

PREVENTION OF *BORRELIA BURGdorFERI* TRANSMISSION IN GUINEA PIGS BY TICK IMMUNITY

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Abstract. We examined the effect of repeated infestation of guinea pigs with *Ixodes scapularis* on the capacity of ticks to transmit *Borrelia burgdorferi* infection. Repeated challenges with nymphs or larvae lead to a reduction in duration of nymphal tick attachment and weight of recovered ticks consistent with the development of tick immunity. Only one of 18 *I. scapularis*-immune guinea pigs challenged with *B. burgdorferi*-infected nymphal ticks became infected, whereas 10 of 18 naive guinea pigs similarly challenged became infected. We conclude that tick immunity interferes with borrelial transmission.

Ticks are the most common vector transmitting diseases to humans in the United States.¹ The ixodids, also called hard-bodied ticks, have a complex life cycle involving egg, larval, nymphal, and adult stages. Development of the later three stages requires ingestion of blood. The prolonged period of feeding, 48–96 hr for larvae and nymphs, allows an immune response to develop in certain hosts against tick components. Trager first observed that repeated feeding of larvae or nymphs of *Dermacentor sp.* upon guinea pigs resulted in tick immunity.²

Tick immunity is the capacity of previously exposed hosts to interfere with tick feeding and development. A reduction in tick weight, duration of attachment, number of ticks feeding, size of egg mass, and molting success are parameters to measure immunity. In addition to guinea pigs, tick immunity has been described in cattle and rabbits.^{3–7} While some have reported anti-tick immunity in mice,⁸ others have reported that it did not occur.⁹ Wikel and others have recently reported that BALB/c mice repeatedly infested with pathogen-free *Ixodes scapularis* ticks failed to become infected when subsequently infested with *Borrelia burgdorferi*-infected ticks, even though mean weights of fed ticks and percentage reaching repletion did not indicate development of acquired resistance.¹⁰

Development of tick immunity involves the interactions of tick antigens with host antibodies, T cells, B cells, mast cells, and basophils.¹¹ Langerhans' cells in the skin process and present tick antigens to lymphocytes that develop into sensitized lymphocytes and plasma cells secreting antibodies of various isotypes; among these are tissue-binding or homocytotropic antibodies. Antibodies bound to mast cells and basophils through their Fc receptors recognize tick antigens and induce degranulation resulting in development of microvesicles at the attachment site. Also contributing to the various manifestations of tick immunity are the multiple lymphokines, monokines, and chemokines released at the site of attachment. Basophil accumulation at tick attachment sites characterizes the immune reaction termed cutaneous basophil hypersensitivity,^{12,13} although the relative role of this reaction compared with the various other immunoreactants in producing the elements of tick immunity is not well defined, and may vary in different host species.

The present paper evaluates the response of guinea pigs to repeated *I. scapularis* infestation and, for the first time,

its effect on transmission of *B. burgdorferi*. Guinea pigs were chosen, in spite of the fact that they are not natural hosts for *I. scapularis*, because their immune reactions, especially in the skin, more closely approximate those of humans than do mice. We first established that *B. burgdorferi* infection could be transmitted to naive guinea pigs by tick bite, and then determined whether tick-immune guinea pigs would become infected by challenge with *B. burgdorferi*-infected ticks.

MATERIALS AND METHODS

Animals. Two hundred to five hundred gram female Hartley guinea pigs were housed in individual hanging cages at the Yale University animal facilities. Guinea pigs were killed by CO₂ inhalation.

Spirochetes. Cloned cultures of the N40 strain of *B. burgdorferi* were obtained from S. Barthold. (University of California, Davis, CA).

Ticks. After feeding on uninfected CD 1 mice, naive *I. scapularis* larvae were molted to nymphs in an environmental chamber at 22–24°C. Infected nymphs were obtained from larvae fed on B31- or cN40-infected CD 1 mice or N40 infected guinea pigs.¹⁴ Infection rate was calculated by macerating nymphs, staining with fluorescein isothiocyanate (FITC)-conjugated goat anti-*B. burgdorferi* polyclonal antibody (Kirkegaard & Perry, Gaithersburg, MD), and examining the preparations under a Zeiss (Carl Zeiss, Oberkochen, Germany) fluorescent microscope.

Sensitization. Guinea pigs were anesthetized with 1–1.5 ml of a ketamine:xylazine:phosphate-buffered saline (PBS) mixture prepared in a 1:1:2 ratio and containing approximately 37 mg of ketamine and then challenged with 50–100 larvae or 5–50 uninfected nymphs. We placed a water pan underneath the individual cages and covered the edges of the pan with Vaseline® (Chesebrough-Ponds, Greenwich, CT) to contain ticks. The water pan was examined and changed daily until all ticks that fed to repletion had been recovered. Guinea pigs were rested for 3–4 weeks before rechallenge with similar numbers of ticks. In some experiments guinea pigs were rested and rechallenged a third time. We did not observe any difference in the duration of attachment or resultant tick weights whether the animals were sensitized two or three times.

