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VECTOR COMPETENCE IN LYME DISEASE : STUDIES ON *IXODES SCAPULARIS*, *DERMACENTOR VARIABILIS*, AND *AMBLYOMMA AMERICANUM*

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VECTOR COMPETENCE IN LYME DISEASE :

STUDIES ON *IXODES SCAPULARIS*,

DERMACENTOR VARIABILIS,

AND *AMBLYOMMA AMERICANUM*

BY

MIN-TSUNG YEH

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE

REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

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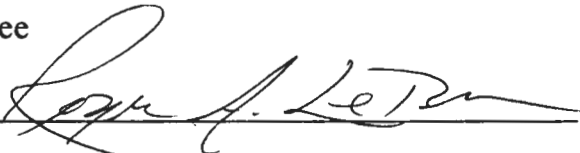UNIVERSITY OF RHODE ISLAND

1994

DOCTOR OF PHILOSOPHY DISSERTATION

OF

MIN-TSUNG YEH

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ABSTRACT

Ticks in the *Ixodes ricinus*-species complex have been implicated as vectors of Lyme disease spirochetes (*Borrelia burgdorferi*). A variety of other ticks, notably American dog ticks (*Dermacentor variabilis*) and Lone Star ticks (*Amblyomma americanum*) in the U.S., appear incapable of transmitting Lyme disease spirochetes despite ingesting these bacteria. In this study, I confirmed that deer ticks, but not dog ticks or Lone Star ticks became infected with Lyme disease spirochetes after feeding on infected hosts. In addition, I assessed several potential physiologic factors that could affect the ability of ticks to acquire, maintain, and transmit *B. burgdorferi*. One factor in particular, found in tick saliva, appears responsible for preventing spirochete killing in *I. scapularis* but not *D. variabilis* or *A. americanum*.

In one study, I assessed anti-microbial activity of the tick's immune system. In particular, I determined whether ticks exhibited measurable phenoloxidase and anti-microbial activity. Phenoloxidase activity in the hemolymph of larvae of the greater wax moth (*Galleria mellonella* L) was compared to phenoloxidase activity in *I. scapularis, D. variabilis* and *A. americanum*. Although activity was detected in wax moth hemolymph, no phenoloxidase activity was detected in three species of ixodid ticks. In addition, *Enterobacter cloacae* was used in an attempt to induce anti-microbial activity in punched cuticle of three species of ixodid ticks. Using this assay, no anti-microbial activity was detected in ticks. Moreover, no anti-microbial substances were found in the midgut of *I. scapularis, D. variabilis* and *A. americanum*. Factors associated with the tick's humoral immunity do not appear to play a role in vector competence for Lyme disease spirochetes.

In another study, host-associated borreliacidal factors were examined *in vitro*. Spirochetes survived in the presence of pre-immune rabbit serum but were killed in the

presence of immune rabbit serum. Heat-inactivation (56 °C, 1 hr) destroyed the killing effect of immune serum but the borreliacidal activity of heat-inactivated immune serum could be restored by the addition of pre-immune serum. Using a similar assay, spirochetes survived in tick midgut extract cultures when ticks were removed from pre-immune hosts. Spirochetes also survived in *I. scapularis* midgut extract cultures, but not in *D. variabilis* or *A. americanum* midgut extract cultures when ticks were removed from immune hosts. As it did in immune serum, heat-inactivation destroyed the killing effect of *D. variabilis* and *A. americanum* midgut extract cultures. An indirect immunofluoresence assay indicated that anti-*B. burgdorferi* antibody concentrations were similar in both the host and the tick midgut extract. Taken together, these experiments suggest that borreliacidal activity of host immune serum is mediated by the activity of complement. Furthermore, this same activity is found in the midgut of *D. variabilis* and *A. americanum* feeding on immune hosts but is not found in *I. scapularis*.

Since vector competence appeared to be related to the activity of host antibody and complement in the tick midgut, I determined whether substances existed in *I. scapularis* that might inhibit or inactivate antibody or complement. We tested for the presence of an antibody-cleaving enzyme in the gut extract of *I. scapularis* and were unable to demonstrate any activity in several dilutions of gut extract after 30 minutes at 37°C. A spirochete survival assay was performed in cobra venom, which possesses powerful anti-complement activity, and in the saliva collected from *I. scapularis*, *D. variabilis* and *A. americanum*. Spirochete survival of over 95% was observed in immune serum with cobra venom and *I. scapularis* saliva but no spirochete survival was found in cultures containing saliva from *D. variabilis* and *A. americanum*. We suggest that an anti-complement factor in the saliva of *I. scapularis* inactivates complement in the midgut of engorged ticks.

Finally, the duration of tick attachment may serve as a useful predictor of risk for acquiring various tick-transmitted infections such as Lyme disease and babesiosis. We measured three tick engorgement indices (EI) at known time intervals after tick attachment and used these indices to determine the length of time that ticks were attached to tick-bite victims in selected Rhode Island and Pennsylvania communities where the agents of Lyme disease and human babesiosis occur. Regression equations developed correlate tick engorgement indices with duration of feeding. More than 60% of tick-bite victims removed adult ticks by 36 hours of attachment, but only 10% found and removed the smaller nymphal ticks within the first 24 hours of tick feeding. A table containing specific EI prediction intervals were calculated for both nymphs and adults allowing practitioners or clinical laboratories to use easily-measured tick engorgement indices to predict transmission risk by determining the duration of feeding by individual ticks.

ACKNOWLEDGMENTS

PREFACE

This dissertation is presented in manuscript format; it contains four separate manuscripts and two appendices. The first three manuscripts cover the possible factors for the mechanism of vector competence in Lyme disease and the fourth manuscript describes a method to determine the duration of deer tick attachment to tick-bite victims. Our findings may explain a mechanism for vector competence in Lyme disease. Our methods may also help to predict the risk of Lyme disease in tick-bite victims.

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INTRODUCTION

Insects and ticks are well known vectors of human and animal pathogens, transmitting a wide variety of microorganisms. These microorganisms replicate in vertebrate and invertebrate hosts. The invertebrate host is a blood-sucking arthropod that is competent to transmit the pathogen between susceptible animals. Microorganisms transmitted by ticks must adapt to the peculiar physiological and behavioral characteristics of ticks, particularly with regard to blood feeding, bloodmeal digestion, and molting (Nuttall et al. 1994). What is still poorly understood, however, is why certain of these arthropods are incompetent as vectors of the pathogen which they encounter during bloodfeeding. Innate barriers to infection resulting in a potential vector's inability to transmit ingested pathogens have largely been characterized in ambiguous terms such as midgut or salivary gland barriers. Despite considerable research on such important infections as mosquito-transmitted malaria and tick-transmitted rickettsiae and viruses, we cannot yet identify a single molecule responsible for vector competence.

Ticks in the *Ixodes ricinus* species complex have been implicated as vectors of Lyme disease spirochetes (*Borrelia burgdorferi*) worldwide. Other varieties of ixodid ticks, notably dog ticks (*Dermacentor variabilis*) and Lone Star ticks (*Amblyomma americanum*) in the U.S. have been shown in the laboratory to be incompetent vectors for Lyme disease spirochetes (Piesman & Sinsky 1988, Mather & Mather 1990a, Lindsay et al. 1991, Mukolwe et al. 1992, Ryder et al. 1992). Although *D. variabilis* and *A. americanum* may be capable of acquiring spirochetes from the host, infection disappears and is not detectable after molting. *Ixodes scapularis*, however, is capable of maintaining the infection and efficiently passing *B. burgdorferi* transstadially. Since it has been speculated that *D. variabilis* and *A. americanum* may possess some factor inside their gut

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that destroys the spirochetes, while *B. burgdorferi* survives in the midgut during *I. scapularis* transstadial transformation, it would appear that factors promoting vector competence for this bacteria reside within the midgut (Piesman & Sinsky 1988). *Ixodes scapularis* possess potent pharmacological armaments in their saliva, including apyrase, a kininase, an anaphylatoxin-destroying capacity, vasodilatory prostaglandins and an anticomplement protein. It has been well described how these substances serve the tick in successful blood feeding, and perhaps in modifying the tick feeding site so as to facilitate spirochete infection of the host (Ribeiro 1987a).

Further research was needed on the adaptation and replication of *B. burgdorferi* in gut and saliva of ticks to determine the effect of pharmacological factors in gut and saliva on spirochetes. These factors may be involved in the mechanism of vector competence for Lyme disease.

LITERATURE REVIEW

Lyme disease was first recognized as a clinical entity in 1909 by a Swedish dermatologist and was termed erythema migrans. The first recognized American case of Lyme disease was reported in Wisconsin in 1969 by a physician who was familiar with the European literature (Scrimenti 1970). He sought spirochetes in biopsy material and used penicillin to effect a cure. The first recognized epidemic of Lyme disease appeared in coastal Connecticut in 1975. The affluent suburban community of Old Lyme gave its name to the disease then afflicting so many of its inhabitants. Some 51 residents of the site were found to be suffering from an atypical arthritic condition that was generally preceded by an annular rash. *Ixodes* ticks immediately became suspect as vectors for this disease (Steere et al. 1977). *Ixodes dammini* was recognized as a distinct species in 1979 (Spielman et al. 1979). Outbreaks of infection seem to occur solely where *I. dammini* is abundant, especially in the northeastern and north central portion of the United States, the regions in which cases cluster most intensely. However, Oliver et al. (1993) suggested that *I. dammini* Spielman, Clifford, Piesman & Corwin, 1979, is not a valid species separate from *I. scapularis* Say, 1821. No major divergence could be demonstrated between *I. scapularis* and *I. dammini* in experiments involving hybridization, assortative mating, morphometrics, chromosomes, isozymes, life cycles, host preferences, vector competencies, and DNA sequences. The pathogen of this disease has been isolated and named *Borrelia burgdorferi* (Burgdorfer et al. 1982, Johnson et al. 1984b). From 1986 to 1990, Lyme disease cases accounted for 81 % of all reported cases of arthropod-transmitted diseases in the United States (Centers for Disease Control 1992). Lyme disease is now the most important arthropod-transmitted disease in this country.

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Pathogen (Spirochete)

The genus *Borrelia* is composed of 21 species, the majority of which are asociated with relapsing fever illnesses that are transmitted by soft-bodied ticks (Argasidae) (Johnson et al. 1984a, 1987; Kelly 1984). In contrast to all the other species of tick-associated borreliae, *B. burgdorferi* is transmitted by hard-bodied ticks (Ixodidae), namely the *Ixodes ricinus* complex (Hoogstraal 1981), such as *I. scapularis, I. ricinus, I. pacificus,* and *I. persulcatus* (Steere & Malawista 1979; Burgdorfer et al. 1982, 1983, 1985; Burgdorfer & Gage 1986; Ai et al. 1988). *Borrelia burgdorferi* is morphologically similar to *Treponema*. Cells are irregularly coiled with tapered ends and possess 6 to 8 axial fibrils located beneath the outer membrane; the cell diameter is about 0.2 um and ranges from 4 to 30 um in length (Steere et al. 1983b). The spirochetes tend to remain in the midgut of unfed ticks, where they are sequestered in the microvillar brushborder and intercellular spaces of the epithelium (Burgdorfer 1989a).

The most important immunodominant proteins of *B. burgdorferi* are the 94 KDa, 60 KDa, 41 KDa (flagellin), 34 KDa (OSPB), 31 KDa (OSPA), 30 KDa, 21 KDa (OSPC), and 17/18 KDa proteins. Whereas the 60 KDa, 41 KDa, and 34 KDa constituents reveal a marked cross-antigenicity with other spirochetes and even more distantly related bacteria, antibodies against the 94 KDa, 31 KDa, and 21 KDa protein are largely species-specific. The early immune response in Lyme borreliosis is triggered mainly by the flagellin. In the later stage a wide range of immunogenic proteins is involved, with the 94 KDa antigen being the best marker for late immune response (Barbour et al. 1985, Craft et al. 1986, Coleman and Benach 1987, Zoller et al. 1993). Five to seven HSPs (heat shock proteins) of *B. burgdorferi* were detected and HSP may result in an autoimmune reaction causing arthritis (Carreiro et al. 1990).

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The major proteins of *Borrelia* isolated from *I. scapularis* collected on Shelter Island, New York were similar to proteins in *Borrelia* from *I. pacificus*, wild animals, and humans in the United States (Anderson et al. 1983, Benach et al. 1983, Bosler et al. 1983, Steere et al. 1983a, Burgdorfer et al. 1985), from *I. ricinus*, wild animals, and humans in Europe (Burgdorfer et al. 1982, Asbrink et al. 1984, Hovmark et al. 1988), and from *I. persulcatus* and humans in Asia (Ai et al. 1988). Variants with different major outer surface proteins from the seminal B 31 strain have frequently been isolated from humans and *I. ricinus* in Europe (Barbour et al. 1985; Stanek et al. 1985; Wilske et al. 1985, 1986, 1988; Anderson et al. 1986a), but in the United States the isolates from humans, rodents, and most ticks have been remarkably similar to one another and the B 31 strain (Barbour et al. 1985). Exceptions have included isolates from the songbird (*Catharus fuscescens*), Cottontail rabbits (*Sylvilagus floridanus*), *I. dentatus*, *I. scapularis*, *I. neotomae*, and *I. pacificus* (Barbour et al. 1985; Bisset & Hill 1987; Anderson et al. 1988, 1989; Lane & Pascocello 1989). While major proteins vary considerably among European *Borreliae* isolated from humans, specific illnesses linked to particular variants have not been documented.

Animal Reservoir:

Borrelia burgdorferi has been isolated from or detected in tissues of three domestic and wild mammals and eight birds in United States, three rodents in Europe, and one rodent in Asia (Anderson & Magnarelli 1993). The white-footed mouse is a particularly important host for the spirochete in the northeastern and midwestern United States (Anderson et al. 1983, 1985, 1986c, 1987b; Bosler et al. 1983, 1984; Levine et al. 1985; Bosler & Schulze 1986; Donahue et al. 1987; Callister et al. 1989). These mice

apparently harbor the spirochete throughout their lives (Bosler & Schulze 1986, Donahue et al. 1987), and where Lyme disease is prevalent 70 % to 80 % or more of the mice may be infected. Prevalence of infection is highest in summer, following peak feeding by nymphs, and lowest in winter, when immature ticks are inactive (Anderson et al. 1987a). Other species of rodents, such as eastern chipmunks, may also be important reservoirs, but these animals have not been extensively studied (Anderson et al. 1985, Mather et al. 1989). *Apodemus* may be an important reservoir in Europe and Asia (Hovmark et al. 1988, Miyamoto et al. 1991).

Deer were reported as reservoir incompetent (Telford et al. 1988). Antibody to *B. burgdorferi* has been detected in deer (Magnarelli et al. 1984b, 1986a) and these animals are extensively parasitized by *I. scapularis* (Piesman et al. 1979, Anderson & Magnarelli 1980, Main et al. 1981). Serologic surveys by IFA and ELISA also have confirmed extensive exposure of animals to borrelial antigens in the United States; they include dogs, cats, horses, cows, white-tailed deer, axis deer (*Axis axis*), Columbian black-tailed deer (*Odocoileus hemionus*), fallow deer (*Dama dama*), Virginia opossum (*Didelphis virginiana*), black-tailed jack-rabbit (*Lepus californicus*), Barbary sheep (*Ammotraqus lervia*), desert cottontail (*Sylvilagus audubonii*), brush rabbit (*S. bachmani*), cottontail rabbit, coyote (*Canis latrans*), deer mouse (*Peromyscus maniculatus*), and pinon mouse (*P. truei*) reported from areas of the United States where ticks are common (Magnarelli et al. 1984a,b,c, 1985, 1987, 1988, 1990; Marcus et al. 1985; Lane & Burgdorfer 1986, 1988; Rawlings 1986; Godsey et al. 1987; Schulze et al. 1987; Teitler et al. 1988; Burgess & Windberg 1989; Lane 1990b; Lane et al. 1991; Levine et al. 1991). Birds also are parasitized by *I. scapularis* and are naturally infected with *B. burgdorferi* (Anderson & Magnarelli 1984, Anderson et al. 1986b, Schulze et al. 1986). Only one isolate of this bacterium has been obtained from a field-caught bird, a veery (*Catharus fuscescens*)

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(Anderson et al. 1986), and this was antigenically different from strain B 31 (Barbour et al. 1985). Larval ticks feeding on birds, as on mammals, often harbor *Borreliae* (Fish & Daniels 1990 and Anderson et al. 1990).

Tick vectors:

The principal vectors of Lyme disease are ticks belonging to the *I. ricinus* complex. Members of this group occur on nearly every continent (Hoogstraal 1981). North American species include *I. scapularis* (=*I. dammini* [Oliver et al. 1993]) and *I. pacificus*. The Eurasian species is identified as *I. persulcatus* and the European species is *I. ricinus*.

The two species in North America tend to occur in different geographical areas. *Ixodes pacificus* extends along the west coast of the United States from the California-Mexico border northward into southwestern Canada (Bishopp & Trembley 1945, Cooley & Kohls 1934, Gregson 1956, Arthur & snow 1968, Easton et al. 1977, Keirans & Clifford 1978, Furman & Loomis 1984, Anderson 1989, Burgdorfer 1989b). *Ixodes scapularis* extends northward from Virginia into New Hampshire, Vermont, and Maine and westward through southern Ontario to its westernmost border in Minnesota and Iowa, including southern Illinois, and southward from Florida to central Texas and the southeastern United States (Cooley & Kohls 1934; Bishopp & Trembley 1945; Keirans & Clifford 1978; Spielman et al. 1979, 1985; Schulze et al. 1984; Coan & Stiller 1986; Anderson et al. 1987c, 1990a; Ginsberg & Ewing 1988; Wilson et al. 1988; Anderson 1989; Burgdorfer 1989b; Pinger & Glancy 1989; Smith et al. 1990; Bouseman et al. 1990; Levine et al. 1991).

Ixodes ricinus extends from England to about 50° to 55° east longitudinally (Arthur 1966, Snow & Arthur 1970, Doss et al. 1978, Anderson 1989, Burgdorfer 1989b). It has been collected in North Africa and as far north as 65° latitude. *Ixodes persulcatus* overlaps with *I. ricinus* in eastern Europe and is found in a relatively broad band across Asia (Arthur 1966, Doss et al. 1978, Anderson 1989, Burgdorfer 1989b).

