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Infestation with Pathogen-Free Nymphs of the Tick *Ixodes scapularis* Induces Host Resistance to Transmission of *Borrelia burgdorferi* by Ticks

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Female BALB/c mice were infested four times with pathogen-free *Ixodes scapularis* nymphs prior to infestation with nymphs infected with *Borrelia burgdorferi* B31. Each infestation was separated by a 14-day tick-free period. Mean weights of fed ticks and percentage reaching repletion did not indicate development of acquired resistance. Only 16.7% of mice repeatedly infested with pathogen-free ticks prior to infected *I. scapularis* nymph challenge became positive for *B. burgdorferi*. One hundred percent of control mice infested only with infected ticks were culture positive for *B. burgdorferi*.

The tick-host-pathogen interface is characterized by dynamic interactions among all three elements of the relationship. Tick feeding induces a complex array of host immune regulatory and effector responses involving antigen-presenting cells, cytokines, immunoglobulins, complement, and T lymphocytes (1, 6, 24, 27, 29). Immunologically based acquired resistance to ticks reduces engorgement, egg production, and viability (24, 25). In turn, ticks have developed countermeasures to host immune defenses (18, 25–28). Tick-induced host immunosuppression reduces both T-lymphocyte in vitro responsiveness to mitogens (23, 25) and antibody production (26) and impairs the elaboration of macrophage and Th1-lymphocyte cytokines (18). Tick modulation of host immune defenses likely facilitates tick feeding and transmission of vector-borne disease-causing agents (27, 28).

Transmission of *Borrelia burgdorferi* by the tick vector results in a different array of host immune responses than needle inoculation of the spirochetes (13, 21). Antibodies to the outer surface proteins (Osp) A and B did not develop in animals infected by tick transmission of *B. burgdorferi*, while animals infected by needle inoculation of cultured spirochetes developed high titers of antibodies to both proteins. The antibody response to needle inoculation of *B. burgdorferi* is dependent upon the number of spirochetes inoculated (3). Administration of greater than 10^4 spirochetes to mice induces an anti-OspA response, while needle inoculation of fewer viable spirochetes did not stimulate the production of OspA-reactive immunoglobulins for up to 2 weeks postinjection.

B. burgdorferi, causative agent of Lyme borreliosis, is transmitted by ticks of the genus *Ixodes*, and rodents serve as reservoir hosts (2, 14). *Ixodes scapularis* is the primary vector of Lyme borreliosis in the eastern and central United States and *Ixodes pacificus* is the primary vector in the western United States (2, 14, 15).

Canines experimentally infested with adult *I. scapularis* developed acquired resistance, which reduced tick viability and oviposition with each successive exposure (11). Acquired resistance to the vector might reduce transmission of spirochetes. *Clethrionomys glareolus* (bank vole), a natural host of *Ixodes ricinus*, which is the most important European vector of Lyme borreliosis, acquired resistance to *I. ricinus* with repeated infestations (10). Acquired antitick resistance of *C. glareolus* reportedly interferes with *I. ricinus* transmission of *B. burgdorferi* (9).

Acquired resistance to tick infestation has been shown to alter transmission of another tick-borne pathogen, *Francisella tularensis* (4). Rabbits pre-exposed to pathogen-free *Dermacentor andersoni* were partially protected when exposed to *F. tularensis*-infected nymphs. Those rabbits infested with pathogen-free *D. andersoni* also had heightened delayed cutaneous reactivity upon intradermal inoculation of homogenates of whole tick larvae, nymphs, or adults, indicating cell-mediated immune reactivity to tick antigens (4).

The objective of this study was to determine whether repeated infestation with pathogen-free *I. scapularis* nymphs alters the subsequent transmission of *B. burgdorferi* by infected nymphs. Toward this aim, laboratory-reared nymphal *I. scapularis* ticks used in this study were obtained from a colony established in the Department of Entomology, Oklahoma State University, Stillwater. Ticks were maintained in cotton-plugged vials held over water in a desiccator at 15°C with a 14:10 (light to dark) photoperiod. Laboratory colonization involved feeding larvae and nymphs on BALB/c mice and feeding adults on guinea pigs. Host animals were fed a commercial diet and water ad libitum while being housed at 22°C in the Laboratory Animal Resources facility, Oklahoma State University. *I. scapularis* ticks were infected with the B31 strain of *B. burgdorferi* as previously described (16). Only feedings that resulted in a ≥80% infection rate in unfed nymphal ticks on darkfield examination were included in this colony.