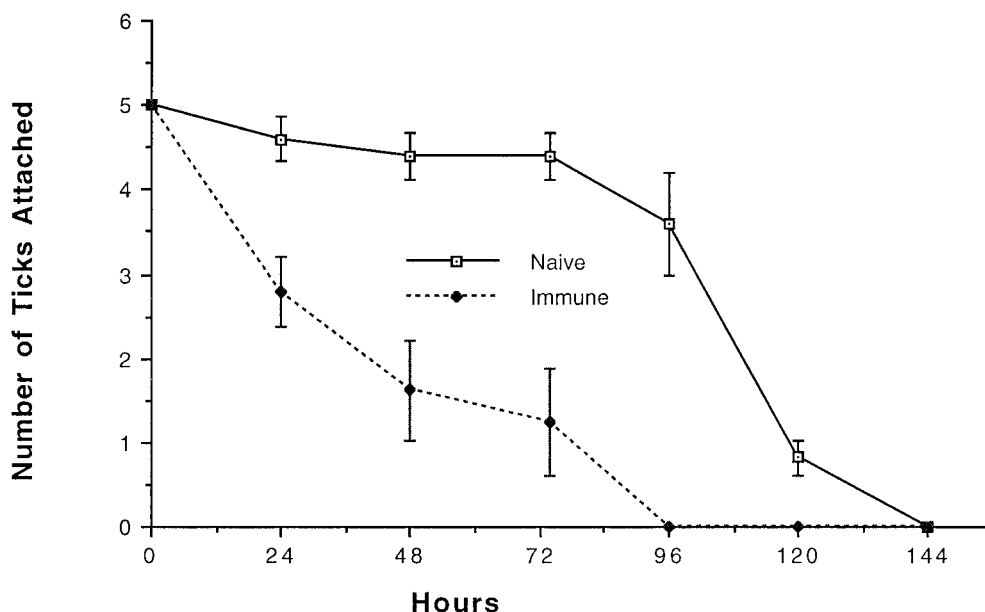


FIGURE 1. Duration of attachment of *Ixodes scapularis* nymphal ticks to tick-immune or -naive guinea pigs. Each point represents the mean \pm SE of five animals.

Challenge. After two or three cycles of sensitization, we anesthetized, shaved, and prepared each animal as described for the sensitization protocol. Five to ten infected or uninfected nymphs were applied per guinea pig and allowed to attach. We examined the skin at attachment sites and counted the number of ticks remaining every day as well as collected ticks from the water pan underneath each cage. Ninety to a hundred percent of the ticks were recovered after feeding.

Infection and disease. We performed 2–3-mm skin punch biopsies from tick attachment sites two, four, and seven weeks after tick challenge. Guinea pigs were shaved and the skin was cleaned well with Betadine® (Purdue Frederick Co., Totowa, NJ) and 70% ethanol. Skin punches were incubated 2–3 min in Betadine® solution, rinsed in 70% alcohol, and put into Barbour-Stoener-Kelley¹¹ medium. Culture tubes were incubated at 33°C for 14 days. Four to eight weeks after tick challenge, guinea pigs were killed, serum was collected, and a sample of blood, bladder, skin, and spleen were cultured. In some cases, skin punch biopsies at sites of tick attachment were obtained. Sections were fixed with Karnovsky fixative¹⁵ and stained with Giemsa for optimal visualization of basophils.

Immunoblots. The N40 spirochetes were grown to a concentration of 10^7 spirochetes/ml, washed three times in PBS, and lysed in water. The lysate was reduced with mercaptopurine and the equivalent of 10^6 spirochetes per lane were separated on a 12% polyacrylamide gel by electrophoresis. Proteins were transferred to nitrocellulose, blocked with bovine serum albumin, and probed with serum from tick-challenged guinea pigs or monoclonal antibodies against outer surface protein (Osp) A, CIII.78, flagellin X116B, NS-1-p39 (generously provided by Smith, Kline Beecham Laboratories, Rixensart, Belgium) and polyclonal antibody against recombinant Osp C. Alkaline phosphatase-conjugated goat anti-mouse immunoglobulin (Hyclone, Logan UT) was used as a secondary antibody. Nitroblue tetrazolium and 5-bromo-

4-chloro-indolyl phosphate obtained from Promega (Madison, WI) were used as substrates.