Each tick feeds three times in its life, initially as a larva, then as a nymph, and finally as an adult. The duration of the life cycle is a year or more. *Ixodes scapularis* and *I. pacificus* may take one to two years to complete their lives (Rogers 1953, Arthur & Snow 1968, Furman & Loomis 1984, Spielman et al. 1985). *Ixodes ricinus* and *I. persulcatus* often complete their life cycles in two years. The seasonal distribution of the four life stages of *I. scapularis* is as follows (Spielman et al. 1985): larvae hatch from eggs predominately in midsummer, and after feeding fully from a host they detach and drop to the ground. Larvae either molt into nymphs or remain in an engorged state in the duff layer of the soil throughout the winter (Yuval & Spielman 1990). Blood-fed larvae molt into nymphs the following spring. Host-seeking nymphs become abundant in late spring and early summer. After ingesting blood from animals, they fall to the ground and molt into adults. Males and females seek large mammals. The fully engorged females lay the eggs and repeat the cycle. Seasonal abundance of the three feeding stages of the other species differs from *I. scapularis*. *Ixodes pacificus* may be collected throughout the year in California (Arthur & Snow 1968, Furman & Loomis 1984, Westrom et al. 1985, Lane & Loye 1989). In unfed *Ixodes* ticks the Lyme disease spirochetes are usually found sequestered within the tick's midgut. In fed ticks, infection is disseminated; spirochetes might be found in saliva, hemolymph, or other tick tissue. Disseminated infection is rare in flat ticks (Burgdorfer et al. 1983, Benach et al. 1987, Ribeiro et al. 1987b, Ewing et al. 1994). The direct inoculation of spirochetes into vertebrates via

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regurgitated midgut contents has been suggested as one possible route of transmission (Burgdorfer 1984) but has not been critically examined. However, *B. burgdorferi* have been directly observed in the salivary gland (Burgdorfer et al. 1989b, Zung et al. 1989), e.g. in pilocarpine-stimulated salivary secretions collected from infected ticks, specifically feeding nymphal and adult *I. scapularis*, thus documenting that spirochetes are inoculated via saliva (Ribeiro et al. 1987b, Ewing et al. 1994). Following a tick's attachment to its host, spirochetes multiply (Benach et al. 1987) and may congregate in intercellular pits near the microvillar brushborder of midgut epithelial cells (Burgdorfer et al. 1989b), where some leave the midgut by both inter- and intracellular penetration of the gut epithelial cells (Zung et al. 1989). Once free of the gut's basement membrane, spirochetes disseminate, traveling throughout the tick's hemocoel via hemolymph (Ribeiro et al. 1987b). Disseminated spirochetes may infect various tick tissues, including salivary glands and ducts, ovaries, and the central ganglion (Zung et al. 1989). However spirochetes were observed in saliva only of ticks having hemolymph infection (Ribeiro et al. 1987b). In adult ticks, spirochetes were observed in the hemolymph of gut-infected adult *I. scapularis* as early as 24 hours after tick attachment and spirochetes were observed in saliva as soon as 72 hours following tick attachment. Spirochetes were frequently observed within the salivary glands of nymphal ticks after 48 hours of attachment and less frequently in nymphs attached for only 24 hours (Zung et al. 1989). So, the duration of tick attachment on animals is an important factor which allows spirochetes to be transmitted from ticks to animals. The ability of *I. scapularis* to transmit *B. burgdorferi*, for example, is obtained only after a certain amount of feeding. Transmission of this infectious agent is most efficient when ticks feed to repletion (Mather et al. 1990b). Spirochetes were rarely transmitted sooner than 24 hours in nymphal ticks feeding on white-footed mice and hamsters (Piesman et al. 1987b) and

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were rarely transmitted sooner than 36 hours in adult ticks feeding on rabbits (Piesman et al. 1991). It is important to investigate the time of attachment in order to predict the risk of Lyme disease and removing attached ticks within 24 hours is an important strategy in the management of Lyme disease.

The prevalence of spirochete infection (tick infection rate) varies widely among distinct populations of vector ticks. In *I. scapularis*, adult ticks generally display about twice the rate of *B. burgdorferi* infection as do nymphs. In highly endemic areas, spirochete prevalence in populations of adult *I. scapularis* typically ranges between 35 % and 75 %, while that for nymphs is between 15 % and 35 % (Anderson et al. 1983; Bosler et al. 1983; Piesman et al. 1986b, 1987a; Schulze et al. 1986; Falco & Fish 1988). Spirochete-infected host-seeking larval ticks are rare due to the apparent inefficiency of transovarial infection in *I. scapularis*. Spirochetes were detected in just 2 of 274 host-seeking larvae (less than 1 %) collected in Massachusetts (Piesman et al. 1986a), but none were found among 148 examined in Connecticut (Steere et al. 1983a).

In contrast to *I. scapularis* in the eastern United States, spirochete prevalence in the western *I. pacificus* is quite low. *Borrelia burgdorferi* typically infect between 0 % to 5.9 % of adult ticks collected from vegetation in northern California and Oregon (Burgdorfer et al. 1985, Bisset & Hill 1987, Lane & Lavoie 1988). However, transovarial passage of spirochetes appears to occur more efficiently in *I. pacificus* than in *I. scapularis*. One of three field-collected female *I. pacificus*, whose ovaries contained spirochetes, successfully infected 100 % of its progeny (Lane & Burgdorfer 1987). Furthermore, these same larval ticks maintained their spirochetal infections, passing them transstadially into the nymphal and adult stages. These adult ticks eventually passed their infection transovarially, also with great efficiency.

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Ticks not belonging to the *I. ricinus* complex have only rarely demonstrated competence as vectors of *B. burgdorferi* (Schulze et al. 1986, Magnarelli et al. 1986a, Anderson et al. 1985, Teltow et al. 1991). Despite their ability to become infected by ingesting spirochetes during feeding, ticks such as *Dermacentor variabilis, Amblyomma americanum, I. holocyclus,* and *Haemaphysalis leporispalustris* appear to lose their infection either before or during transstadial passage (Piesman & Sinsky 1988, Telford & Spielman 1989, Mather & Mather 1990a, Lindsay et al. 1991, Mukolwe et al. 1992, Ryder et al. 1992).

Determinant factors influencing vector competence are yet unknown. Many factors, including physiological factors, probably influence the ability of ticks to become infected with, transstadially pass, and ultimately transmit pathogens. As a physiological factor, vector-competent *Ixodes* may possess a spirochetal growth-promoting factor that other groups of ticks lack. Spirochetes can be observed in preparations made from several species of ticks as well as other arthropods, such as mosquito and fleas, soon after the ingestion of infected blood (Magnarelli et al. 1986b, Rawlings 1986). Spirochetes have also been shown to multiply rapidly in *I. scapularis* during the first two weeks following ingestion (Benach et al. 1987, Piesman et al. 1990). Spirochete survival in vector-incompetent arthropods is ephemeral, however, usually lasting less than two to four weeks. As an alternative hypothesis, vector-incompetent ticks may possess spirochete growth-inhibitory factors that may not occur, or occur at lower concentrations, in the vector-competent *Ixodes*.

Ixodes scapularis secretes copious amounts of saliva containing a variety of antihemostatic, anti-inflammatory, immunosuppressive properties, and an anti-complement factor (Ribeiro et al. 1985 & 1987b). Active immunosuppressive chemicals, including prostaglandin E2 (PGE_2), block macrophage activation and neutrophil activity

and may inhibit T-cell activation, all early precursors in the cascade of cellular events leading to antibody production and antigen processing. Thus, salivary components injected by *I. scapularis* may promote infection of the host by Lyme disease spirochetes. By deactivating macrophages, PGE_2 may serve to protect *B. burgdorferi* during its initial phase of adaptation in the skin of infected hosts, allowing spirochetes to escape into tissues. Anti-complement may serve to interrupt the complement pathway and spirochetes can survive even when exposed to the host's humoral immune system. All these pharmacological factors may play a role in vector competence for *I. scapularis*.

Among the principal and potential vectors of *B. burgdorferi*, it is perhaps more important to consider intrinsic vector competence. Internal physiological ability to become infected with and transmit spirochetes and other innate behavioral characteristics promoting infection or transmission are major factors in vector competence (Mather & Mather 1990a) . For example, in laboratory studies, both *I. scapularis* and *I. pacificus* are highly competent vectors of *B. burgdorferi* (Lane & Burgdorfer 1987, Piesman & Sinsky 1988), while the natural prevalence of infection among these ticks in the southeastern and western United States is relatively low (less than 4 %) (Burgdorfer et al. 1985). Because immature stages of *I. scapularis* and *I. pacificus* frequently infest lizards (Lane & Loye 1989a), which are inhospitable hosts for *B. burgdorferi* (Lane 1990a), it is most likely that perceived differences in the vector potential of these ticks when compared with that of *I. scapularis* result more from their predilection for feeding on noninfective hosts than on any physiological vector incompetence. Thus, the intrinsic vector competence and, more specifically in this case, the feeding behavior exhibited by biologically competent vectors, may significantly influence the prevalence of infection in vector populations.

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ABSTRACT

Deer ticks (*Ixodes scapularis*) but not American dog ticks (*Dermacentor variabilis*) or lone star ticks (*Amblyomma americanum*) become infected with and transmit Lyme disease spirochetes (*Borrelia burgdorferi*). We evaluated factors associated with the midgut of these three ixodid ticks that might affect their ability to serve as vectors for this spirochete.

When larvae of all three species of ticks ingested blood from a spirochete-infected rabbit, only nymphal deer ticks (*I. scapularis*) derived from these larvae retained the infection (infection rate = 82%). *In vitro* experiments demonstrated that spirochetes survived in the presence of pre-immune rabbit serum but were were killed in the presence of spirochete-immune rabbit serum. Moreover, spirochetes also survived in the presence of immune rabbit serum heated to $56^{\circ}C$ for 1 hour to remove the activity of serum complement. Additionally, spirochetes survived in the presence of midgut extracts from all three species of ticks fed on pre-immune rabbits, and in the presence of deer tick gut extract from ticks fed on immune rabbits. However, spirochetes were killed in the presence of dog tick (*D. variabilis*) and lone star tick (*A. americanum*) gut extract when these ticks fed on immune hosts. Heat inactivation of the gut extract restored spirochete survival regardless of the tick species. The α-*B. burgdorferi* antibody titer in immune serum and tick gut extract was similar (1:1024), even after incubation at $37^{\circ}C$ for up to 90 minutes, suggesting that these ticks lack an antibody-cleaving enzyme in their midgut. Thus, inactivating serum complement appeared to be critical for spirochete survival.

Using a borreliacidal microassay composed of immune serum and spirochetes, the addition of as little as 2 μg protein of cobra venom (a known complement inhibitor) resulted in >95% spirochete survival. Spirochetes failed to survive in the presence of as

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much as 20 µl of either dog tick or lone star tick saliva. Spirochete survival increased from 5 to 95% in the presence of increasing volumes of deer tick saliva. Taken together, these experiments indicate an *I. scapularis* anti-complement factor (*Isac*) present in this tick's saliva that would allow spirochetes to survive in the tick's midgut even in the presence of ingested immune serum. The apparent absence of a similar factor in dog tick and lone star tick saliva suggests that *Isac* serves as the basis for vector competence in Lyme disease.

INTRODUCTION

Insects and ticks are well known vectors of human and animal pathogens, transmitting a wide variety of microorganisms. What is still poorly understood, however, is why certain of these arthropods are incompetent as vectors of the parasites or pathogens which they encounter during bloodfeeding. Innate barriers to infection resulting in a potential vector's inability to transmit ingested pathogens have largely been characterized in ambiguous terms such as midgut or salivary gland barriers. Despite considerable research on such important infections as mosquito-transmitted malaria and tick-transmitted rickettsia and virus, we cannot yet identify a single molecule responsible for vector competence.

The saliva of vector arthropods contains a vast array of pharmacologically-active substances which facilitate bloodfeeding by antagonizing or modifying the host inflammatory response (Ribeiro 1989). For instance, virtually all hematophagous species studied possess salivary anti-platelet activity, usually associated with the enzyme apyrase, which degrades ADP, an important mediator of platelet aggregation. Other, vasodilatory peptides have been found that likely increase blood flow to capillary hemorrhages surrounding vector mouthparts. Arthropod adaptation to overcoming host inflammation and homeostasis appears to have reached a zenith in the diversity of activities found in vector saliva.

Ticks in the *Ixodes ricinus*-species complex have been implicated as vectors of Lyme disease spirochetes (*B. burgdorferi*) worldwide. Several other ixodid ticks, notably American dog ticks (*Dermacentor variabilis*) and Lone Star ticks (*Amblyomma americanum*) in the U.S., are largely incapable of transmitting Lyme disease spirochetes despite ingesting these bacteria (Piesman & Sinsky 1988, Mather & Mather 1990,

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Lindsay et al. 1991, Ryder et al. 1992, Mukolwe et al. 1992). Because *B. burgdorferi* survive in the midgut during *I. scapularis'* transstadial transformation, it would appear that factors promoting vector competence for this bacteria reside within the tick midgut. However, these ticks also possess a potent pharmacological armament in their saliva, including apyrase, a kininase, an anaphylatoxin-destroying capacity, vasodilatory prostaglandins and an anticomplement protein (Ribeiro 1987, 1988; Ribeiro et al. 1985; Ribeiro & Spielman 1986). It has been well described how these substances serve the tick in successful blood feeding, and perhaps in modifying the tick feeding site to facilitate spirochete infection of the host. It may be that re-ingested saliva also exhibits some activity in the tick's midgut. In particular, we observed antagonism of the *in vitro* borreliacidal activity of serum from immune hosts after heat treatment, treatment with a known complement inhibitor (cobra venom), midgut extracts from engorged *I. scapularis*, as well as with *I scapularis* saliva. Anti-complement activity in the saliva of *I. scapularis* may well serve to determine this tick's ability to become infected with *B. burgdorferi*.

MATERIALS AND METHODS

In vivo experiments

To assess vector competence for Lyme disease spirochetes (*B. burgdorferi*) in *Ixodes scapularis*, *D. variabilis* and *A. americanum* feeding on rabbits, larval ticks of all three species derived from a spirochete-free laboratory colony were allowed to feed simultaneously on the ears of a spirochete-infected New Zealand white rabbit (Charles River Laboratories, Wilmington MA). The rabbit had been infected three weeks previously by allowing more than 50 field-collected female *I. scapularis* to engorge to repletion. Field-collected ticks were from Prudence Island, Rhode Island, where the prevalence of infection in adult *I. scapularis* was 40%. All immature ticks in the laboratory colony were derived from adult ticks also collected on Prudence Island. Engorged larvae were collected in cloth bags affixed to the rabbit's ears, and then stored in plastic vials at 23°C and >95% relative humidity until molting into nymphs. Nymphs of all three species, derived in this manner, were examined for the presence of *B. burgdorferi* by dissecting midgut tissues onto a glass slide and treating them directly with fluorescein isothiocyanate-conjugated (FITC) antibodies to *B. burgdorferi* in a direct fluorescent antibody assay (Piesman et al.1986, Mather et al 1989).

To prepare tick midgut homogenates, field-collected adult *I. scapularis*, *D. variabilis* and *A. americanum* were fed to repletion in groups of 30 on the ears of NZ white rabbits. Engorged ticks were collected following detachment, weighed and then dissected to remove all midgut tissue. Pools composed of midguts from five ticks were stored at -70° C until used in an assay.

To obtain serum from pre-immune and *B. burgdorferi*-immunized rabbits, blood was collected by venapuncture either just before allowing ticks to feed, or 4 weeks following tick feeding. Blood was allowed to clot for 1 hour at 4°C, and then was centrifuged at 2000 g for 15 min. Serum was immediately removed and stored at -70°C. Antibody titers were assessed by an indirect immunofluorescent assay (Magnarelli et al. 1984).

Tick saliva was collected by allowing field-collected adult *I. scapularis*, *D. variabilis* and *A. americanum* to partially engorge on NZ white rabbits. Ticks were harvested on the fifth through seventh day of engorgement (just prior to their rapid engorgement phase), rinsed in distilled water and then prepared for collecting saliva as described previously (Tatchell 1967, Ewing et al. 1994). Briefly, ticks were affixed to glass slides using double-faced tape and their mouthparts inserted into a sterile, glass micropipette. Salivation was induced by topically applying 2 μl of pilocarpine to the ticks' scutum. All saliva collected in about two hours from a group of 15-30 ticks was pooled and stored at -70°C until used.

In vitro experiments

We developed a simple *in vitro* microassay to assess borreliacidal activity of host serum components as well as spirochete survival in the presence of potential borreliacidal antagonists. In this assay, 25 μl of host serum, either pre-immune, immune, or heat-inactivated (56°C, 1 hr) sera from New Zealand white rabbits or laboratory-raised white-footed mice (*Peromyscus leucopus*) was added to an equal volume of BSKII containing 2 X 10^6 actively-growing *B. burgdorferi*. In some experiments, varying amounts of tick midgut extract, tick saliva, or cobra venom (a complement antagonist) were added to these cultures. The mixture was incubated at 33°C for 4-18 hours depending on the

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experiment, at which time cultures were centrifuged (14,000 x g for 12 s), and examined for spirochete survival on the basis of motility, refractivity, and extensive surface blebbing using darkfield and phase contrast microscopy.

To assess the borreliacidal activity of host antibody as compared to serum complement I designed an experiment with three treatments:(1) Two-fold serial dilutions (1:2 to 1:4046) of immune rabbit serum, (2) dilutions of pre-immune rabbit serum in the presence of undiluted, heat-inactivated immune serum (titer: $2048^{1/x}$), and (3) dilutions of heat-inactivated immune serum in the presence of undiluted, pre-immune serum. Spirochete survival was determined after overnight incubation at 33°C. The immune rabbit serum has antibodies and complement, both of which are diluted in treatment (1). In treatment (2), the complement in pre-immune serum is diluted, but antibody concentrations remain high in the undiluted, heat-inactivated immune serum. Antibodies are diluted in treatment (3), but complement concentrations remain high in the undiluted, pre-immune serum.

