoculation of *B. burgdorferi*. A bright field image of the hemolymph sample (left panel) and an immunofluorescent image of spirochetes (green) in the same field (right panel) are displayed in the figure. **a)** *I. scapularis*, 1 h. Note some spirochetes free in the plasma (white arrow) versus others attached to (or possibly internalized) in a hemocyte (white arrowhead); **b)** *I. scapularis*, 24 h. Note numerous fluorescent fragments (white arrowhead), some adjacent to or possibly within hemocytes, but no free *Borrelia*. **c)** *D. variabilis*, 1 h. Note numerous immunofluorescent fragments free in the plasma (white arrowhead) and associated with hemocytes and possibly a single intact *Borrelia* (white arrow). **d)** *D. variabilis*, 24 h no spirochetes were observed. In the bright field images, hemocytes are marked by black arrows. 400 x.

**Figure 2.** Confocal microscopic images confirming internalization of spirochetes in a *D. variabilis* hemocyte. Right hand panel shows a laser image of a spirochete at different levels from the base of the hemocyte. Left hand panel shows Normarski DIC images of the hemocyte at the same level (arrow). The sections are 2  $\mu\text{m}$  apart. 1000 x.

**Figure 3.** Digital images of tick salivary glands from fed female ticks following hemocoelic inoculation of *B. burgdorferi*. Bright field images of the salivary gland (left panel) and immunofluorescent images of spirochetes (green) in the same field (right panel) are displayed in the figures. **a)** *I. scapularis* 1 h; **b)** *I. scapularis* 24 h; **c)** *D. variabilis* 1 h; **d)** *D. variabilis* 24 h. Arrows show (apparently intact) spirochetes. 400 x.

**Figure 4.** Differences in antimicrobial activity of hemolymph plasma collected from ticks, *I. scapularis* and *D. variabilis*, 1 h after challenge with *B. burgdorferi* or saline (sham-treated). The assay was done with *B. burgdorferi* incubated in a mixture of BSK-H plus tick hemolymph plasma. Time (minutes) minutes after introduction of tick hemolymph plasma.

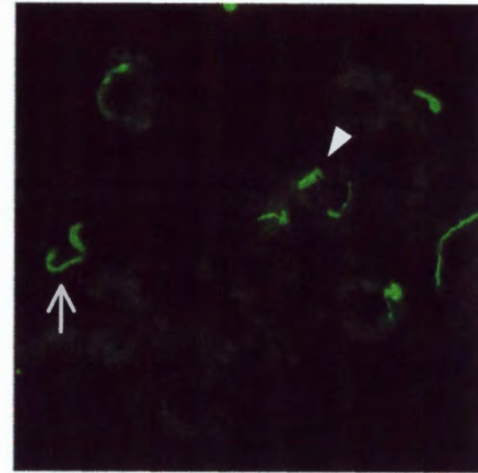
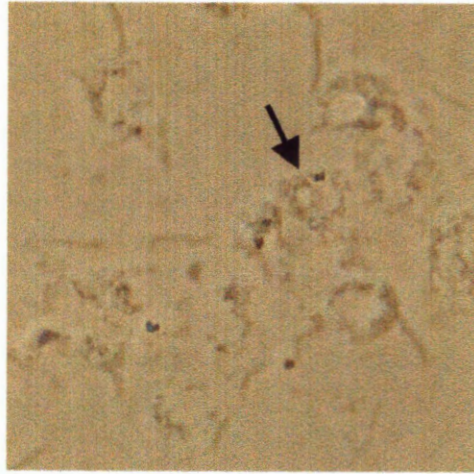
**Figure 5.** Changes in hemocyte populations following direct inoculation of *B. burgdorferi* into the hemocoel of fed female ticks or in untreated controls. a) *I. scapularis*; b) *D. variabilis* (with permission of the *Entomological Society of America*).