RESULTS

Guinea pigs repeatedly sensitized to nymphs developed an area of induration and erythema at tick attachment sites within the first 24 hr after challenge with 10 uninfected *I. scapularis* nymphs. We obtained skin punch biopsies at tick attachment sites from naive and sensitized guinea pigs, prepared sections and stained them with Giemsa. The epidermis of sensitized guinea pigs showed an infiltrate with predominant basophils characteristic of a cutaneous basophil hypersensitivity reaction. This did not occur at tick attachment sites in the skin of naive guinea pigs.

Figure 1 demonstrates a characteristic experiment in which guinea pigs sensitized twice with 10 nymphs each time, were challenged with five nymphs. Ticks detached from nymph-immune guinea pigs two days before their naive counterparts. The average weight of ticks recovered from sensitized guinea pigs (0.93 mg) was significantly reduced as compared with that of ticks feeding on naive guinea pigs (2.79 mg; $P < 0.01$) as shown in Figure 2.

Since the nymph and adult stages of *Amblyomma americanum* and *Dermacentor variabilis* ticks have been shown to share reactive antigens,^{16,17} we determined if *I. scapularis* larval and nymphal stages shared protective antigens as well. Guinea pigs were sensitized with 50 larvae on two occasions. Three weeks later, we challenged them with 50 nymphs per guinea pig. Nymphal ticks fed on guinea pigs sensitized to larvae detached before those feeding on naive guinea pigs as shown in Figure 3, a representative experiment. The weight of the nymphs recovered after feeding on larvae-immune guinea pigs was reduced compared with those feeding on naive hosts. Since larvae-sensitized guinea pigs rejected nymphs, at least some of the antigens inducing

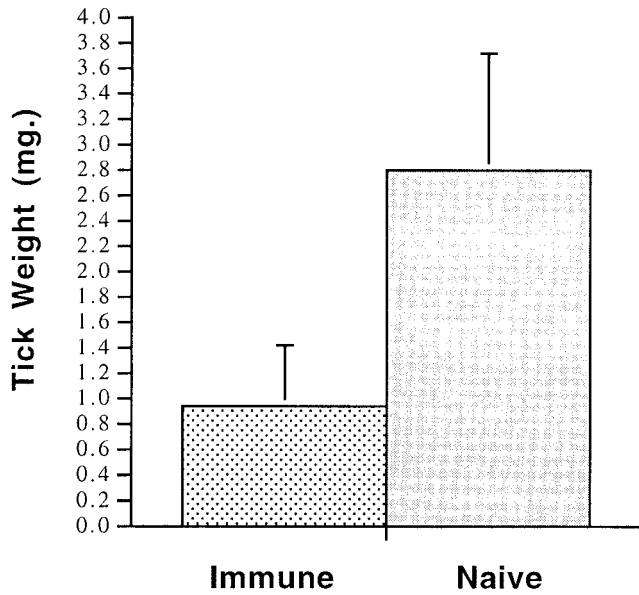


FIGURE 2. Average \pm SE weight of ticks recovered after attachment to the same tick-immune or naive guinea pigs shown in Figure 1.

immunity are shared among larval and nymphal stages of the life cycle.

We then examined the course of infection and disease in naive guinea pigs infected with *B. burgdorferi* by tick bite.

We challenged 500-gram guinea pigs with five nymphs infected with cloned B31 or N40 strains of *B. burgdorferi*. Two, four, and seven weeks after tick challenge, skin punches at sites of tick attachment, and elsewhere on the back of the animal, 2–3 cm from attachment sites, were consistently positive for spirochetes by culture. Blood, bladder, and spleen cultures were consistently negative. Histologic examination of the joints and hearts of infected animals failed to show inflammation.

To confirm infection, we determined that guinea pigs developed an immune response against *B. burgdorferi* eight weeks after tick challenge. Immunoblots of extracts of cloned N40 spirochetes were probed with sera from tick-challenged guinea pigs. Sera from all infected guinea pigs, as determined by positive culture, reacted to flagellin, P39 and, at times, to Osp C, consistent with the antibody response observed early after infection.¹⁸ Sera from guinea pigs challenged with uninfected ticks, and those exposed to infected ticks that failed to become culture-positive failed to reveal such bands. We have therefore demonstrated *B. burgdorferi* infection in tick-challenged guinea pigs by culture and serologic examinations that were always concordant.