To evaluate the effect of tick midgut factors on spirochete survival, extracts were prepared from a homogenate of midguts dissected from pools of 5 rabbit-engorged female ticks. Briefly, frozen midguts were macerated in enough PBS to make a 20% w/vol homogenate, centrifuged (14,000 x g for 12 s) to pelletize the gut tissue, and the supernatant passed through a 0.45 μm filter. Gut extracts from *I. scapularis*, *D. variabilis* or *A. americanum* were added to cultures of high- or low-passaged strains of *B. burgdorferi* (B31 and T15, respectively) growing in BSKII. Similar experiments were conducted where tick midgut extracts were heat-inactivated (56°C, 1 hr) prior to inoculation of the culture. Cultures were checked after 4-6 hours and again after 18 hours by dark field and phase contrast microscopy to determine spirochete survival.

To examine the effect of tick saliva on *in vitro* spirochete survival, 2, 4, 6, 8, 10 and

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20 μl of saliva collected from *I. scapularis*, *D. variabilis* and *A. americanum* was added to 25 μl of *B. burgdorferi* (strain B31) mixed with an equal volume of immune rabbit serum (titer: $2048^{1/x}$) for a final concentration of 10^6 cells/ml. Cultures were incubated at 33°C overnight and examined for spirochete survival as described above. Controls included cultures containing serum alone or 2-20 μl of cobra venom (1μg protein/μl--Sigma Chemical Co., St. Louis MO). Additional controls included spirochetes incubated in the presence of tick saliva or cobra venom (20 μl each).

To determine α-*B. burgdorferi* antibody concentrations in hosts and tick midgut extracts, we developed an indirect immunofluorescent antibody assay. Briefly, *B. burgdorferi* antigen (strain B31) was prepared by centrifuging (35, 000 x g for 45 minutes) 8 ml of a two-week old culture. The resulting pellet was washed three times by resuspending in equal parts of phosphate-buffered saline (PBS). After the final wash, the spirochete concentration was adjusted to 10^7 cells per ml (Magnarelli et al. 1984). One drop of the antigen solution was placed into wells on an eight-well immunofluorescence microscope slide. Slides were air-dried, then fixed in acetone for 10 min. Slides prepared in this way were overlaid with either heated (56°C, 1 hr) or non-heated host serum or tick midgut extract in two-fold serial dilutions, incubated for 30 min at 37°C, then washed in PBS for 20 min. After washing, slides were treated with a goat α-rabbit IgG antibody conjugated to FITC (Sigma Chemical Co., St. Louis, MO) for 30 min at 37°C, then washed again in PBS for 20 min. Prepared slides were air-dried, mounted in buffered glycerol and examined for fluorescence. The antibody titer was determined to be the dilution prior to the fluorescence end-point.

RESULTS

When larvae of deer ticks (*I. scapularis*), dog ticks (*D. variabilis*) and lone star ticks (*A. americanum)* were simultaneously fed on an infected New Zealand white rabbit, *B. burgdorferi* was detected in 82% of the derived nymphal *I. scapularis* but could not be detected in either nymphal *D. variabilis* or *A. americanum* (Table 1). Additionally, deer ticks took more than twice as long to molt from the larval to the nymphal stage than either the dog ticks or lone star ticks.

Spirochetes survived *in vitro* in pre-immune rabbit sera but were killed in sera collected 4-6 weeks after infection of the host by tick bite up to a dilution of 1:32 (Figure 1). In more dilute sera, an increasing proportion of spirochetes survived; 100% survival was noted at a serum dilution of 1:4096. At lower dilutions, little change in the spirochetes could be seen after just 30 min of incubation with immune serum but by 1.5 hr, both spirochetal form and motility were lost in 68.6% of those bacteria counted. Spirochete killing increased by 3 hr and by 4 hrs of incubation all spirochetes were killed. Heat-inactivation ($56^{o}C$, 1 hr) of immune sera prevented spirochete killing, but borreliacidal activity was restored by the addition of pre-immune rabbit sera (Table 2). Heat-inactivation of the serum appeared to have no effect on antibody titer (Table 3).

The assay for spirochete survival in immune serum showed the following survival 0 %, 33.9 %, 91.5 %, 97.9 %, and 100 % at 1 : 16, 1 : 32, 1 : 64, 1 : 128, and 1 : 256, respectively. The last dilution for 0 % survival of the serial dilution of pre-immune serum mixed with undiluted heat-inactivated immune serum was 1 : 16, but the last dilution for 0 % survival of the serial dilution of the heat-inactivated immune serum mixed with undiluted pre-immune serum was 1 : 256. The results showed that the killing of spirochetes was more efficient in undiluted pre-immune serum mixed with the serial

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dilution of heat-inactivated immune serum, however 1 : 2048 dilution, the three treatments all had 100 % survival (Figure 2). It seemed that the killing of spirochetes required a large quantity of complement but only a small quantity of antibody.

Spirochetes survived when incubated with midgut extracts from all three species of ticks bloodfed on pre-immune rabbits (Table 4). However, spirochetes incubated with extracts from *A. americanum* and *D. variabilis* taken from immune rabbits were killed while those incubated with extracts from *I. scapularis* survived. Spirochetes survived exposure to heat-inactivated midgut extracts from all three tick species fed on immune rabbits. Serum antibody titers in pre-immune and immune rabbits, and in tick gut extracts from ticks derived from those rabbits were comparable (Table 5). Pre-immune IFA α-*B. burgdorferi* IgG titers in rabbit sera and tick midgut extracts were 1:32 while immune titers in both sera and gut extracts were 1:4096 by week 6 post infection. It would appear that both *A. americanum* and *D. variabilis* possess a heat-labile, borreliacidal factor in their midgut that is not shared with *I. scapularis*.

We also evaluated *in vitro* spirochete survival in the presence of tick saliva. Spirochete survival increased with the addition of increasing amounts of *I. scapularis* saliva to mixtures of spirochetes and immune serum. Nearly 50% survival was observed in the presence of as little as 10 μl of saliva (Figure 3). No spirochetes survived in similar assays where saliva from *A. americanum* or *D. variabilis* was used. Cobra venom, a powerful α-complement substance also "protected" spirochetes from the borreliacidal action of serum complement at concentrations as low as 2 μg protein. Neither tick saliva nor cobra venom appeared to have any direct effect on the spirochetes in the absence of immune serum.

DISCUSSION

The time necessary to kill spirochetes in immune rabbit serum provided us with the information that after 4 hours (Figure 1), spirochetes would be killed; therefore we were able to check the survival rate in the experiment. Lovrich et al. (1991) reported similar results in immune hamster serum.

In vitro, a comparison of treatments 1 and 2 showed different results. Spirochetes survived in three kinds of ixodid tick gut extracts fed on pre-immune rabbits, but for treatments 2 and 4, spirochetes only survived in *I. scapularis* gut extract and were killed in *D. variabilis* and *A. americanum* gut extract fed on immune rabbits. These data suggested that the blood source from an infected or a non-infected host is very important in determining spirochete survival or killing. The factor which killed spirochetes in gut extract of *D. variabilis* and *A. americanum* in treatment 2 may be related to temperature and may be heat labile, since the spirochetes survived in the heat treated gut extract of both ixodid ticks, even when fed on immune rabbits. These data may explain why *in vivo* the spirochetes could not be found in *D. variabilis* and *A. americanum* using DFA when three kinds of ixodid larval ticks were fed on spirochete infected rabbits at the same time.

In treatments 5, 6, 7, and 8, the spirochetes were killed in immune rabbit serum and pre-immune rabbit serum mixed with heat-inactivated immune rabbit serum. Complement may be involved in the killing of spirochetes, because the antibody of heat-inactivated immune rabbit serum was $2048^{1/x}$ and was the same titer as non-heat treated serum with complement intact. The only difference was that heat treated serum may have the heat-labile complement destroyed. It was reported that immune hamster serum failed to kill spirochetes in the absence of complement (Lovrich et al. 1991). In treatment 7, the heat-

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inactivated immune rabbit serum (Antibody) was restored by the addition of pre-immune rabbit serum (Complement) to kill the spirochetes.

The antibody titer of tick gut extract depends on the antibody titer of the host. In our experiments, there were no changes in the antibody titer of rabbit serum and gut extract of three kinds of ixodid ticks during the first feeding and the second feeding. It is interesting that the spirochete can be killed by immune rabbit serum *in vitro*, but the larval ticks of *I. scapularis* still picked spirochete up from the immune rabbit in xenodiagnosis. This may suggest that the spirochete has a mechanism to protect itself from an effective borreliacidal response and to evade host defenses (Duray & Johnson 1986, Johnson et al. 1988, Barbourr et al. 1986, Fikrig et al. 1990, Lovrich et al. 1991).

The other interesting factor which may be involved in vector competence was some component in the gut extract of *I. scapularis*. This factor might allow the spirochetes to survive *in vitro* and *in vivo* in this species. In experiments 1 to 8, we concluded that the killing of spirochetes required antibody and complement in rabbit serum. Similar results were reported in human and hamsters; immune serum was able to kill *B. burgdorferi* when the presence of complement (Kochi & Johnson 1988, Lovrich et al. 1991). It also seems that the killing of spirochetes needs a great amount of complement, compared to antibody (Figure 2). It has been reported that *B. burgdorferi* activated both the alternative and classical complement pathways in normal human serum (NHS), however, the killing of spirochetes was demonstrated through the classical complement pathway. It is not clear why the alternative pathway does not participate in the killing mechanism. It was suggested that the failure of the alternative pathway to mediate killing in the presence of the antibody must ultimately be related to inappropriate interaction of the membrane attack complex (MAC) with the bacterial surface as a consequence of a different localization of the alternative pathway C_5 convertase. Thus, the alternative pathway C_5

convertase may result in C_{5b-9} complexes that are lytically inefficient because of deposition of C_{5b} at inappropriate sites not susceptible to the action of the antibody (Kochi & Johnson 1988, Kochi et al. 1991 & 1993) (Appendix I). If antibody and complement were involved in killing of spirochetes, a factor in *I. scapularis* may inhibit or cleave the antibody or complement allowing the survival of spirochetes. One possible factor is an enzyme in *I. scapularis* which may cleave the antibody very quickly; this enzyme is also heat-labile. In this manner, the spirochetes could survive even in ticks fed on immune rabbits. The test for digesting antibody from gut extract of *I. scapularis* at different concentrations showed that antibody titers were the same in different treatments at 37°C for 30 minutes, compared to the control group. These data indicated that the enzyme for digesting antibody may not be the factor allowing the survival of spirochetes. The other possible factor is anti-complement in the gut of *I. scapularis*. The complement from rabbit serum in tick gut extract may lose the ability to kill the spirochetes through the addition of an anti-complement factor from *I. scapularis*. In this case, the spirochetes could survive, therefore anti-complement may be involved in vector competence.

The saliva-activated transmission (SAT) factor was demonstrated in the salivary glands of competent vectors of Thogoto virus, but not in a limited number of non-competent vectors (Jones et al. 1992). An SAT factor may exist in the saliva of *I. scapularis* which determines the vector competence of *I. scapularis*. Ribeiro (1987) reported that anti-complement activity was found in the saliva of *I. scapularis*. The anti-complement activity may exist in the gut of *I. scapularis*, too, because the channel for saliva secreted by the salivary gland passing through feeding ticks is the same channel in which the blood meal is ingested (Odhiambo 1982). The saliva, with anti-complement, may possibly mix with the blood meal in the feeding channel of the tick and pass through to the gut or get added into the blood of the cavity which is formed in the host's tissue

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during feeding by *I. scapularis*. When spirochetes appear in the blood cavity, the blood mixed with anti-complement may be sucked into the gut. The immune serum will fail to kill spirochetes when anti-complement exists in the blood. In the spirochete survival assay, spirochete survival reached over 95 % at an antibody titer of 2048x in immune rabbit serum with the presence of 20 ul of cobra venom and the saliva of *I. scapularis* (Figure 3). If anti-complement exists in the gut homogenate of the tick, the function of complement will be lost and spirochetes can survive in the gut homogenate. It has been reported that cobra venom and the saliva of *I. scapularis* contain anti-complement factors. The cobra venom factor has been used extensively in the depletion of complement (Nelson 1966, Cochrane et al. 1970, Drake et al. 1974, Rudofsky et al. 1975, Wikel & Allen 1977). The addition of cobra venom to immune serum resulted in the depletion of complement; therefore spirochetes could survive. Anti-complement in the saliva of *I. scapularis* that prevents C_3 hydrolysis even after C_3 is fixed to a surface has been described. The complement pathway may be disrupted with consequent and inhibition of the associated C_3 convertase (Ribeiro 1987). The interruption of the classical complement pathway may prevent the formation of the MAC and result in the survival of spirochetes. It has not been reported that anti-complement exists in *D. variabilis* and *A. americanum*. There was no spirochete survival after addition of 20 ul of the saliva of *D. variabilis* and *A. americanum* to immune serum. A salivary enzyme of *D. variabilis* was reported to cleave the C_5 complement fraction of the complement cascade leading to the production of anaphylatoxins (Berenberg et al. 1972). These anaphylatoxins may be the C_{5a} products generated during the activation of and C_5. The activation of C_5 may help the formation of MAC, therefore it could enhance the killing of spirochetes. Future research should be done to confirm any anti-complement activity in *D. variabilis* and *A. americanum*.

Ixodes scapularis also secretes copious amounts of saliva containing salivary apyrase, a kininase, an anaphylatoxin inactivating activity, and vasodilatory prostaglandins (Ribeiro et al. 1985a, Ribeiro 1987). Active immunosuppressive chemicals, including prostaglandin E2 (PGE_2) and anaphylatoxin inactivating activity, block macrophage activation and neutrophil activity and may inhibit T-cell activation, all early precursors in the cascade of cellular events leading to antibody production and antigen processing. Thus, salivary components injected by *I. scapularis* may promote infection of the host by Lyme disease spirochetes. By deactivating macrophages, PGE_2 may serve to protect *B. burgdorferi* during its initial phase of adaptation in the skin of infected hosts and spirochetes could escape into tissues. Anti-complement may serve to interrupt the complement pathway and spirochetes can survive even exposed to the host's humoral immune system. We suggested that all these pharmacological factors may play a role in vector competence for *I. scapularis*. An anti-complement factor may be an especially important factor for determining the vector competence of *I. scapularis*, *D. variabilis*, and *A. americanum* for Lyme disease.

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ABSTRACT

We compared the growth of spirochetes incubated at various temperatures, both *in vivo* in *I. scapularis*, *D. variabilis* and *A. americanum*, and *in vitro* in cultures containing midgut extracts from those ticks. Temperature significantly affected the *in vitro* growth of spirochetes. Spirochete concentrations in midgut extract cultures and BSKII controls grown at 28°C and 33°C were significantly higher than those grown at 18°C and 23°C ($P < 0.05$). Not surprisingly, spirochete concentrations in the *I. scapularis* extract cultures were higher than spirochete concentrations in gut homogenates from *D. variabilis* and *A. americanum* at 28°C and 33°C ($P < 0.05$). Spirochetes failed to grow in *D. variabilis* and *A. americanum* midgut extract cultures incubated at 18°C and 23°C.

Spirochetes could be detected in nearly all of the nymphal *I. scapularis* derived from larvae bloodfed on an infected rabbit, and prevalence of infection was similar regardless of the tick's incubation temperature. With one exception, spirochetes were not detected in any nymphal *D. variabilis* or *A. americanum;* three spirochetes were observed in 1 of 57 *A. americanum* held at 18°C. Although extrinsic incubation temperature did affect the time required for ticks to molt, it appears to have no effect on vector competence in Lyme disease.

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INTRODUCTION

Environmental temperature can have a significant effect on pathogen transmission by mosquito vectors (Davis 1932, Kramer et al. 1983, Turell et al. 1985, Turell & Lundstrom 1990). However, little is known about the effect of environmental temperature on pathogen replication and transmission in ticks. The rate of *Theileria parva parva* transmission to cattle by *Rhipicephalus appendiculatus* was more rapid at higher rather than lower ambient temperatures (Ochanda et al. 1988). However, Venezuelan equine encephalomyelitis (VEE) virus replication was not significantly different among infected *Amblyomma cajennense* held at different temperatures (Dohm & Linthicum 1993). The effect of temperature on vector competence in ticks may vary according to species and pathogen.

The deer tick, *Ixodes scapularis*, is the principal vector of Lyme disease spirochetes (*Borrelia burgdorferi*) in the Eastern United States. The American dog tick, *Dermacentor variabilis* and the lone star tick, *Amblyomma americanum* also have been implicated as potential secondary vectors (Anderson et al. 1985, Magnarelli et al. 1986, Schulze et al. 1986). However, in studies directly comparing their ability to become infected, *I. scapularis* readily are infected but *D. variabilis* and *A. americanum* fail to maintain *B. burgdorferi* beyond the transtadial molt (Piesman & Sinsky 1988, Mather & Mather 1990, Lindsay et al. 1991, Mukolwe et al. 1992, Ryder et al. 1992). It has been suggested that *I. scapularis* possesses a potent α-complement protein in their saliva that disables complement-mediated spirochete killing in the tick's gut (Yeh et al., unpublished-Manuscript 1). Both *D. variabilis* and *A. americanum* lack such a mechanism.

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In this study, we compared replication of *Borrelia burgdorferi* growing in culture with tick midgut homogenates, and in infected ticks at different ambient temperatures. Specifically, we investigated whether temperature affected the borreliacidal activity of serum antibody and complement in the presence of tick midgut extracts and *in vivo*. Other innate midgut factors besides immune serum may affect the growth of spirochetes in a temperature-dependent manner.

MATERIALS AND METHODS

In vivo experiments:

To assess the effect of the temperature on replication success of Lyme disease spirochetes (*B. burgdorferi*) in *I. scapularis*, *D. variabilis* and *A. americanum*, larval ticks of all three species derived from a spirochete-free laboratory colony were allowed to feed simultaneously on the ears of a spirochete-infected New Zealand white rabbit (Charles River Laboratories, Wilmington MA). The rabbit had been infected three weeks previously by allowing more than 50 field-collected female *I. scapularis* to engorge to repletion. Field-collected ticks were from Prudence Island, Rhode Island, where the prevalence of infection in adult *I. scapularis* was 40%. All immature ticks in the laboratory colony were derived from adult ticks also collected on Prudence Island. Engorged larvae were collected in cloth bags affixed to the rabbit's ears, and then stored in plastic vials at 18°C, 23°C, 28°C, and 33°C and >95% relative humidity until molting into nymphs. Nymphs of all three species, derived in this manner, were examined for the presence of *B. burgdorferi* by dissecting midgut tissues onto a glass slide and treating them directly with fluorescein isothiocyanate-conjugated (FITC) antibodies to *B. burgdorferi* in a direct fluorescent antibody assay (Piesman et al. 1986, Mather et al 1989).