1A

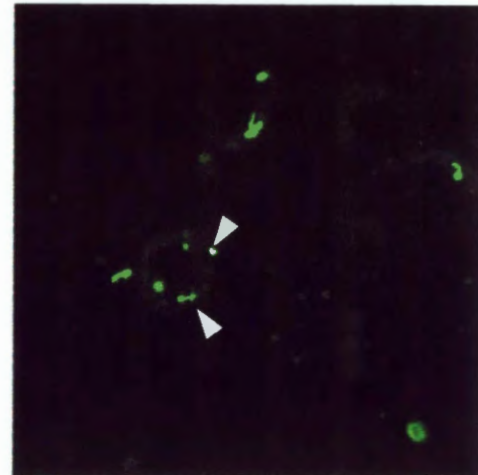
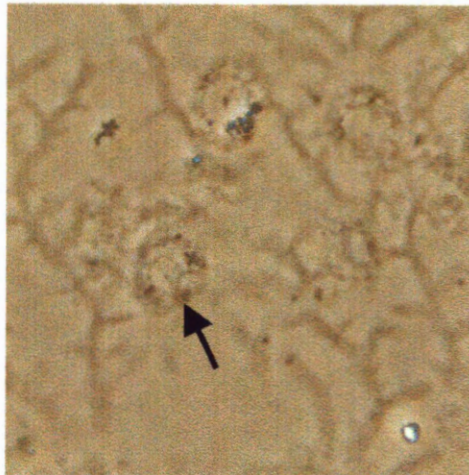
Brightfield

FITC anti *Borrelia*

a



b



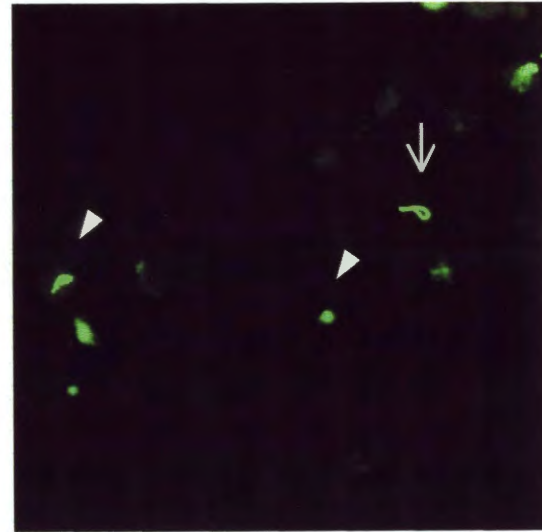
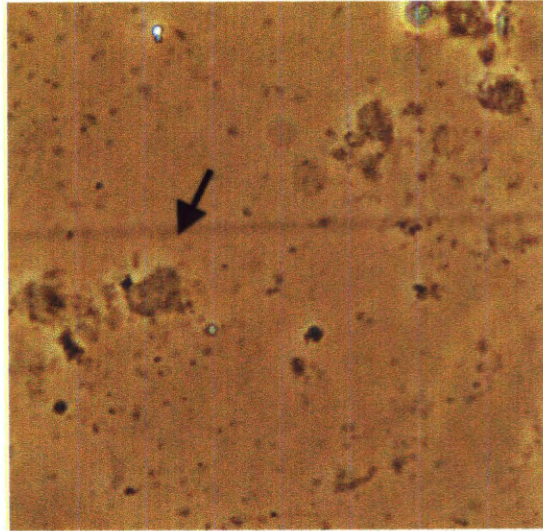