Experiments were initiated with 10- to 12-week-old female BALB/c mice weighing between 20 and 25 g (Jackson Laboratories, Bar Harbor, Maine). Mice were maintained at 22°C and fed a commercial diet and water ad libitum.

This study was repeated twice. The first experiment consisted of 6 mice each in both the experimental and control groups, and the second experiment utilized 12 mice in each

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group. The experimental group was exposed to four infestations with pathogen-free *I. scapularis* nymphs, followed by a fifth infestation with *B. burgdorferi* B31-infected *I. scapularis* nymphs. The first three infestations were with 10 nymphs per animal, while the fourth pathogen-free infestation consisted of 8 nymphs per mouse. The infected-tick challenge was with six nymphs per mouse. Control mice received one infestation with *B. burgdorferi* B31-infected *I. scapularis* nymphs. Both experimental and control mice were infested with spirochete-infected ticks at the same time. Each infestation was allowed to proceed until the nymphs engorged and detached or for 10 days, at which time ticks were removed. A 14-day tick-free period was maintained between each infestation. Each tick-free period began 10 days after initiation of the preceding infestation, regardless of the time when ticks were removed from the host. All pathogen-free or *B. burgdorferi*-infected ticks used in a given experiment were derived from a common pool of ticks which were handled in a similar manner.

The percentage of ticks completing feeding and the mean engorgement weights were determined for each infestation group. An ear punch biopsy was obtained from each mouse, 4 weeks after termination of the infestation with infected ticks, and cultured for spirochetes as described below. The surface of the ear was swabbed with 70% ethanol and allowed to air dry before a sterile punch biopsy was obtained. Tissue was placed into sterile Barbour-Stoenner-Kelly medium according to the method of Sinsky and Piesman (22). Cultures were maintained at 34°C and examined for spirochetes by darkfield microscopy beginning on the 3rd day and ending on the 14th day of culture.

B. burgdorferi infections in *I. scapularis* ticks, which molted to adults, were detected and densities were estimated by a two-monoclonal antibody (MAb) OspA enzyme-linked immunosorbent assay (ELISA), which was modified slightly from one described previously (7). Briefly, wells of a polyvinylchloride ELISA plate were coated overnight at 4°C with 0.17 μg of MAb H5332 in 50 μl of phosphate-buffered saline (PBS). All subsequent steps were performed at room temperature. The next morning, wells were washed three times with a solution containing PBS and 0.05% Tween 20 (PBS-T) before being blocked with 200 μl of 2.5% nonfat dry milk blocker (Bio-Rad Laboratories, Richmond, Calif.) in PBS-T for 1 h (blocking solution). Nymphs were prepared for ELISA by homogenization in 50 μl of 0.25% Nonidet P-40 in PBS after which PBS-T was added to a final volume of 300 μl. After the wells were washed as described above, 50 μl of tick homogenate was added to each well for 90 min. Wells were again washed, and 8 ng of MAb 1-15 (a gift from Pfizer Animal Health) conjugated to horseradish peroxidase in 50 μl of blocking solution was then added to each well for 1 h. MAb 1-15 reacts with a nonprotective epitope of *B. burgdorferi* OspA. Wells were washed again prior to addition of 100 μl of 2,2′-azino-bis(3-ethylbenzthiazoline-sulfonic acid) per well. A_{405}s were read after 1 h. Spirochete numbers were estimated by comparing absorbances generated from the culture samples with a standard curve of known low-passage B31 spirochetes.

Statistical significance was determined by analysis of variance followed by mean separation by the Newman-Keuls method (12) or by chi-square.

The percentages of nymphs feeding to repletion (± standard deviation) were determined for ticks collected from mice either infested four times with pathogen-free ticks or uninfested prior to challenge with *B. burgdorferi*-infected *I. scapularis* nymphs. In the first experiment, the percentage of ticks feeding to repletion recovered after each of the repeated infestations did not differ significantly ($P < 0.05$, Newman-Keuls method, 35 degrees of freedom) among each other or from ticks feeding on control mice. Percentages (± standard deviation) of ticks feeding to repletion for the first through fourth infestations with pathogen-free ticks were 65.0 ± 10.5, 50.0 ± 6.3, 81.7 ± 11.7, and 52.1% ± 20.0%, respectively. The percentages of infected ticks feeding to repletion among the fifth infestation of the experimental group and the first infestation of control mice were 66.7 ± 23.6 and 55.6% ± 32.8%, respectively. During the second experiment, the percentages of *B. burgdorferi*-infected ticks completing feeding during the fifth infestation of repeatedly infested mice and during infestation of the previously uninfested mice were significantly less than ($P < 0.05$, 69 degrees of freedom) the percentages of pathogen-free ticks feeding to repletion during each of the initial four exposures of the previously infested group. Repletion percentages for the first four infestations of the experimental group were 56.7 ± 21.0, 60.0 ± 23.0, 55.0 ± 15.7, and 61.5% ± 21.0%, respectively. The repletion percentage of infected ticks for the experimental group was 23.6% ± 26.1%, and for the control mice it was 26.7% ± 21.1%.