In vitro experiments

To evaluate the effect of temperature on replication success of *B. burgdorferi* in gut homogenates of *I. scapularis*, *D. variabilis* and *A. americanum*, extracts were prepared

from midguts dissected from pools of 5 engorged female ticks bloodfed on New Zealand white rabbits. Briefly, frozen midguts were macerated in enough PBS to make a 20% w/vol homogenate, centrifuged (14,000 x g for 12 s) to pelletize the gut tissue, and the supernatant passed through a 0.45 μm filter. Gut extracts were added to cultures of *B. burgdorferi* B31 and diluted with BSKII media. The final tick gut homogenate dilution was 1: 200 which was determined to be the 50% killing endpoint of spirochetes in immune serum. The final concentration of spirochetes was $3.0 \pm 1.0 \times 10^5$ in each tube. Each treatment incubated at 18°C, 23°C, 28°C, and 33°C was replicated three times. Experimental controls for each trial were *B. burgdorferi* growing in BSKII. Spirochete cell concentrations were determined by dark field and phase contrast microscopy after 8 days of incubation using a Petroff-Hausser counting chamber (Kochi and Johnson 1988).

To compare spirochete replication in cultures containing midgut extracts incubated at different temperatures we used a 5x4 factorial design with two-way analysis of variance (Keppel 1991). Spirochete infection rates of the three tick species held at 18°C, 23°C, 28°C, and 33°C were compared using the Chi-Square test (SAS institute 1990).

RESULTS

Spirochetes were detected in nearly all of the nymphal *I. scapularis* derived from larvae bloodfed on an infected rabbit. Prevalence of infection was similar (Chi-square test, $P > 0.05$) regardless of the tick's incubation temperature (Table 6). With one exception, spirochetes were not detected in any nymphal *D. variabilis* or *A. americanum* simultaneously bloodfed on the same rabbit. Three spirochetes were observed by direct fluorescent antibody assay in the gut preparation from one of 57 *A. americanum* held at 18°C. Engorged larval *D. variabilis* and *A. americanum* molted in about one week when held at 33°C whereas *I. scapularis* took 18-19 days to molt at that temperature (Table 6). All three tick species required longer to molt from larvae to nymphs at lower ambient temperatures.

Spirochete growth *in vitro* was influenced by both temperature and the type of gut homogenate. A significant two-way interaction between temperature and gut homogenate ($F_{12, 40} = 128.6$, $P < 0.05$) indicated that the effect of gut homogenate differed at different temperatures. Spirochete growth in original cultures, control cultures, and *B. burgdorferi* mixed with gut homogenate of *I. scapularis*, *D. variabilis*, and *A. americanum* was compared at each temperature. There were no significant differences at 18°C, $F_{(4, 40)} = 0.008$, $P > 0.05$ or at 23°C, $F_{(4, 40)} = 1.526$, $P > 0.05$. In contrast, there were significant differences between cultures at 28°C, $F_{(4, 40)} = 338.9$, $P < 0.05$ and at 33°C $F_{(4, 40)} = 357.1$, $P < 0.05$.

A simple comparisons test (Tukey) indicated that the mean of the cell concentration for the control and *B. burgdorferi* mixed with gut homogenate of *I. scapularis* group were significantly greater than *B. burgdorferi* mixed with gut homogenate of *D. variabilis* and *A. americanum* at 28°C and 33°C (Figure 4).

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DISCUSSION

Ticks required less time to molt from engorged larvae to nymphs at higher temperatures. Enzymatic activity involved in the molting process is likely enhanced at higher temperatures, and other studies have shown temperature to be a major determinant affecting the molting process (Sonenshine 1991). The length of time required for the transstadial molt in *I. scapularis* did not appear to affect the tick infection rate, as no statistical differences were found in infection prevalence among ticks taking more than twice as long to become nymphs.

Our experiment confirms that *A. americanum* and *D. variabilis* are unable to maintain infection with *B. burgdorferi* following the transstadial molt regardless of the ambient temperature in which they are held. However, Spirochetes grown in midgut extracts from all three tick species were able to increase in cell concentration when incubated at higher temperatures ($28^{\circ}C$ and $33^{\circ}C$), albeit at a lower rate than spirochetes growing in BSKII without gut extracts. Little or no growth was observed in cultures incubated at lower ambient temperatures.

In this study, we grew spirochetes in cultures containing midgut extracts from *I. scapularis*, *D. variabilis* and *A. americanum* fed on immune rabbits. Previously, we showed that although some spirochetes survived to grow when cultured with midgut extracts from *I. scapularis*, spirochetes were killed *in vitro* in the presence of *D. variabilis* and *A. americanum* midgut extracts when ticks were derived from immune hosts (Manuscript 1). Thus, in order to evaluate temperature effects on spirochete growth in these midgut homogenates, it was necessary to dilute the extracts, especially those of *A. americanum* and *D. variabilis*, so that some spirochetes would survive. To

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this end, we cultured spirochetes in midgut extract concentrations that would result in 50% survival of spirochetes after 4 hours of incubation at 33°C.

Spirochete-immune serum in tick gut extracts may kill up to half of the spirochetes inoculated into a culture, but the surviving spirochetes were able to replicate and increase in cell concentration at higher temperatures where they were unable to at lower temperatures. Cell concentrations in *I. scapularis* midgut extract were significantly higher than those in extracts of *D. variabilis* and *A. americanum* grown at 28°C and 33°C suggesting the protective effect of *I. scapularis'* salivary α-complement activity. However, spirochete growth in *D. variabilis* and *A. americanum* gut homogenates suggest that under certain conditions, where tick infection occurs without concomitant ingestion of immune serum components, it may be possible for these ticks to vector *B. burgdorferi*. Further study would be required to assess whether such conditions might occur in nature.

Although three spirochetes were observed in one of 57 derived nymphal *A. americanum* at 18 °C by DFA, there was no statistical difference, compared to the infection rate at 23°C, 28°C, and 33°C. Experiments have been performed using white-footed mice (Mather & Mather 1990), Syrian hamsters (Ryder et al. 1992), and New Zealand white rabbits (Mukolwe 1992), with similar results. In our experience, larval *A. americanum* had difficulty feeding on white-footed mice and Syrian hamsters. Mather & Mather (1990) reported immature stages of *A. americanum* have never been observed on white-footed mice. White-footed mice and Syrian hamsters may not be the natural host for *A. americanum*, but we found that larval *I. scapularis*, *D. variabilis* and *A. americanum* could feed on New Zealand white rabbits simultaneously in this experiment. Although these three species of larval ticks were fed on spirochete infected rabbits, the infection rate was high only in derived nymphal *I. scapularis*. The spirochetes survived in

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derived nymphal *I. scapularis* and were killed in derived nymphal *D. variabilis* and *A. americanum*. We suggest that as soon as the rabbit was infected with spirochetes, the immune response began with antibody production, while spirochetes were simultaneously replicating in the tissue of the rabbit, but that some mechanism protects the spirochete from borreliacidal antibody, and thus the bacteria can evade host defenses (Duray & Johnson 1986, Johnson et al. 1988, Barbourr et al. 1986, Fikrig et al. 1990, Lovrich et al. 1991).

When three species of larval ticks were fed on the infected host, they should have had the same possibility of acquiring spirochetes from the host. But the spirochetes may have mixed with antibody in the blood, and in this manner, spirochetes may be killed in the blood cavity which is formed in the host's tissue during feeding, or spirochetes may have passed through the feeding channel and were killed in the gut of *D. variabilis* and *A. americanum* by borreliacidal antibody. A factor may exist in the gut of *I. scapularis* preventing killing by antibody. An anti-complement factor in saliva of *I. scapularis* which interrupts the complement pathway was reported by Ribeiro (1987). The anti-complement factor in saliva may mix with body fluid in the blood cavity during feeding on the host and this factor travels to the gut of *I. scapularis* allowing the survival of spirochetes.

From *in vitro* and *in vivo* results, we conclude that a factor in *I. scapularis* may inhibit killing of *B. burgdorferi* by spirochete immune serum of rabbits. Information from this experiment combined with the experiments in manuscript I, suggest that anti-complement factor may play a role in determining vector competence for Lyme disease among these three species of ticks.

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ABSTRACT

Phenoloxidase activity in whole body homogenates *Ixodes scapularis, Dermacentor variabilis* and *Amblyomma americanum* adult females was compared to the activity in hemolymph of greater wax moth larvae (*Galleria mellonella* L). No phenoloxidase activity was detected in three ixodid ticks compared to significant activity in the larvae of *Galleria mellonella* L from 20 minutes over baseline activity at 0.5, 5, and 10 minutes.

Enterobacter cloacae was used to induce antimicrobial activity in abraded cuticle in three species of ixodid ticks. *Escherichia coli* pop 3 was used in an antimicrobial assay; no inhibition zones were observed in all homogenates from three species of ticks.

Although three species of ixodid tick have hemocytes (prohemocytes, granulocytes, and plasmatocytes) that are morphologically similar by microscopy to insect hemocytes, no phenoloxidase or antimicrobial activity was observed.

INTRODUCTION

There is little information available on the immune mechanisms in chelicerate groups such as the Acari and the Araneidae. Most results concerning invertebrate immunity were obtained with insects and crustaceans. Because some tick species have hemocytes that are morphologically similar to insects (Kuhn 1994), we examined the hemocytes of three tick genera to confirm any morphological similarities with insects. We were especially interested in the presence of granulocytes because they involved in prephenoloxidase activity of insects and crustaceans, (Ashida and Ohnishi 1967; Ashida and Dohke 1980; Unestam and Nylund 1972; Soderhall 1981; Soderhall 1982; Soderhall and Ajaxon 1982; Soderhall and Smith 1983). The phenoloxidase system is roughly equivalent to the vertebrate complement system, directing and working in concert with the humoral and cytological portions of the immune system to initiate phagocytosis, encapsulation and nodule formation (Appendix II). As with any enzymatic system it is possible to measure the amount of activity initiated by particular non-self stimuli depending on the chemical composition and quantity of antigenic material (Ratcliffe et al. 1982, Soderhall 1982, and Gupta 1986). The immue response of the ticks may be similar to insects and crustaceans when stimulated by pathogen invasion.

A standard phenoloxidase activity assay was used to determine the presence of phenoloxidase in whole homogenates of non-engorged adult female *I. scapularis, D. variabilis,* and *A. americanum* and compared to activity in the larvae of the greater wax moth (*G. mellonella* L.) as a positive control.

In addition, antimicrobial factors in the hemolymph have been demonstrated for a number of arthropods (Chadwick and Dunphy 1986, Brey et al. 1993) and some ixodid ticks (Podboronov 1991) infected by pathogens. An antimicrobial assay would reveal

antimicrobial factors in the form of homogenous substances and together with phenoloxidase activity, help gain a better understanding of immune factors in tick vector competence.

MATERIALS AND METHODS

Hemocyte preparation:

Ticks were bled by amputating the distal portion of one or more legs with dissecting scissors (Burgdorfer 1970). The wounded ticks were placed on a slide containing one drop of PBS, were covered with a cover slip and examined under bright field, phase contrast, and differential interference contrast (Nomarski method) microscopy.

Assay of Phenoloxidase Activity:

Phenoloxidase activity was assayed according to the method described by Pye (1974). *Galleria mellonella* larvae (Grubco Co., Hamilton, Ohio) were used as positive controls to standardize minimum significant incubation periods. Hemolymph from fifteen *G. mellonella* larvae was pooled on ice. After 0.5, 5, 10, 15, 20, 30, 40, 60, and 90 minutes, a 20 ul aliquot of hemolymph from each time interval was added to a mixture of 50 ul 100 mM 4-methyl-catechol, 200 ul 50 mM 4-hydroxyproline ethyl ester and one ml 100 mM sodium phosphate, pH 6.0, for 5 minutes, in a 30^OC water bath. Twenty microliters of 1 M thiourea were added to stop the enzyme-catalyzed reaction. The increase in absorbance at 520 nm was measured with a DU-8B spectrophotometer (Beckman Instruments, Inc., Irvine, CA.) One-way analysis of variance (ANOVA) (SAS Institute 1990) was performed on three replicates to compare optical density at each time interval. Phenoloxidase activity is expressed as the change in absorbance at 520 nm caused by 20 ul hemolymph/5 min (A_{520}-20ul^{-1}-5 min^{-1}).

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F 2 adult ticks were derived from parental adults collected by flagging on Prudence Island , Rhode Island during the spring and fall of 1993. Ticks were raised in a 23 °C incubator under relative 95% humidity. Thirty adult female ticks were weighed and macerated to form a 50 % w/v homogenate in 100 mM sodium phosphate, pH 7.2. The homogenate was then centrifuged at 14,000 rpm in an Eppendorf centrifuge 5415 (Brinkmann Instruments, Inc., Westbury, N.Y.) for 12 seconds to pellet the tissue. The supernatant was collected and incubated on ice for 30 minutes for the phenoloxidase activity assay as described above. The positive control was *G. mellonella* hemolymph from larvae of *G. mellonella* and the negative control contained no hemolymph or tick homogenate. Both control treatments were incubated on ice for 30 minutes. The experiments were repeated three times.

Preparation of tick homogenate:

Ixodes scapularis, D. variabilis, and *A. americanum* adult ticks were blood fed in groups of 12 mating pairs on the ears of New Zealand white rabbits (Charles River Laboratories, Wilmington, MA). Cloth ear bags were taped to the rabbits' ears to prevent tick escape. An Elizabethan collar was placed on each rabbit to prevent grooming. After the partial engorgement of ticks on the right ear of the rabbit, at the fourth day of attachment on *I. scapularis*, at the fifth day of attachment on *D. variabilis*, and at the sixth day of attachment on *A. americanum*, a 28 G 1/2 micro-fine IV needle (Becton Dickinson & Co., Franklin Lakes N.J.) with *E. cloacae* (cell concentration: 10^9/ml) was used to punch a hole inside the tick body behind the scutum. Ticks on the left ear of the rabbit were punched only with a needle as a control group. After treatment for 24 hours, the partially engorged female ticks were collected, weighed and macerated in PBS to

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make a 25% w/v homogenate, then centrifuged at 14,000 rpm 12 sec (Eppendorf, centrifuge 5415) to pelletize the gut tissue. The supernatant from each type of tick gut homogenate was passed through a 0.45 um filter for the antimicrobial activity assay.

Antimicrobial activity assay:

Escherichia coli pop 3 (Institut Pasteur, Paris, France) were cultured in 5 ml nutrient broth. After two days, the *E. coli* pop 3 were mixed with streptomycin sulfate, 1 mg/ml in NaCl (0.9 % solution), and 15 ml of nutrient agar in a water bath at 45°C. This mixture was swirled to mix and poured into a petri dish. When the agar had solidified, three mm wells were punched using a sterile pasteur pipette and a laboratory suction line. Fifteen microliters of 50 % tick homogenate was placed in each of six wells per species. All detection plates were placed at 4°C for 30 minutes and then incubated at 30°C for 24 hours, at which time inhibition zones were scored. A clear zone (> 1 mm from the edge of the well) inhibiting bacterial growth is considered indicative of antimicrobial activity (Hultmark 1982, Brey et al. 1993).

RESULTS AND DISCUSSION

Based on morphological studies, there are at least three types of hemocytes in ticks that are structurally similar to insects (Brehelin & Zachary 1986; Gupta 1986, 1991; Ball et al. 1987; Nardi & Miklasz 1989; Suzuki et al. 1991; Kobayashi et al. 1991; Suzuki & Funakoshi 1992). There are prohemocytes (Figure 6), plasmatocytes (Figure 7), and granulocytes (Figure 8) and granulocytes degranulate by exocytosis *in vitro* (Figure 9). Similar findings were described in *Ixodes ricinus* (Brinton & Burgdorfer 1971; Binnington & Obenchain 1982; Amosova 1983; EI Shoura 1986,1989; Sonenshine 1991; Kuhn & Haug 1994). Although three species of ixodid ticks have hemocytes morphologically similar to insects, phenoloxidase and antimicrobial activity was not detected.

The F values of optical densities of hemolymph of the larvae of greater wax moths incubated at different times had overall significance, $\underline{F}$ (8,18) = 12.17, at $\underline{P}$ < 0.05 by the Tukey multiple range. Optical density was significant after incubation of hemolymph on ice for 20 minutes ($\underline{m}$ = 0.05), compared to 0.5 minutes ($\underline{m}$ = 0.04) (Figure 5). This information supplied us with the proper incubation time for the phenoloxidase activity assay for ticks. Compared to the positive controls, there was no phenoloxidase activity detected in three species of ixodid ticks (Table 7). Similar results were reported by Schwagerl (1991). The absence of activity suggest either the absence of phenoloxidase, the existence of factors inhibiting phenoloxidase or the inability to detect phenoloxidase activity by this method.

Antimicrobial activity was also not detected using our methods. Either no antimicrobial factors are present or the method is not effective. For example, blood fed ticks diluted whatever factors were present prior to challenge with *E. cloacae*.

Podoronov (1991) used an unspecified number of unfed ticks which may have provided enough test material to detect antimicrobial activity.