1B

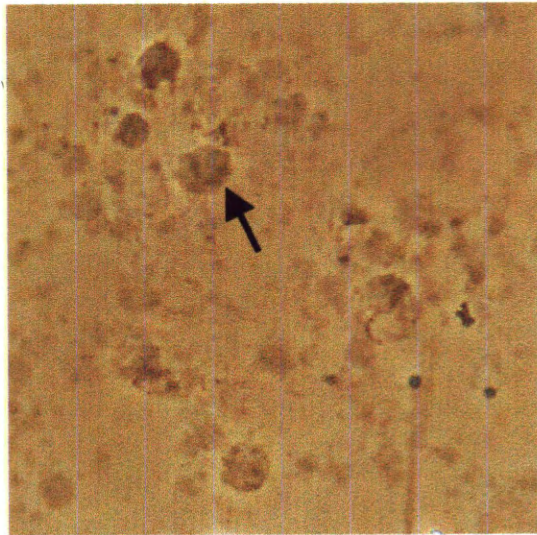
Brightfield

FITC anti *Borrelia*

c



d



M00 088

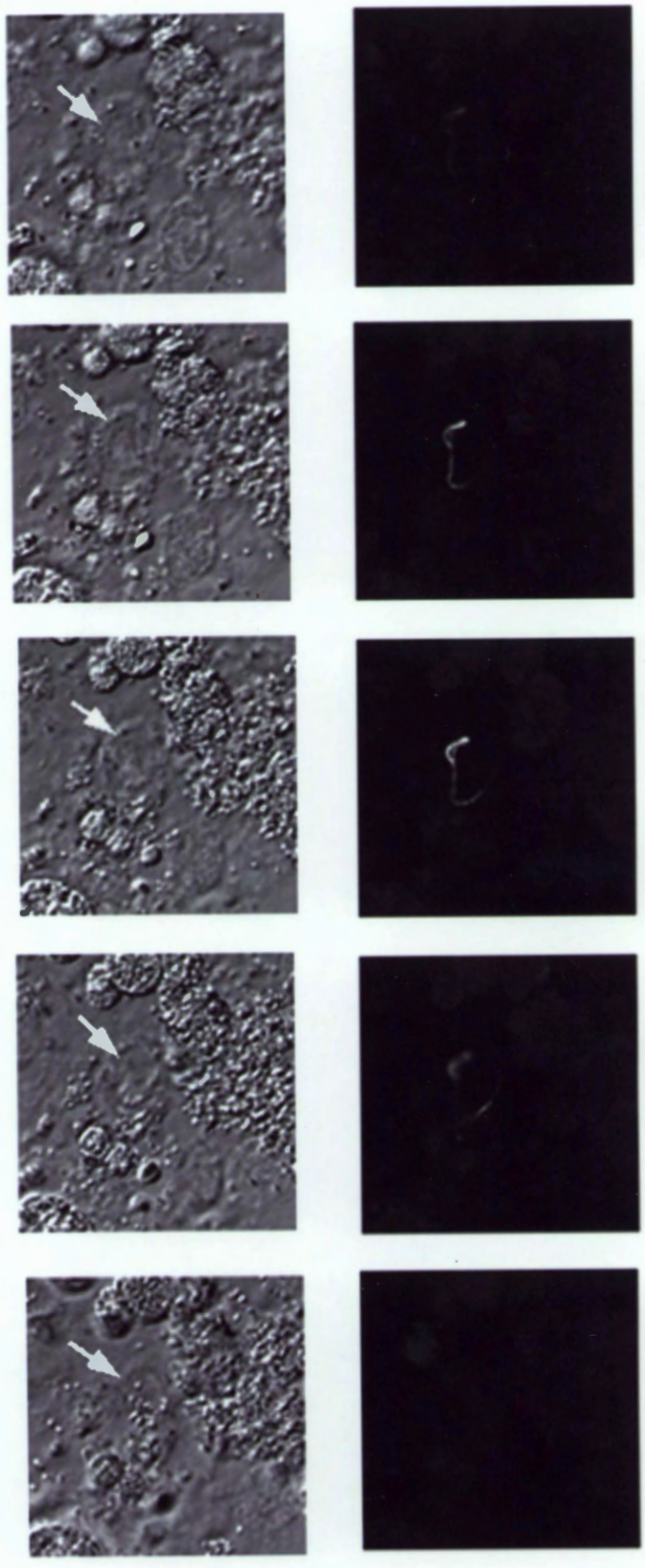
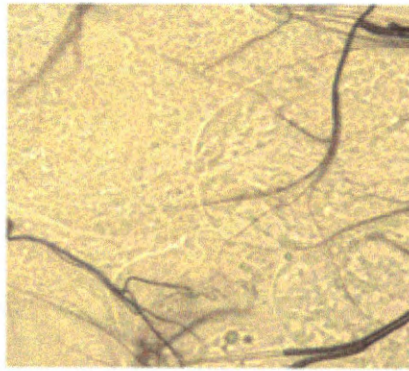