TABLE 1. Postinfestation *B. burgdorferi* infection status of *I. scapularis* nymphs determined by OspA antigen capture ELISA

Group	No. of infected ticks/no. of ticks tested[a]	
	First expt	Second expt
Pathogen-free ticks	0/7	0/22
B. burgdorferi-infected ticks		
Previously infested hosts	5/7*	1/6**
Previously uninfested hosts	6/6*	9/9**

[a] *, chi-square = 0.43, 1 degree of freedom; $P = 0.5142$. **, chi-square = 7.81, 1 degree of freedom; $P = 0.0052$.

Mean engorgement weights in milligrams (± standard deviation) were determined for ticks obtained from each mouse at the end of every infestation. Mean engorgement weights decreased significantly ($P < 0.05$, 35 degrees of freedom) with successive infestations during the first experiment but not during the second experiment. Mean engorgement weights for the first experiment for pathogen-free tick exposures one through four were 3.0 ± 0.3, 2.5 ± 0.3, 2.5 ± 0.3, and 1.9 mg ± 0.5 mg, respectively, while for the infected-tick infestation (fifth exposure) mean engorgement weight was 1.9 mg ± 0.5 mg. Mean engorgement weight for the infected ticks infesting the control mice was 1.9 mg ± 0.2 mg.

Ear punch biopsies from mice that became infected after infestation with *B. burgdorferi*-infected ticks were positive for spirochetes by the fourth day of culture in BSK-II medium. During both experiments, repeated infestation with pathogen-free *I. scapularis* nymphs resulted in resistance to subsequent acquisition of *B. burgdorferi* infection when mice were fed upon by infected ticks. During both the first and second experiments 16.7% of mice previously infested with pathogen-free ticks became infected when given a challenge infestation with *B. burgdorferi*-infected *I. scapularis* nymphs. Ear punch biopsies of 1 of 6 and 2 of 12 mice were BSK II culture positive at 4 weeks postinfestation with infected ticks during the first and second experiments, respectively. During both experiments, 100% of control mice infested with only infected ticks became infected with *B. burgdorferi*. Differences between these two treatment groups were statistically significant for both the first (chi-square = 5.49, 1 degree of freedom, $P = 0.0192$) and second (chi-square = 13.89, one degree of freedom, $P = 0.0002$) experiments.

Results of OspA antigen capture ELISA provided in Table

1 confirm the postchallenge presence of *B. burgdorferi* in the infected *I. scapularis* nymphs. The sample population of pathogen-free ticks assayed postinfestation was negative for *B. burgdorferi* infection. All ticks collected from control mice which received their only infestation with *B. burgdorferi*-infected ticks were OspA positive (Table 1). Five of seven and one of six infected ticks obtained at the termination of the challenge infestation (fifth exposure) of mice repeatedly infested with pathogen-free ticks were OspA antigen capture positive. Differences between the number of OspA antigen-capture-positive challenge ticks infesting previously exposed versus initial exposure animals were significantly different during the second experiment (chi-square, $P < 0.05$, 1 degree of freedom).

This study demonstrated that BALB/c mice repeatedly infested with pathogen-free *I. scapularis* nymphs were resistant to subsequent infection with tick-transmitted *B. burgdorferi*. Transmission-blocking immunity was first described by Bell et al. (4) for rabbits resistant to tick transmission of highly virulent *F. tularensis* after repeated infestation with pathogen-free adult *D. andersoni*. In another study, bank voles (*C. glareolus*) expressing acquired resistance to *I. ricinus* did not become infected with *B. burgdorferi* after infestation with infected ticks, while bank voles not resistant to tick feeding became infected with *I. ricinus*-transmitted spirochetes (9). The yellow-necked mouse, *Apodemus flavicollis*, did not develop resistance to infestation with *I. ricinus*, indicating possible host species specificity of resistance to the vector and vector-borne pathogen (8, 9). This study describes a laboratory model for characterization of vector-blocking immunity.