Agglutination activities of lectins in the hemolymph of four tick species, *Ixodes ricinus, Ornithodoros tartakovskyi, Ornithodoros papillipes*, and *Argas polonicus* have been reported (Grubhoffer et al. 1991) and it is possible that these tick lectins play an important role in the processes of self and non-self recognition and defense reactions. The absence of phenoloxidase coupled with the degranulation of cells by exocytosis and the presence of lectins suggests that the tick immune system may more closely resemble that of *Limulus polyphemus* than that of insects. *Limulus* has a system functionally analogous to the prophenoloxidase cascade of insects. Exposure of granulocytes of *Limulidae* to nonself molecules causes rapid release of their contents (Levin & Bang 1964, Levin 1979, Armstrong & Rickles 1982, Armstrong 1985). Like the phenoloxidase system, *Limulus* immune activity depends on a divalent cation to stimulate proteolytic cleavage of a serine protease which in turn converts zymogen precursors sequestered in granulocytes to active forms. The proteolytic cascade in *Limulus* is best stimulated by bacterial lipopolysaccharide with granulocyte aggregation mediated by a lectin. Furthermore, the resulting protein monomers polymerize like those formed in the prophenoloxidase cascade to form the mesh of the clot (Gupta 1991). *Limulus* is more closely related to Chelicerates than to other arthropods (Barth & Broshears 1982). Hemocytes of ticks are structurally similar to insects but functionally are analogous to *Limulus,* therefore future research should determine the degree of homology between tick and *Limulus* immune systems.

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ABSTRACT

Objectives: The duration of tick attachment is one factor associated with risk for human infection caused by several tick-borne pathogens. We measured tick engorgement indices (EI) at known time intervals after tick attachment and used these indices to determine the length of time that ticks were attached to tick-bite victims in selected Rhode Island and Pennsylvania communities where the agents of Lyme disease and human babesiosis occur.

Methods: The total body length and width as well as the length and width of the scutum was measured on nymphal and adult northern deer ticks (*Ixodes scapularis = I. dammini*) removed from laboratory animals at 0, 12, 24, 36, 48, 60, and 72 hours after their attachment. Three engorgement indices were calculated at each time interval. In addition, EI measurements were recorded for 504 ticks submitted to a commercial laboratory for pathogen detection testing between 1990-1992.

Results: No detectable change was observed in the average EI's for either nymphal or adult ticks between 0 and 24 hours of attachment using any of the engorgement indices (P > 0.05). After 24 hours of tick attachment, all EI's continuously increased; average indices for nymphs attached 36, 48 and 60 hours were significantly different from those attached ≤ 24 hours and from each other (P < 0.05). Similarly, average EI's for adult ticks attached ≤ 36 hours were significantly different from those attached for 48 hours or more (P < 0.05). More than 60% of tick-bite victims removed adult ticks by 36 hours of attachment, but only 10% found and removed the smaller nymphal ticks within the first 24 hours of tick feeding.

Conclusions: The duration of tick attachment may serve as a useful predictor of risk for acquiring various tick-transmitted infections such as Lyme disease and babesiosis. Regression equations developed herein correlate tick engorgement indices with duration

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of feeding. A table containing specific EI prediction intervals calculated for both nymphs and adults will allow the practitioner or clinical laboratory to use easily-measured tick engorgement indices to predict transmission risk by determining the duration of feeding by individual ticks.

INTRODUCTION

The risk of infection with several tick-borne pathogens, including the agents of Lyme disease, human babesiosis and Rocky Mountain spotted fever, depends directly on the duration of vector tick attachment. For example, before transmission of *Borrelia burgdorferi* can occur, nymphal and adult *Ixodes scapularis* (= *I. dammini*) usually must be attached to a host for more than 24 and 36 hours, respectively (Piesman et al.1987, Piesman et al.1991). Sporozoites of *Babesia microti* generally require a minimum of 36-48 hours to complete maturation in salivary glands of nymphal *I. scapularis,* and babesial infections in hamsters were transmitted most efficiently after 54 hours of tick attachment (Piesman & Spielman 1980). *Rickettsia rickettsii* become "reactivated" during the tick feeding process and infectious forms are transmitted by *Dermacentor andersoni* only after a short feeding period (usually 8 - 10 hrs) (Spencer & Parker 1924, Hayes & Burgdorfer 1982). These examples of transmission or reactivation delays between tick attachment and transmission may partially explain why the risk of acquiring Lyme disease, and perhaps other tick-borne infections, by people with recognized tick bites is less than might be expected based on the relatively high infection prevalence in ticks (Falco & Fish 1988, Costello et al. 1989, Shapiro et al. 1992, Mather 1993). Ticks can be removed prior to pathogen transmission, and many probably are.

During tick feeding, the alloscutal length and width of ixodid ticks increases markedly while the dimensions of their hard scutal plate remain unchanged. Tick engorgement indices have been created from ratios that compare scutal and full-body dimensions, and have been used in previous studies to describe the time course of tick feeding in relation to parasite development and transmission to animals (Obenchain et al.1980, Piesman & Spielman 1980). Thus, it may be possible to assess risk for human infection with various

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tick-transmitted agents, especially those with lengthy delays between tick attachment and transmission, by determining a tick's engorgement index. Accordingly, we evaluated various engorgement indices of *I. scapularis* and compared them with the length of time a tick was attached to a host. In particular, we developed regression equations explaining the relationship between the duration of attachment and various engorgement indices for both nymphal and adult ticks fed on hamsters and rabbits, respectively. We then used these equations to predict the duration of attachment of ticks removed by tick-bite victims in two communities in Rhode Island and Pennsylvania.

MATERIALS AND METHODS

Host-seeking, adult ticks of *I. scapularis* used for experimental infestations were collected from clothing after walking through vegetation (Ginsberg & Ewing 1989) at a heavily tick-infested site located in South Kingstown (Washington Co.), RI, during the spring of 1993. All ticks were separated by sex into different vials and stored at 4^oC under relative humidity > 95% until used. Nymphal *I. scapularis* for experimental infestations were derived from larval ticks blood-fed on laboratory-raised white-footed mice (*Peromyscus leucopus*). The larval ticks were from field-derived adult females collected on Prudence Island, RI during the spring and fall of 1993. Engorged larvae and subsequently-derived nymphs were held in vials at 23^oC under relative humidity > 95%.

To obtain partially-engorged adult *I. scapularis*, ticks were placed onto the ears of a New Zealand white rabbit (Charles River Laboratories, Wilmington, MA) in two groups of 50 mating pairs. Ticks were contained on the rabbit's ears using cloth bags affixed at the ear base with tape. An Elizabethan collar was placed on the rabbit to prevent excessive grooming. After allowing 2-3 hours for tick attachment, the ear bags were opened and all non-attached ticks were removed. At time intervals of 12, 24, 36, 48, 60, and 72 hours after ticks were attached, ten ticks were removed from the rabbit's ear by traction using fine-pointed forceps, taking care not to damage the ticks' mouth parts. Partially-engorged nymphal *I. scapularis* were obtained in a similar way, except that they were attached to three Syrian golden hamsters (Charles River Laboratories, Wilmington, MA) held in small, wire-mesh restraining cages and wrapped in paper. Totals of 100 nymphs were placed on hamsters; after 2-3 hours, all non-attached ticks were removed, and animals were placed singly into larger cages with wire-mesh bottoms held over pans

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of water. At time intervals of 12, 24, 36, 48, and 60 hours following their attachment, 30 nymphs were removed (10 nymphs per time interval from each hamster).

All ticks removed from hosts were anesthetized by transferring them to a 2 ml vial containing cotton and a drop of halothane (Halocarbon Laboratories, North Augusta, S. C.). Ticks were weighed, then moved onto a glass microscope slide and covered lightly with a glass coverslip to flatten the body (as much as the weight of the coverslip would do). Tick body measurements were made with the aid of a micrometer held in the eye piece of a dissecting stereo microscope. Measurements included: (1) total body length, defined as the distance on the mid-line between the anterior edge of the scutum and the posterior tip of the opisthosoma; (2) scutal length, defined as the mid-line distance between the anterior edge and the posterior tip of the scutum; (3) maximum scutal width, measured the widest point on the scutum; and (4) maximum alloscutal width, measured at the widest point on the alloscutum (see Figure 10).

Engorgement indices (EI's) were computed as the ratios between total body length and scutum length (Index 1), total body length and scutum width (Index 2), and alloscutum width and scutum width (Index 3). One-way analysis of variance was performed to compare the three scutal indices of pre-attached adult and nymphal *I. scapularis* with those of ticks removed from hosts at each 12-hour time interval (SAS Institute 1990). Duncan's multiple range tests were used to compare mean engorgement indices for each time interval. Step-wise regression analysis with true stepping (α to enter = .15, α to remove = .20) was used to evaluate relationships between the known duration of tick attachment and each engorgement index. Seventy-five percent prediction intervals (for predicting outcomes from individual tick EI's) were calculated (Runyon 1985 & SAS Institute 1990).

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To predict the duration of attachment of ticks removed by tick-bite victims, the total body length and scutum length (Index 1) was obtained from 128 adult female (101 from RI, 27 from PA) and 377 nymphal *I. scapularis* (206 from RI and 170 from PA), submitted to a commercial laboratory for detection of *B. burgdorferi* (CBR Laboratories, Boston, MA). For our analysis, we included measurements of only those ticks sent from patients residing in either Washington County, RI or Bryn Athyn (Montgomery Co), PA since these two localities represented the largest sources of ticks submitted to the laboratory. Duration of tick attachment was determined using the regression of Index 1 on length of tick attachment to animals in the laboratory.

RESULTS

Both the total body length and alloscutal width of nymphal and adult ticks increased with duration of feeding while their corresponding scutal length and width remained relatively constant (Figure 14). Actually, scutal length measurements decreased during the 72 hr observation period (nymphs--F = 101.01, d.f. = 5, 174, P < 0.05; adults--F = 5.64, d.f. = 6, 63, P < 0.05), but we believe this decrease was only in the appearance of scutal length and could be attributed to scutum tilting as the alloscutum became engorged. The angle at which the engorged tick scutum tilted was calculated using the cos^{-1} of the ratio between the measured (apparent) scutal length and the mean scutal length of 30 unfed nymphs or adults (Figure 13). Thus, the mean angle of tilt for nymphal ticks was $16.9^o \pm 7.8^o$, $24.0^o \pm 4.9^o$, and $39.3^o \pm 5.5^o$ at 36, 48, and 60 hrs of attachment, respectively, while the angle of tilt for adult ticks at 72 hrs of attachment was $24.5^o \pm 6.2^o$. Scutal widths of both nymphal and adult ticks remained unchanged throughout the entire period of observation (nymphs--F = 0.438, d.f. = 5, 174, P > 0.05; adults--F = 1.82, d.f. = 6, 63, P > 0.05).

Engorgement indices of both nymphal and adult *I. scapularis* increased with increasing duration of attachment to hamsters and rabbits. Significant overall differences were noted for both adult female (Index 1: F = 29.01, d.f. = 6, 63, P < 0.05; Index 2: F = 23.74, d.f. = 6, 63, P < 0.05; Index 3: F = 31.76, d.f. = 6, 63, P < 0.05) and nymphal ticks (Index 1: F = 216.33, d.f. = 5, 174, P < 0.05, Index 2: F = 173.09, d.f. = 5, 174, P < 0.05, Index 3: F = 160.08, d.f. = 5, 174, P < 0.05) when EI's at various time intervals of tick attachment were compared. No differences were observed in any of the nymphal or adult engorgement indices for ticks attached $\leq$ 24 hrs. However, mean EI's of nymphal ticks attached $\leq$ 24 hrs were significantly different from those attached $\geq$ 36, 48 and 60

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hrs of attachment. By 72 hrs of attachment, 78.8% of nymphal ticks were fully engorged and had actually detached from their host. For adult female ticks, mean EI values calculated using Index 1 or 3 for 0, 12 and 24 hrs of attachment were significantly different from EI's determined at 36, 48, 60 and 72 hrs of attachment. Using Index 2 we were unable to distinguish between adult ticks attached for $\leq$ 24 hrs and those attached for 36 hrs, although ticks attached for $\geq$ 48 hrs could be distinguished from those attached for shorter duration.

We used stepwise-multiple regression to determine relationships between duration of tick attachment and engorgement indices. Time of attachment explained a substantial amount of variation in each engorgement index. However, the derived equations differed for each EI and life stage (<u>nymphal ticks</u>: Index 1 = 1.37 $*10^{-7}$ *hours of attachment^4 + 1.52, $\underline{R}^2$ = 0.86, $\underline{F}$ = 1081.22, <u>d.f.</u>, = 1, 178, $\underline{P}$ < 0.001; Index 2 = 5.7 $*10^{-6}$ *hours of attachment^3 + 2.01, $\underline{R}^2$ = 0.83, $\underline{F}$ = 858.94, <u>d.f.</u>, = 1, 178, $\underline{P}$ < 0.001; Index 3 = 1.8 $*10^{-4}$ *hours of attachment^2 + 1.25, $\underline{R}^2$ = 0.82, $\underline{F}$ = 816.80, <u>d.f.</u>, = 1, 178, $\underline{P}$ < 0.001; <u>adult ticks</u>: Index 1 = 2.9 $*10^{-6}$ *hours of attachment^3 + 1.74, $\underline{R}^2$ = 0.73, $\underline{F}$ = 179.23, <u>d.f.</u>, = 1, 68, $\underline{P}$ < 0.001; Index 2 = 3.5 $*10^{-8}$ *hours of attachment^4 + 2.10, $\underline{R}^2$ = 0.68, $\underline{F}$ = 144.49, <u>d.f.</u>, = 1, 68, $\underline{P}$ < 0.001; Index 3 = 1.1 $*10^{-4}$ *hours of attachment^2 + 1.39, $\underline{R}^2$ = 0.71, $\underline{F}$ = 164.29, <u>d.f.</u>, = 1, 68, $\underline{P}$ < 0.001). For each 12 hour period we estimated the 75% prediction interval for all engorgement indices. Because we observed no significant difference in EI's for ticks attached $\leq$ 24 hours we combined prediction intervals for 0, 12 and 24 hrs (Table 8).

In addition to increasing engorgement indices with duration of attachment, both nymphal and adult ticks increased in body weight, and adult ticks changed colors as they blood-fed. The average body weights for 30 nymphs at 0, 12, 24, 36, 48, and 60 hours of

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attachment were 0.13, 0.20, 0.23, 0.33, 0.53, and 0.77 mg., respectively. Average body weight for 30 adult ticks attached for ≤ 24 hours was 1.5 mg., and body weights (n = 10) gradually increased to 2.7, 3.5, 3.8, and 6.5 mg. at 36, 48, 60, and 72 hours of attachment, respectively. In female *I. scapularis*, the alloscutum was red (10R 4/6-4/8--Munsell color charts, Kollmorgen Instruments Corp. Newburgh, NY) in unfed ticks and in ticks feeding for 12 or 24 hours. The alloscutum became yellowish red (5YR 5/6-4/6) after 36 hours and 48 hours of attachment. After 60 to 72 hours, the color of these ticks changed to light brown (7.5YR 6/4-5/4). It was difficult to measure the EI of fully-engorged ticks, but following detachment from the host, fully engorged adult females were greenish gray (5GY 5/1) to dark greenish gray (5GY 4/1). It took at least 5 days for adult female *I. scapularis* to become fully engorged, at which time their mean body weight was 261 mg.

Using the tick stage-specific regression equations for engorgement index I, we determined the duration of attachment for nymphal and adult ticks removed by tick-bite victims from communities in Rhode Island and Pennsylvania. Most people (64%, n=128) found and removed adult *I. scapularis* before 36 hrs of attachment, and by 48 hrs nearly all adult ticks were removed (79%) (Figure 15). In contrast, few people found and removed nymphal ticks by 24 hrs of attachment (10%, n=377), although more nymphs were removed by 36 hrs (41%).

DISCUSSION

Several reports have been published showing that the risk of contracting Lyme disease following a tick bite is exceptionally low, despite relatively high rates of *B. burgdorferi* infection in tick populations. Prospective studies of human infection following a tick-bite, from Connecticut, New York and West Germany, indicate a frequency of human infection with *B. burgdorferi* ranging from 1-4% (Paul et al.1986, Falco & Fish 1988, Costello et al. 1989, Nadelman et al. 1992, Shapiro et al. 1992) while the infection prevalence in ticks, at least in the northeastern U.S., generally ranges from 20-35% for nymphs and 50-75% for adults (Mather 1993). Duration of tick attachment is commonly cited as an important factor determining the probability of infection, although studies have yet to be conducted that relate the length of time that a tick feeds to the incidence of Lyme disease, or any other tick-borne infection.

Lack of a convenient, reliable, and objective measure for determining the duration of tick attachment is often cited by clinicians as a principal limitation to assessing risk of infection following a tick bite (Magid et al.1992 & Shapiro et al. 1992). The notion of using engorgement indices (EI's) to measure duration of tick feeding is not new (Obenchain et al. 1980). However, the missing parameter, for infections transmitted by *I. scapularis* (and for most other vector ticks), has been in comparing length of tick attachment with a particular index of engorgement. In this experiment, we made four simple measurements on ticks (Figure 12) assisted only by a dissecting microscope and ocular micrometer, developed regression equations for three separate EI's at relevant time intervals for both nymphal and adult ticks, and then generated a table presenting the 75% prediction intervals (Table 8). For the practitioner, only one EI need be calculated. There was no real advantage of one EI over any other in their ability to discern

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differences in length of tick attachment. We associated all three measures with their respective attachment times merely to provide flexibility in making at least one accurate measurement in the event that a particular patient's tick was damaged. Commonly, after someone removes a tick, the proximal part of the scutal plate will be missing making accurate measurement of Indices 1 and 2 difficult. In practice, we found Index 1 (total body length/scutal length) to be the simplest and most accurate EI to measure as it required no additional manipulation of the tick once it was on the microscope stage. Furthermore, both measurements for Index 1 are taken along the tick's mid-line making a subjective judgment as to the scutum's widest axis unnecessary.

In practice, it is important to compare the EI of an individual tick to the prediction interval rather than just calculating the duration of attachment from the regression equation. Determining tick attachment time from the regression line alone affords no idea of a given prediction's accuracy for individual ticks removed from patients since it fails to consider errors inherent in fitting the line, or in the population variance. Moreover, confidence intervals that would typically be calculated by standard statistical software packages are only useful in describing the accuracy of the fitted regression line for a population and are inappropriate for predicting the duration of attachment by an individual tick. The prediction intervals calculated herein (Table 8) include consideration of the population variance for tick engorgment around the best-fit regression line.