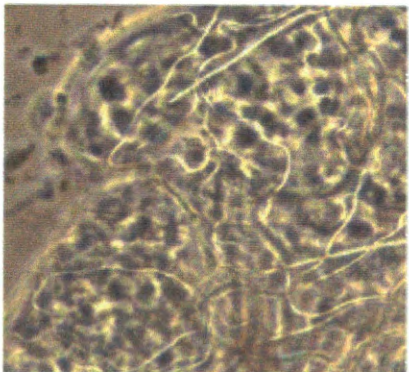
Figure 2

M00-088

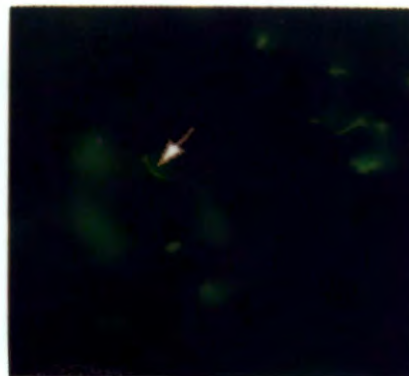
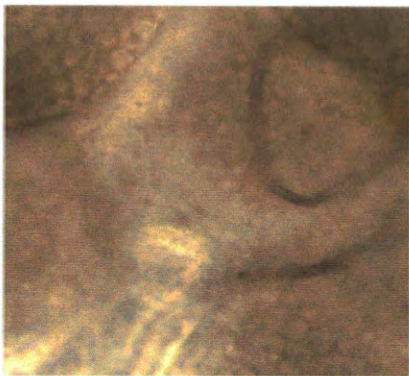
a