Resistance of mice repeatedly infested with pathogen-free *I. scapularis* nymphs prior to infected-tick challenge might be due to an alteration of the ability of the tick to transmit spirochetes and/or a modified host environment into which the spirochetes are introduced. Initiation of feeding stimulates spirochetes to disseminate from the tick gut to the hemocoel and salivary glands for transmission to the host (20). Nymphal *I. scapularis* ticks need to be attached to the host for approximately 48 h for efficient transmission of *B. burgdorferi* (17). Alteration of the events involved in spirochete activation could possibly alter spirochete dissemination within the tick and transmission during feeding. Host antibodies to *B. burgdorferi* OspA in the tick blood meal blocked spirochete dissemination to the tick salivary glands (8).

Using an infestation regimen similar to the one described above, sera were obtained from BALB/c mice which had been repeatedly infested with pathogen-free *I. scapularis* nymphs derived from the colony maintained in this laboratory. BALB/c mice infested with pathogen-free *I. scapularis* nymphs did not develop antibodies reactive on immunoblots with an extract of whole *B. burgdorferi* B31.

Other factors that might affect pathogen transmission and establishment in hosts repeatedly infested with pathogen-free ticks include alterations in the cutaneous environment at the tick attachment site, which would interrupt feeding and/or be deleterious to introduced spirochetes; reduction of duration of attachment to the host; and host modification of pharmacologically active components of the tick saliva.

Tick feeding suppresses innate and acquired immunity of the host (19, 25, 26, 28). Tick-induced suppression of host immune function reduces antibody responses, T-lymphocyte proliferation to mitogens, and elaboration of cytokines by macrophages and Th1 lymphocytes. Greater suppression of host T-lymphocyte in vitro responsiveness was observed at the end of a primary infestation than at the termination of a second exposure (25). Immunosuppressant proteins have recently been described in tick salivary glands (5, 23). Repeated infestation with pathogen-free ticks may induce a host immune response that neutralizes the tick immunosuppressant(s) introduced into the host during feeding and thus enhance resistance to infection with *B. burgdorferi*. More complete expression of host innate and acquired immune effector mechanisms might be a factor in the resistance of repeatedly infested mice to tick transmission of *B. burgdorferi*.

The OspA antigen capture ELISA results indicate that repeated infestation with pathogen-free ticks might impact *B. burgdorferi* in infected challenge ticks. The *B. burgdorferi*-infected ticks recovered after the fifth infestation of repeatedly exposed mice had the lowest incidence of postinfestation infection, as detected by OspA antigen capture. The differences in infection status of ticks collected from control mice receiving a first exposure and repeatedly infested mice are not due to differences in the ability to detect OspA, since the assays were performed on ticks from a common pool at identical physiological ages. The *B. burgdorferi* infection rate of ticks prior to challenge infestation was 100%, as determined by darkfield examination, compared with a 17% infection rate in ticks feeding on mice previously infested with pathogen-free *I. scapularis*. Ticks obtaining a blood meal from hosts repeatedly exposed to pathogen-free tick bites might acquire factors that affect *B. burgdorferi* development, multiplication, dissemination, and/or expression of OspA.

Prior infestation with pathogen-free ticks induces a host transmission-blocking response which provides protection against infection. Tick biology parameters evaluated could not be used to definitively link acquired resistance to protection against tick-transmitted *B. burgdorferi* infection.

Vector-blocking immunity, impairing pathogen transmission and/or establishment within the host, is possibly a manifestation of acquired resistance to tick feeding. Expression of acquired resistance in this study might be masked by serendipitous variations in tick feeding. The host immune response to repeated tick feeding could possibly neutralize immunosuppressive molecules introduced by the tick, allowing for greater expression of host innate and acquired immune defenses against tick-borne pathogens. The magnitude of tick-induced suppression of host T-lymphocyte in vitro proliferative responses to mitogens and elaboration of macrophage and T-lymphocyte cytokines progressively becomes less intense during the course of four repeated tick exposures. A similar phenomenon might have occurred during the repeated infestations with pathogen-free *I. scapularis* nymphs in this study. Reduction of tick immunosuppression of the host might be an effective strategy for enhancing resistance to tick-transmitted disease-causing agents.

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