Animal studies suggest that risk for *B. burgdorferi* transmission is low within the first 24 hours of attachment but increases thereafter. Rodents infested with *B. burgdorferi*-infected nymphal *I. scapularis* for 36 hrs showed a 7% risk of infection, those attached for 48 hrs >50 % risk, while those infested for 72 hrs were universally infected (Piesman et al. 1987 & Piesman 1993). Similarly, adult *I. scapularis* feeding for ≤ 36 hours failed to infect rabbits, whereas two of three rabbits became infected when ticks were attached

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for 48 hours, and five of five rabbits were infected when attachment was >120 hours (Piesman et al.1991). Variability in tick feeding rates in relation to spirochete transmission among different tick hosts has not been determined making it impossible to know if findings from these animal studies are applicable to humans. Similarly designed studies utilizing a wider array of animal hosts may provide some evidence toward the degree of interspecies variation that exists in tick feeding rates.

We propose that the true likelihood of becoming infected can be predicted by (1) the duration of tick attachment, (2) the tick "infection rate", and (3) the efficiency of transmission at a particular stage of tick attachment. In our study, 10% of attached nymphs were removed in $\leq$ 24 hours, 41% in $\leq$ 36 hrs, 65% in $\leq$ 48 hrs, and 87% in $\leq$ 60 hrs. Likewise, 64% of adult ticks were removed in $\leq$ 36 hrs, 79% in $\leq$ 48 hrs, 84% in $\leq$ 60 hrs, and 87% in $\leq$ 72 hrs. Assuming nymphal tick infection rates between 20-35% and transmission efficiencies of 7%, 50% and 100% at 36 hrs, 48 hrs and >60 hrs, respectively, the probability of infection following a nymphal tick bite would likely range from 0.10 where *B. burgdorferi* prevalence in ticks is low to 0.17 where prevalence in ticks is high. Similarly, assuming adult tick infection rates between 50-75% and transmission efficiencies of 0%, 66% and 100% at 36 hrs, 48-72 hrs and > 72 hrs, respectively, risk for human infection following an adult tick bite would likely range from 0.14 where prevalence in ticks is low to 0.21 where prevalence is high. The infection probabilities calculated herein are slightly higher than those observed in the few previous clinical studies available, but tick infection rates or the study participants' tick-finding habits may have differed somewhat from those generalizable to the Rhode Island and Pennsylvania communities reported on here. Moreover, we used transmission efficiencies from animal studies in making our calculations, which may not be quite the same for humans.

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The above model suggests that infection risk is about the same for people bitten by either nymphal or adult ticks. However, numerous epidemiological studies suggest that many more cases of Lyme disease are acquired in the summer when nymphal ticks are most abundant (Piesman et al. 1987, Fish 1993). It may be that human exposure to nymphs is greater making nymph bites more common, as this life stage can be more abundant than adult ticks by an order of magnitude (Fish 1993). Moreover, peak abundance of nymphs during the summer coincides with higher levels of human activity outdoors than during the late fall and early spring when adult *I. scapularis* are most abundant (Piesman et al.1987). Since our findings suggest that the risk of infection following any deer tick bite is similar, it is likely that people are bitten more frequently by nymphs than by adults.

Prophylactic antibiotic treatment of tick bites continues to be controversial, particularly in areas where Lyme disease occurs. Proponents of prophylactic therapy argue that even though *B. burgdorferi* and *B. microti* are mainly transmitted in the latter stages of tick feeding, earlier transmission of a small number of organisms has not been ruled out (Liegner 1990). Moreover, decision analysis has suggested that empirical treatment ("treat all") be recommended when the probability of infection is above 0.01 (Magid et al. 1992). In contrast, opponents of prophylactic treatment cite the apparent low probability of infection even after tick bite and risk of adverse drug reactions in defending their position. In addition, inappropriate tetracycline treatment of a tick-bite leading to Rocky Mountain spotted fever could prove problematic as such treatment may postpone the onset of symptoms perhaps leading to a delay in diagnosis and proper treatment of this serious tick-transmitted infection (Weber & Walker 1991). In this study, we developed a simple and objective means for determining the duration of tick attachment. Since attachment of nymphs for less than 36 hrs and adult ticks for less than

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48 hrs pose relatively little risk, at least for transmission of Lyme disease spirochetes, prophylactic therapy reasonably could be reserved for those patients presenting with a deer tick whose EI suggests a longer period of attachment. Additional testing, to detect infection in the tick, would provide an even better estimate of the infection probability, and thus, is also recommended.

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Appendix I. The activation scheme of the classical and alternative pathways of complement components by the Lyme disease spirochete.
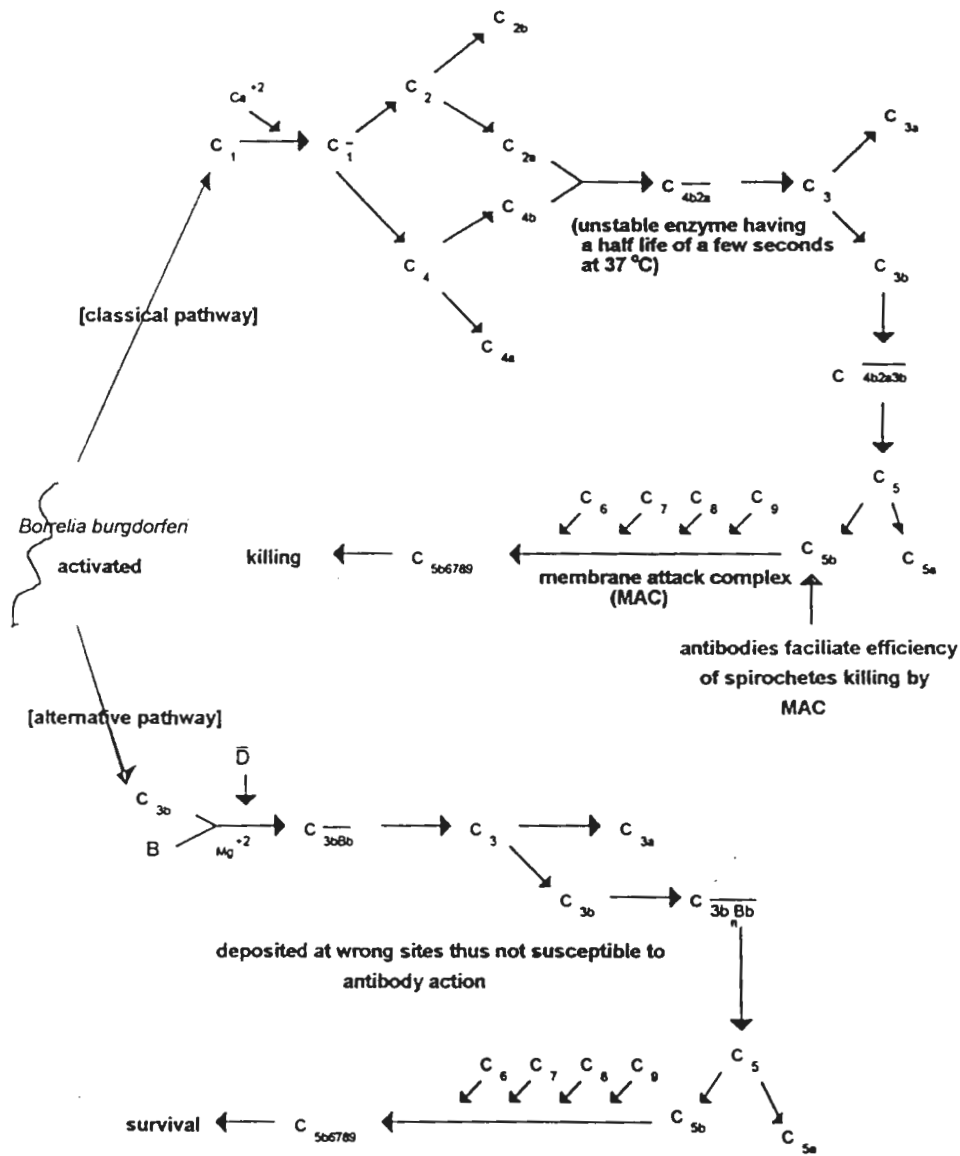
Appendix II. A simplified schematic representation of cellular and humoral immunity of insect responses to pathogens.

pathogen (LPS, B 1,3 - glucan,...)

cytophilic agglutinin

humoral agglutinin

spontaneous activation (low Ca^{+2} conc.)

granules (may contain prophenoloxidase and inactive serine protease)

5-glycosyl transferase
(recognition factor subunit)

serine protease

peptide

active serine protease

polymerize

prophenoloxidase → phenoloxidase

peptide

hexamers

phenol derivatives

attach to molecular foreigner

quinone or melanin

fills in the gaps in the 3-D network

cross linking protein

into 3 diminisional network

sheath

chemotaxis
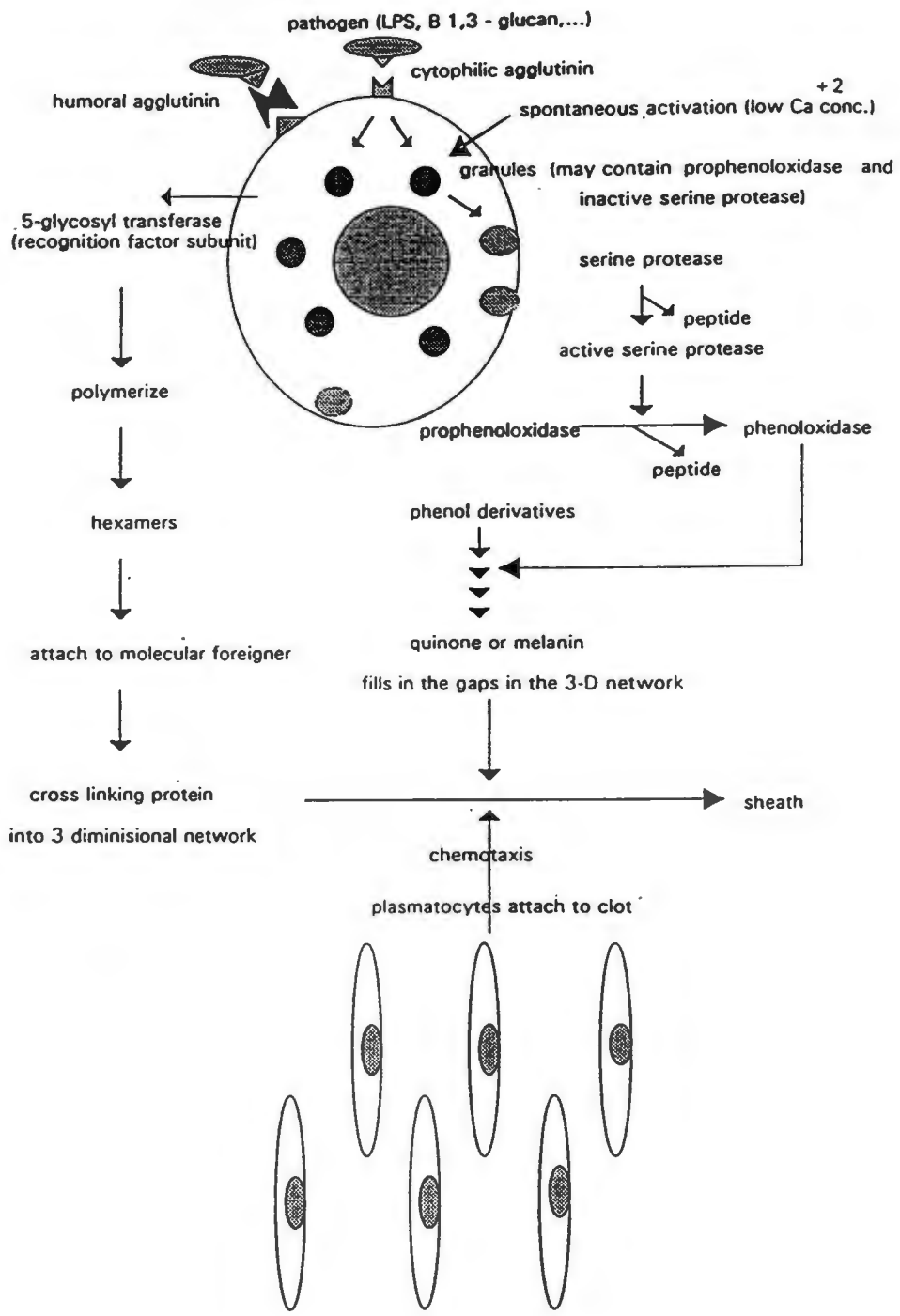
plasmatocytes attach to clot

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Table 1. *Borrelia burgdorferi* infection in nymphal ticks derived from larvae fed simultaneously on a laboratory infected New Zealand white rabbit.

Species	No. examined	Days required for molting	% Infection rate
Ixodes scapularis	50	18 - 19	82.0 %
Dermacentor variabilis	62	6 - 7	0.0 %
Amblyomma americanum	51	7 - 8	0.0 %

Table 2. Experiments demonstrating complement-mediated killing of *Borrelia burgdorferi* (strains B31 and T15) *in vitro*.

Treatment	spirochete survival	
	B31	T15
5. Pre- immune rabbit serum	++	++
6. Immune rabbit serum	--	--
7. Heat-inactivated immune rabbit serum	++	++
8. Tr. #5 + Tr. #7	--	--

* Controls in each treatment: BSK II + spirochetes.

Table 3. Spirochete survival rate and antibody titer in heated and non-heated rabbit serum.

Treatment of serum	Spirochete survival (%)	Antibody titer (x)
56°C, 1hr	100	2048
---	0	2048

Table 4. Experiments demonstrating borreliacidal activity of host serum components in tick midguts

Treatment	spirochete survival		
	I. scapularis	*D. variabilis*	*A. americanum*
1. Tick gut extract from pre-immune rabbit	++	++	++
2. Tick gut extract from immune rabbit	++	--	--
3. Immune rabbit serum + Tr #1	++	--	--
4. Tr.# 2 but heat-inactivated	++	++	++

* Controls for each treatment: PBS + spirochetes.

Table 5. Anti-*Borrelia burgdorferi* antibody titer of the rabbit host and of the tick midgut extract.

Time	Antibody titer (x)			
		In midgut extracts from engorged		
	Rabbit	*I. scapularis*	*D. variabilis*	*A. americanum*
1st feeding				
0 wk	32	32	32	32
2 wk	512	--	--	--
3wk	2048	--	--	--
4wk	1024	--	--	--
2nd feeding				
6wk	4096	4096	4096	4096

Table 6. The effect of temperature on the days for molting and spirochete infection rate in
derived nymphs of three species ixodid ticks.

Treatment	Species	No.	Molting (Days)	Infection rate (%)
18°C	I. scapularis	46	41-42	87.0
	D. variabilis	40	30-31	0.0
	A. americanum	57	36-37	1.8
23°C	I. scapularis	57	29-30	94.7
	D. variabilis	60	15-16	0.0
	A. americanum	60	17-18	0.0
28°C	I. scapularis	48	20-21	95.8
	D. variabilis	42	9-10	0.0
	A. americanum	32	10-11	0.0
33°C	I. scapularis	50	18-19	82.0
	D. variabilis	62	6-7	0.0
	A. americanum	51	7-8	0.0

Table 7. The comparison of phenoloxidase activity in the hemolymph of *G. mellonella* and 50 % w/v homogenate of *I. scapularis*, *D. variabilis*, and *A. americanum*, after 30 minutes incubation on ice.

Scientific name	No.	Total of Body Weight (g)	Phenoloxidase Activity
G. mellonella	6	1.494	++
I. scapularis	30	0.048	--
D. variabilis	30	0.206	--
A. americanum	30	0.107	--

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Table 8. Seventy-five percent prediction intervals of nymphal and adult *Ixodes scapularis* engorgement indices for predicting duration of tick attachment. (Index 1 = body length/scutal length; Index 2 = body length/scutal width; Index 3 = alloscutal width/scutal width).

Tick Stage	Duration of attachment (hrs)	Engorgement Indices		
		Index 1	Index 2	Index 3
Nymph	0-24	1.46 - 1.62	1.91 - 2.18	1.18 - 1.43
	36	1.57 - 1.89	2.12 - 2.44	1.36 - 1.62
	48	1.88 - 2.50	2.32 - 2.96	1.54 - 1.82
	60	2.56 - 3.74	2.83 - 3.67	1.69 - 2.15
Adult	0-24	1.67 - 1.87	1.98 - 2.25	1.30 - 1.53
	36	1.72 - 2.04	1.98 - 2.34	1.42 - 1.64
	48	1.93 - 2.21	2.12 - 2.46	1.58 - 1.70
	60	2.01 - 2.73	2.20 - 2.92	1.64 - 2.02
	72	2.17 - 3.49	2.47 - 3.63	1.69 - 2.21

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Figure 1. Lyme disease spirochete survival rate in immune rabbit serum (titer: 2048X) at 0.5 hour intervals after incubation from 0 to 7 hours at 33 °C.
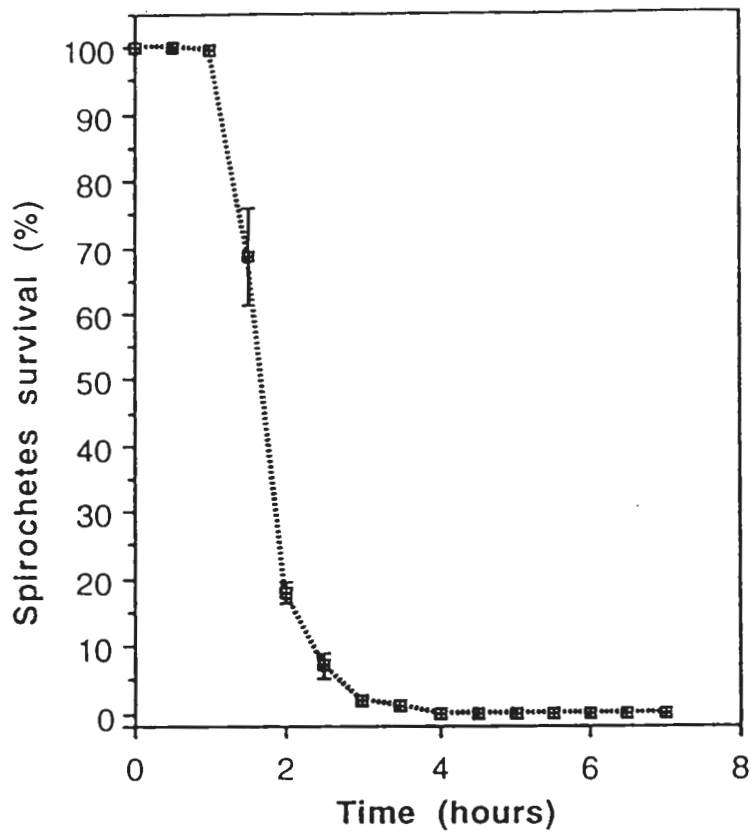
Figure 2. Lyme disease spirochete survival in dilutions of immune serum, and in dilutions of heat-inactivated immune serum containing undiluted pre-immune serum (source of complement) or dilutions of pre-immune serum containing undiluted but inactivated immune serum (sources of antibodies).