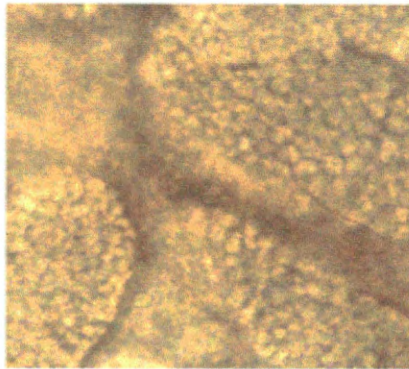
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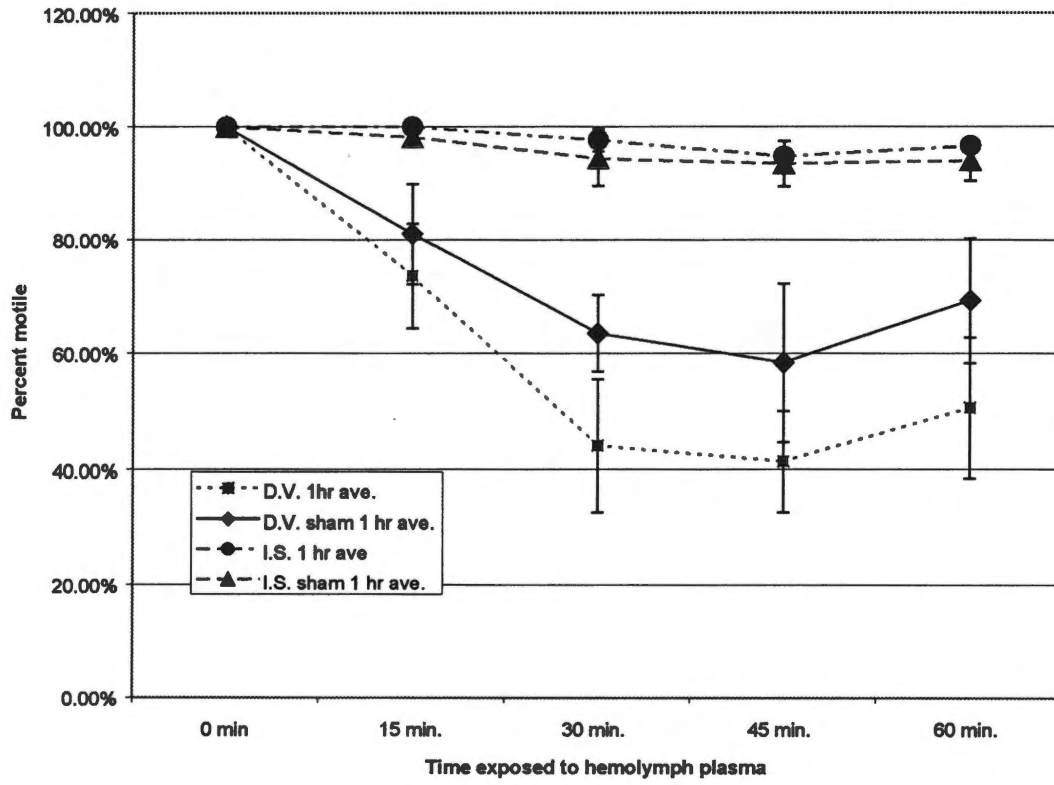
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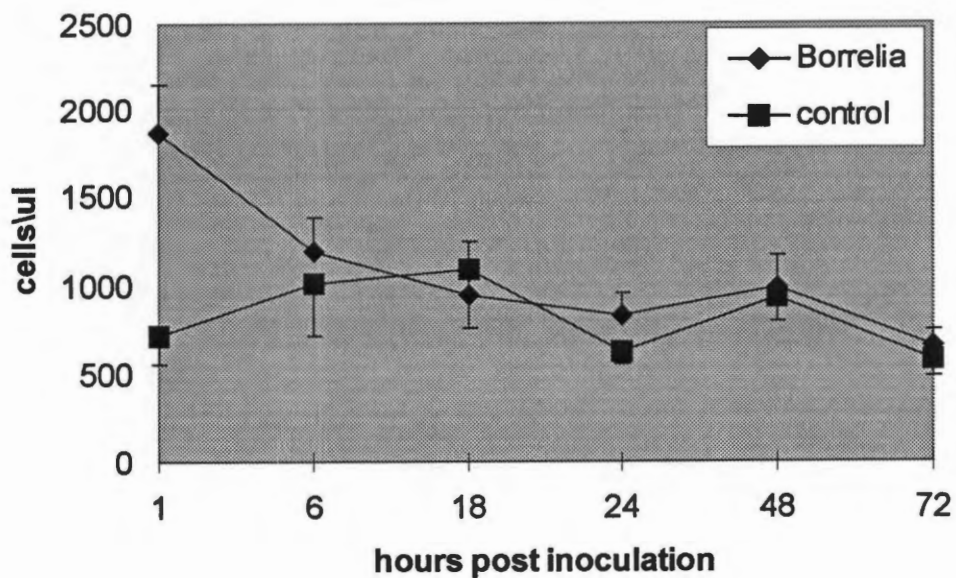


Anti-microbial activity of tick hemolymph for *B. burgdorferi*



A

***Ixodes scapularis* hemocyte count**



B

***Dermacentor variabilis* hemocyte count**