○······ Immune serum

△··· Pre-immune serum in the presence of undiluted heat-inactivated immune serum

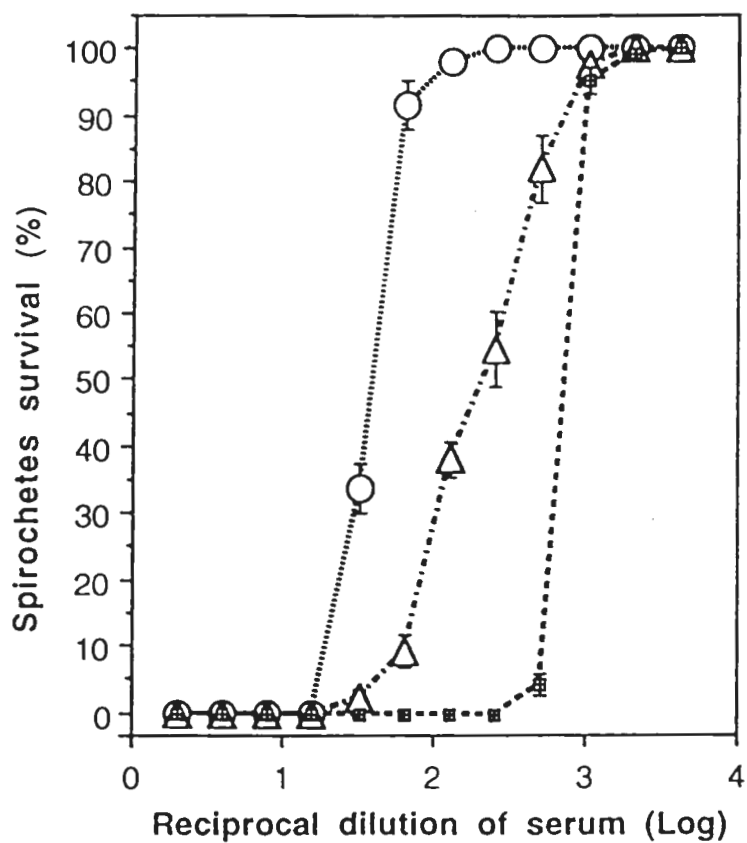⊞··· Heat-inactivated immune serum in the presence of undiluted pre-immune serum

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Figure 3. Spirochete survival in the presence of immune serum and cobra venom, or the saliva from *Ixodes scapularis*, *Dermacentor variabilis*, and *Amblyomma americanum*.
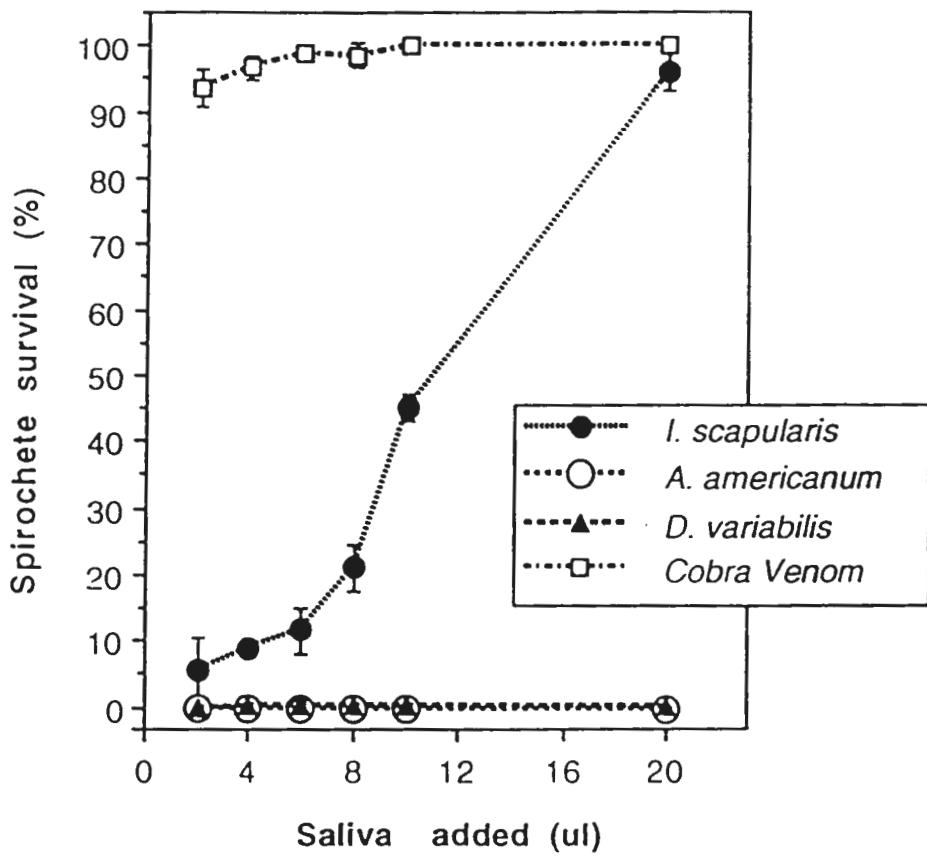
Figure 4. Comparison of spirochete concentrations to original treatment, including *Borrelia burgdorferi* (control), and *B. burgdorferi* mixed with the gut homogenates of *I. scapularis*, *D. variabilis*, and *A. americanum* at 18°C, 23°C, 28°C, and 33°C.
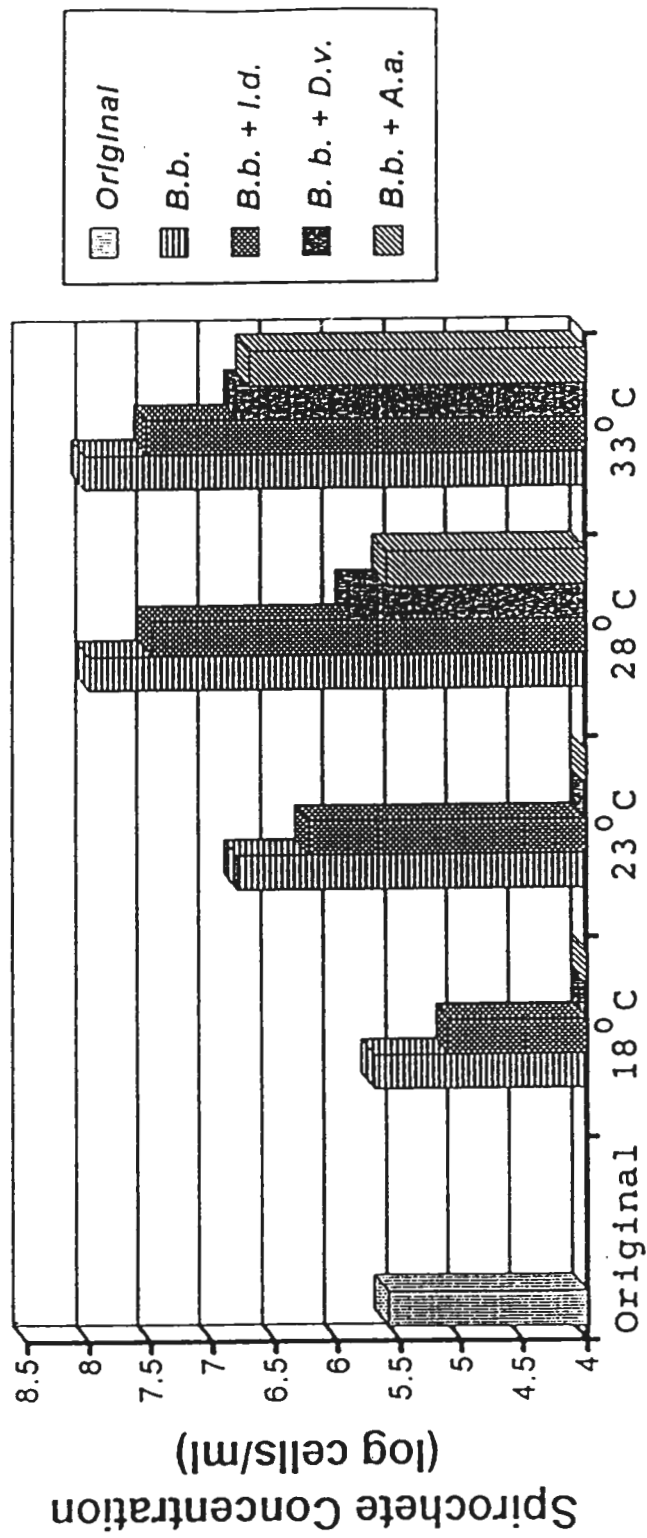
Figure 5. Time course of phenoloxidase activity in the hemolymph of the larvae of *G. mellonella* incubated on ice at different time intervals.

$$y = -1.4760e\text{-}2 + 3.3965e\text{-}2x - 4.5121e\text{-}4x^2 + 2.0705e\text{-}6x^3 \quad R^2 = 0.989$$
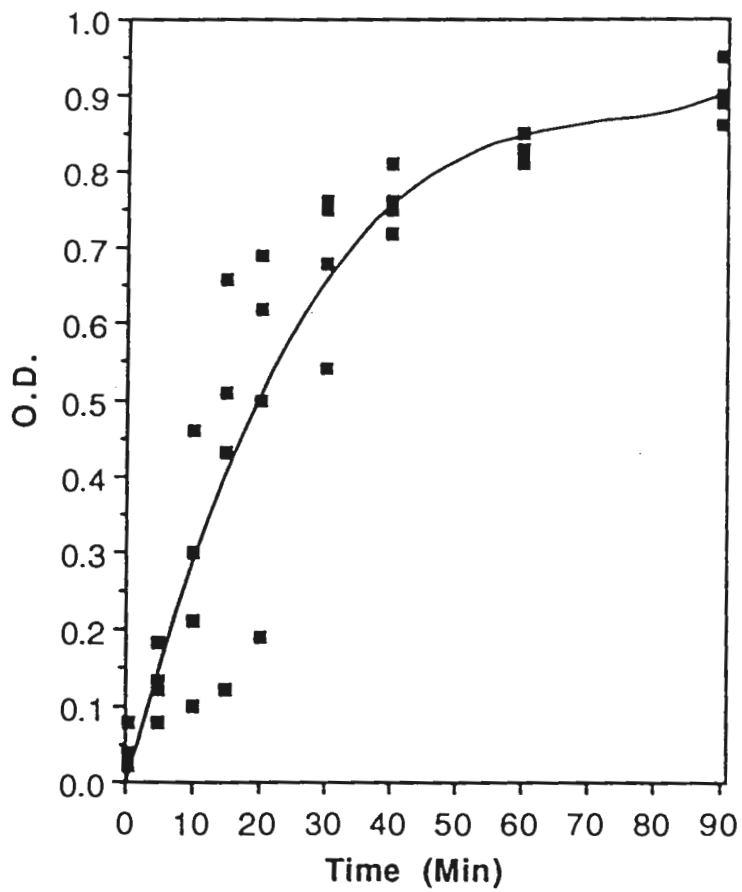
Figure 6. Prohemocytes of *I. scapularis* with nuclei (N) occupying almost all of each cell (Nomarski). Measurement bar, 10 um.

Figure 7. Plasmatocyte of *I. scapularis*, pseudopodia are shown by the arrow (phase contrast). Measurement bar, 10 um.

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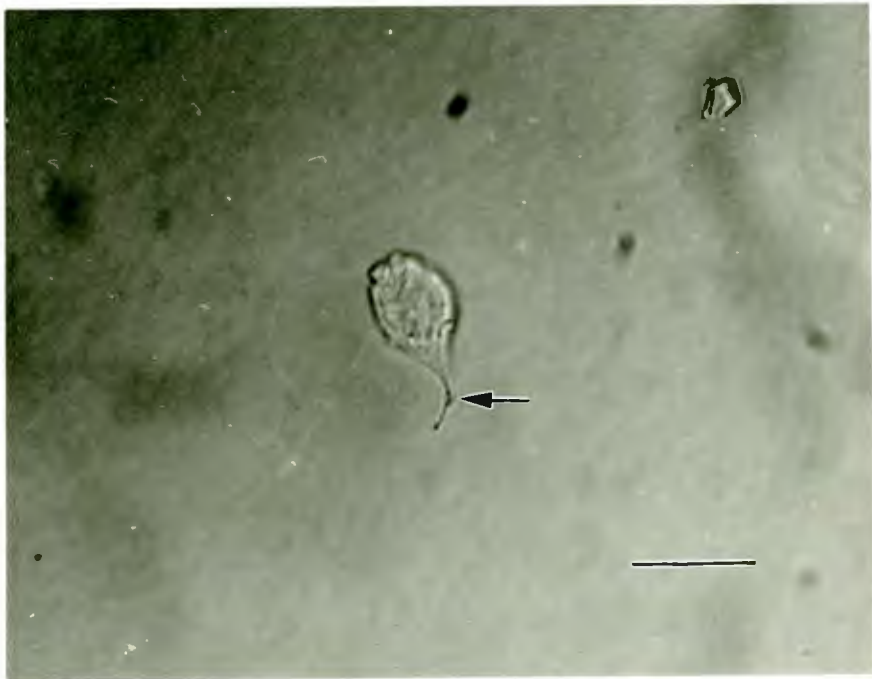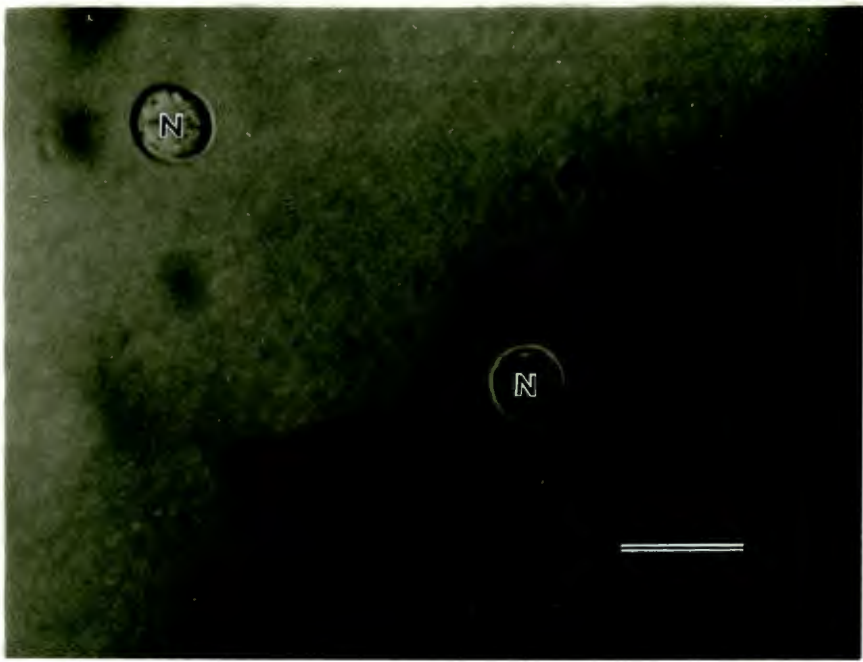
Figure 8. Granulocyte (G) of *I. scapularis*, pseudopodia (P) are shown by the arrow (phase contrast). Measurement bar, 20 um.
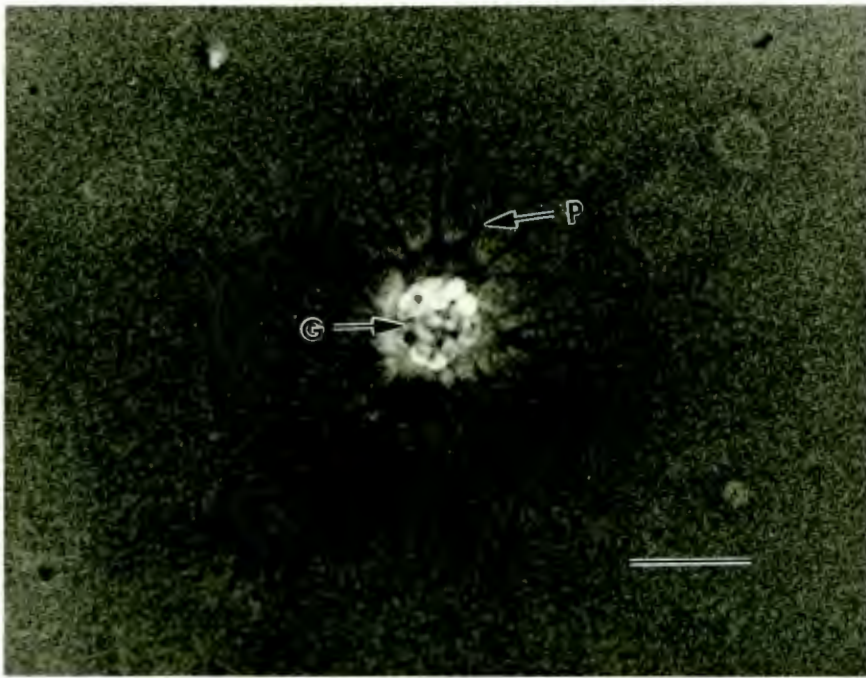
Figure 9. The processes of releasing granules from granulocyte, the granular inclusions were in the cytoplasm on A (Nomarski), but were gradually released granules on B (phase contrast). Measurement bar, 10 um.
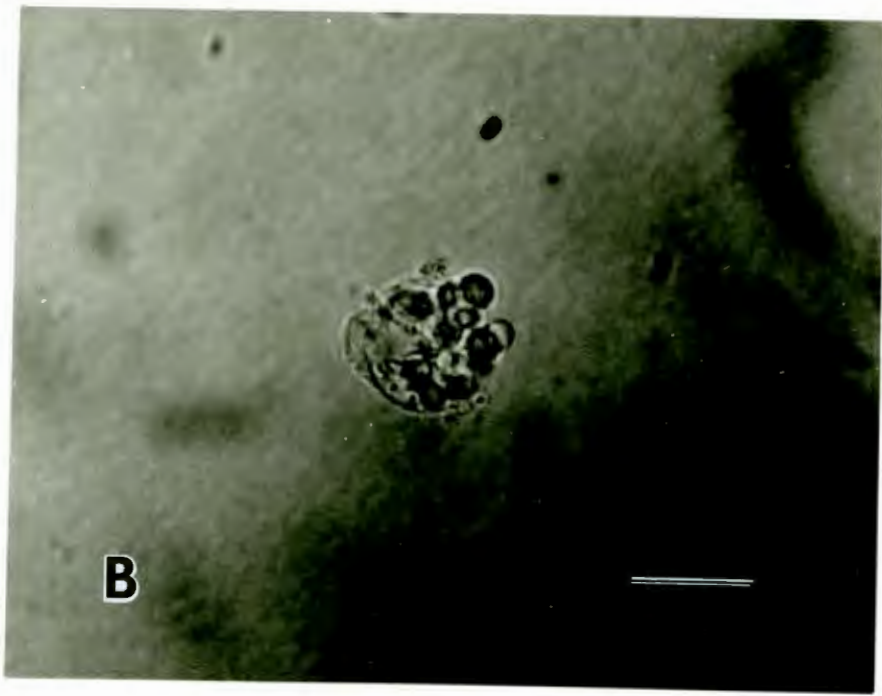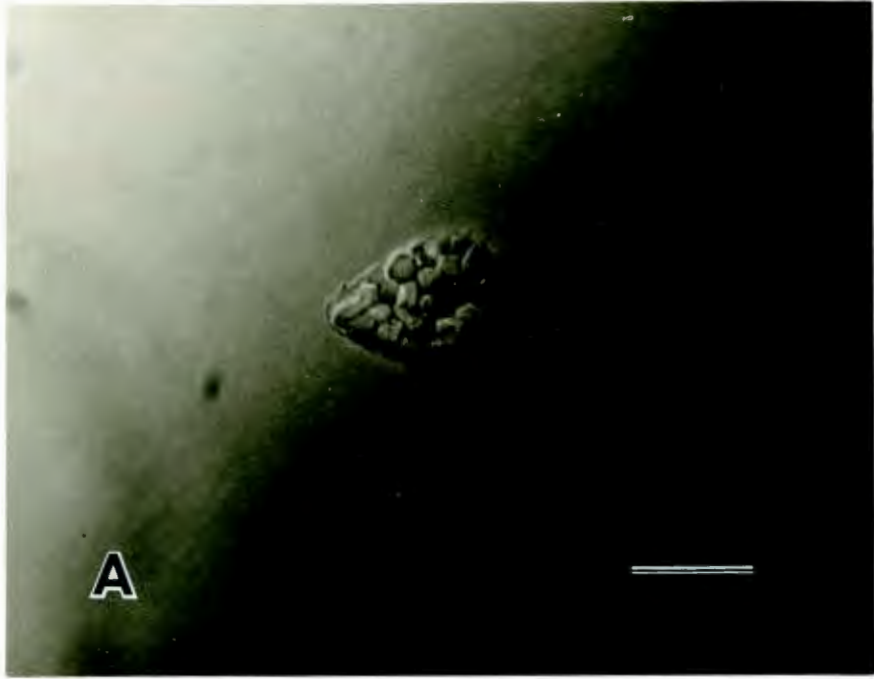
Figure 10. Diagram illustrating external body parts of *Ixodes scapularis* used for measuring engorgement indices 1-3. (SL = scutal length; BL = body length; SW = scutal width; BW = alloscutal (body) width).

Scutum

Alloscutum

SL

BL

SW

BW
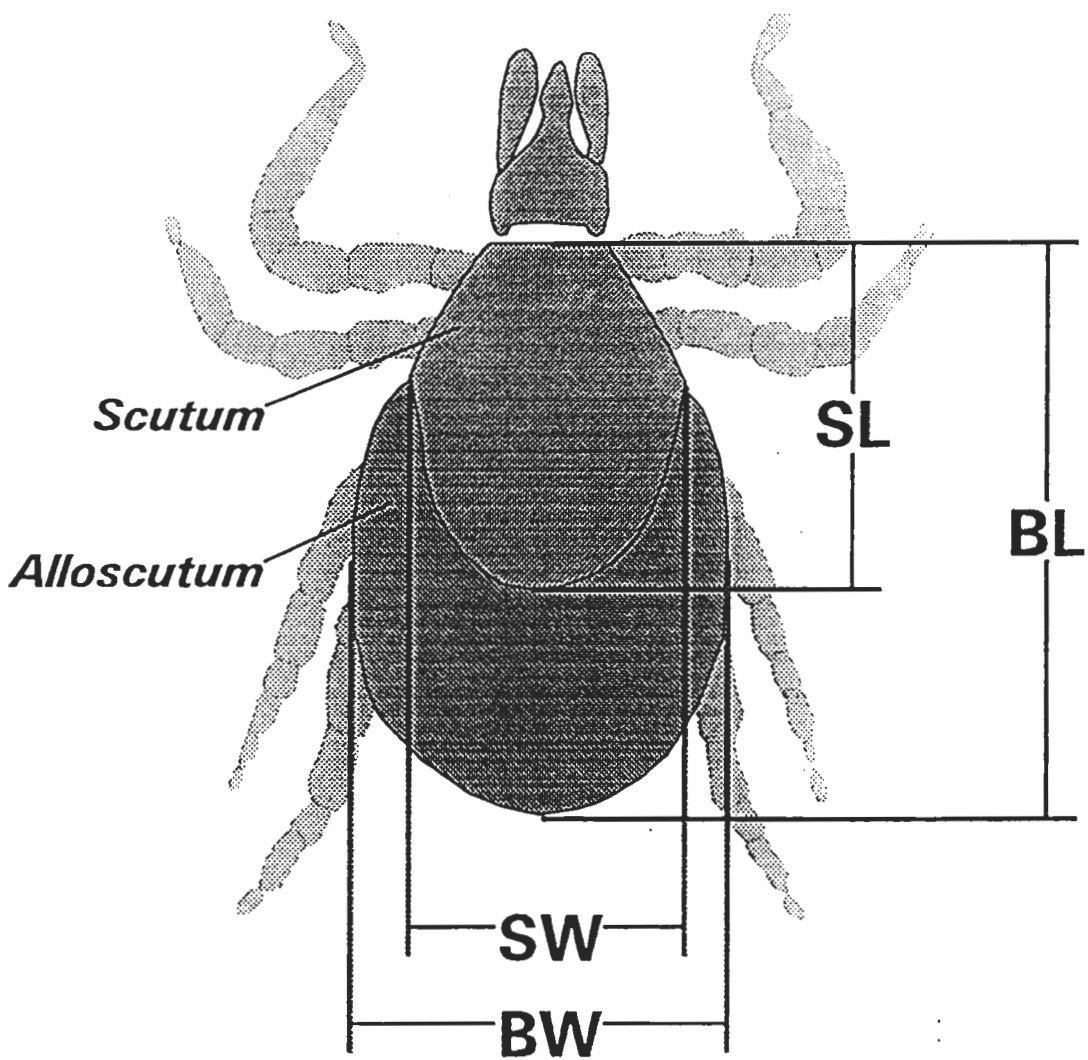
Figure 11. Diagrammatic representation of the tilted scutum on engorged tick body (0 = tilted angle, M = measured apparent scutal length, and A= acutal scutal length).

$$\cos^{-1} \frac{M}{A} = \theta$$

engorged tick
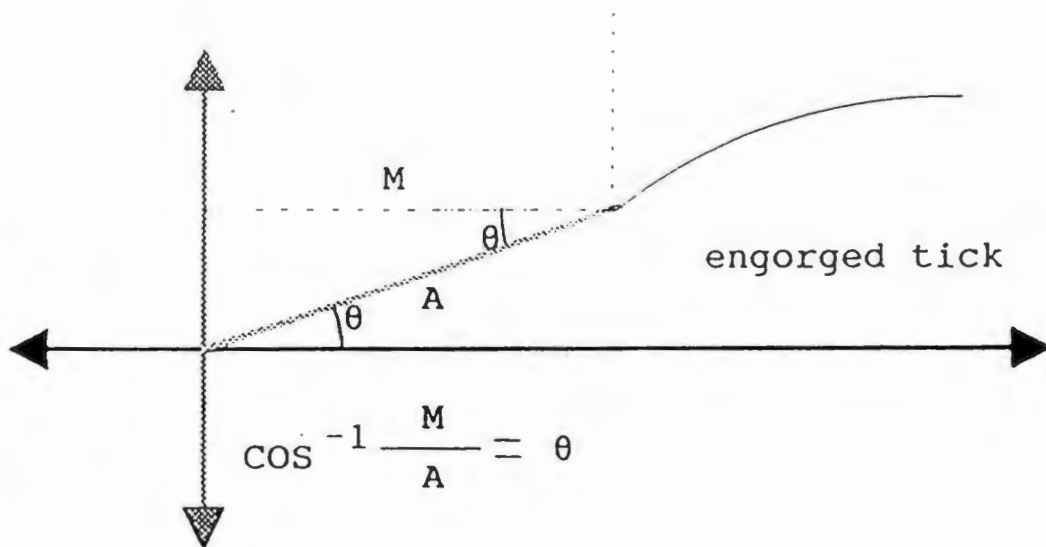
Figure 12. Comparison of mean ($\pm$ s.d.) external body measurements of *Ixodes scapularis* nymphs (A) and adults (B) attached to laboratory hosts for varying lengths of time.

A

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B

Mean body measurement (mm)

Legend:
- S.W. (△)
- S.L. (■)
- B.W. (○)
- B.L. (■---)

Duration of attachment (hrs)
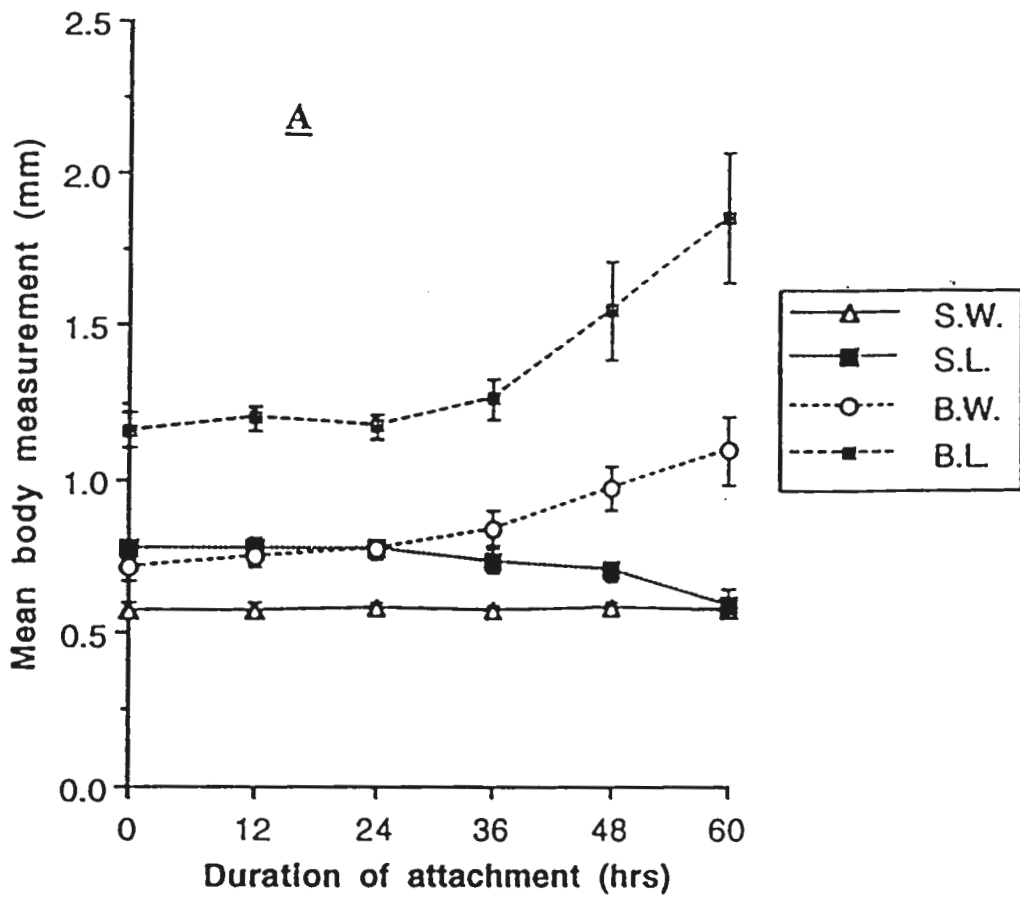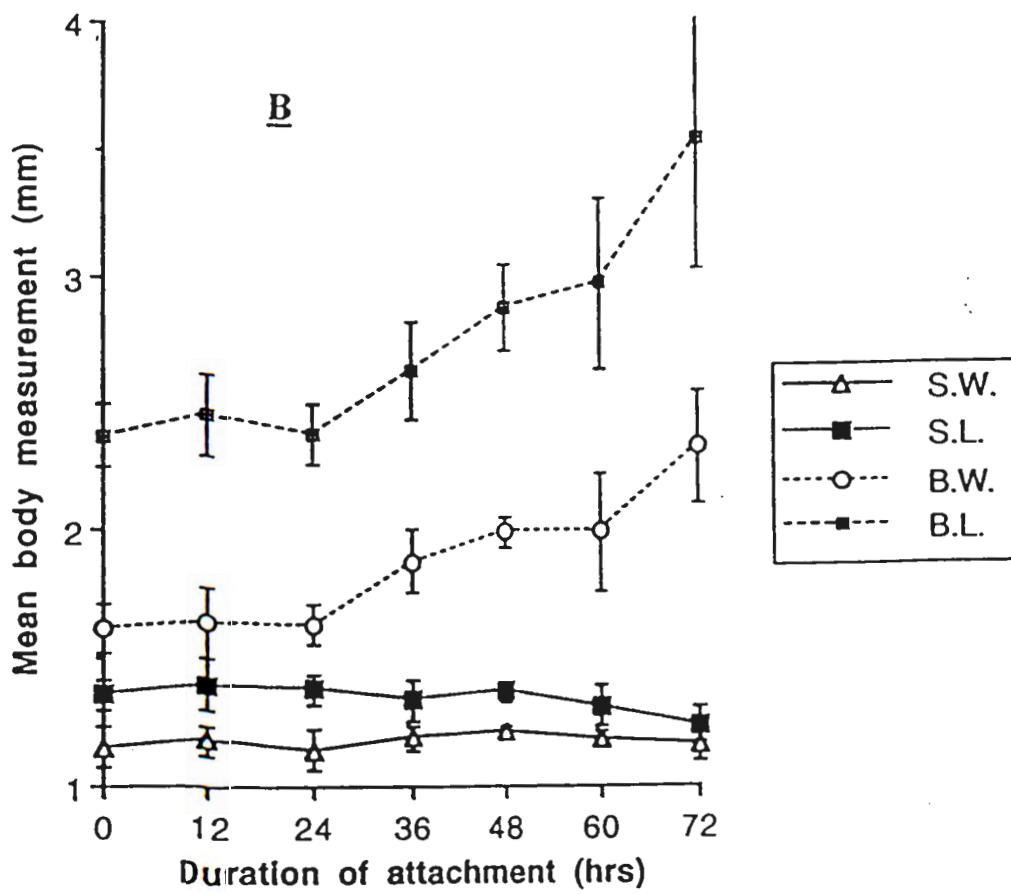
Figure 13. Cumulative frequency distribution (%) showing the duration of nymphal and adult *Ixodes scapularis* attachment to human hosts in Rhode Island and Pennsylvania.
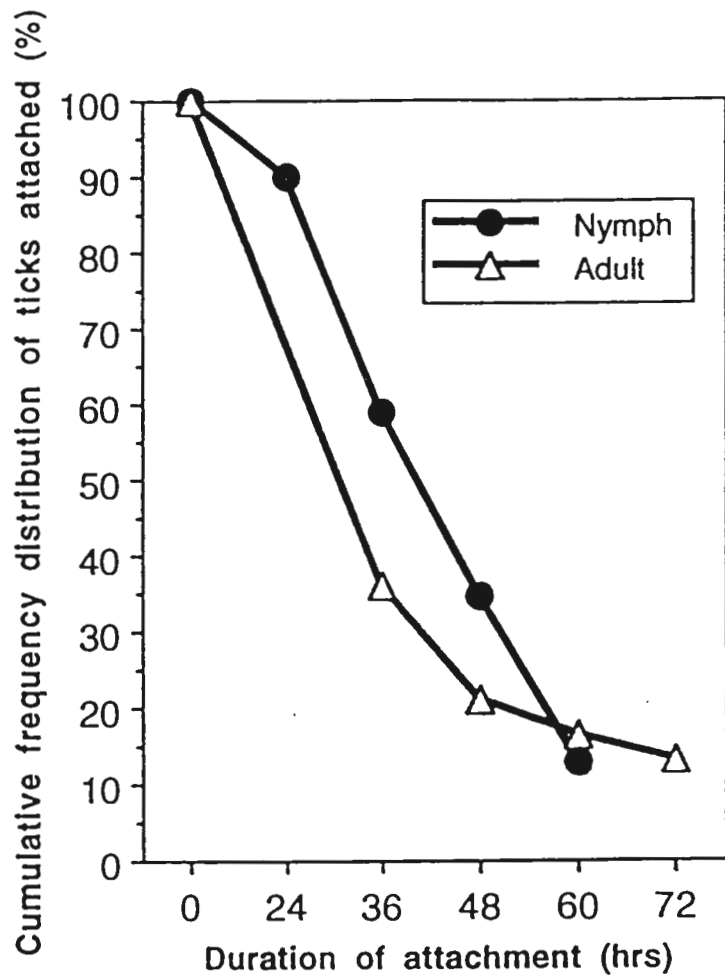
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