We then determined whether tick immunity in host guinea pigs affected the capacity of infected ticks to transmit *B. burgdorferi*. We sensitized guinea pigs with larvae or nymphs and five weeks later challenged with five nymphs from a pool of ticks with a prevalence of 80% infection with cloned N40 spirochetes. We obtained 3-mm skin punch bi-

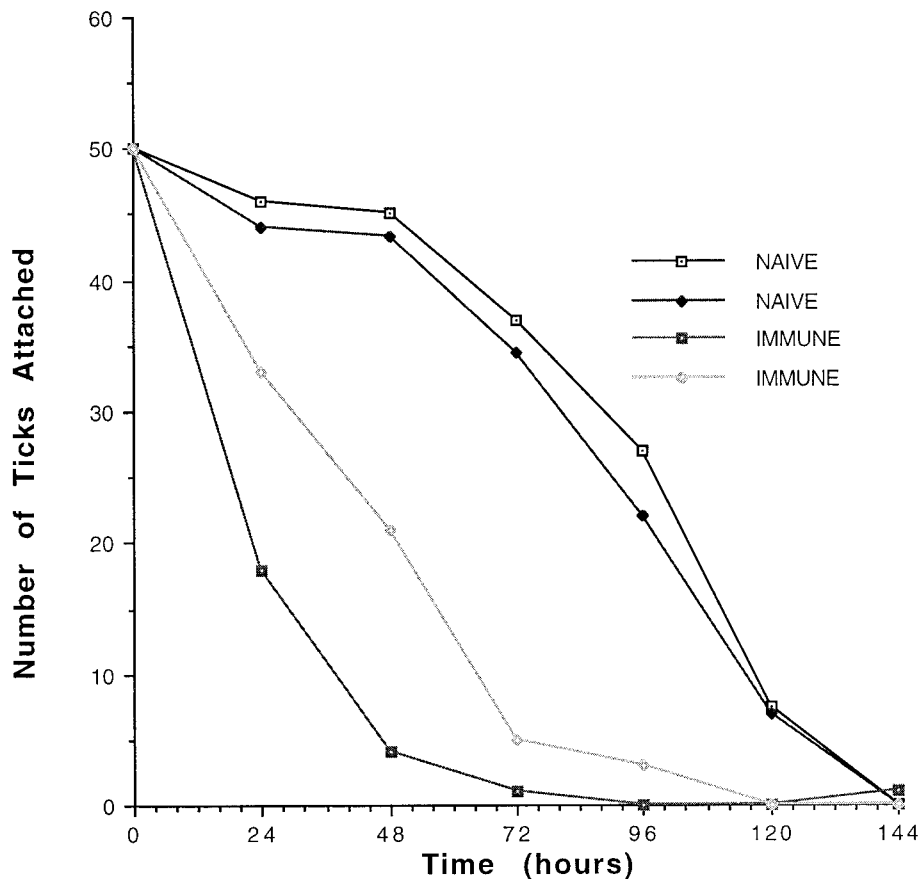


FIGURE 3. Duration of attachment of nymphal ticks on guinea pigs sensitized to *Ixodes scapularis* larvae.

TABLE 1

Prevention of *Borrelia burgdorferi* infection in guinea pigs immune to *Ixodes scapularis**

Guinea pigs	Experiment				Total
	1	2	3	4	
Naive	1/2	2/6	3/5	4/5	10/18
Immune	0/2	0/6	1/5	0/5	1/18

* Individual experiments comparing the rate of infection in tick-immune with that of naive guinea pigs challenged with *B. burgdorferi*-infected nymphal ticks. In experiment 1, strain B31 was used. In all subsequent experiments, strain N40 was used. Infection rate was determined by the number of guinea pigs with positive cultures and development of serologic conversion. In no instance were the culture and serologic data discordant. Values are infected guinea pigs/total guinea pigs exposed to infected ticks.

opsies at tick attachment sites and serum samples at two, four, seven, weeks after tick challenge. At eight weeks after challenge, the animals were killed and samples of blood, bladder, and spleen were cultured. Table 1 summarizes the results of several experiments.

Cultures of skin at tick attachment sites from tick-immune guinea pigs were positive only once in 18 guinea pigs. Skin samples from tick attachment sites of 10 of 18 naive guinea pigs were positive. Cultures of blood, bladder, and spleen were negative in both groups of guinea pigs.

To confirm that *B. burgdorferi* infection had occurred in challenged guinea pigs, we examined guinea pig serum reactivity to extracts of cloned N40 spirochetes. Figure 4 demonstrates that tick-immune guinea pigs challenged with infected ticks failed to develop antibodies to borrelial proteins. In contrast, naive guinea pigs similarly challenged reacted against P39 and flagellin. To ascertain that ticks used for challenge were infected, we stained ticks recovered from both groups of guinea pigs with FITC-conjugated polyclonal antibody against *B. burgdorferi*. Seventy to a hundred percent of the ticks tested were infected depending on the experiment. Tick immunity, therefore, markedly reduced or prevented *B. burgdorferi* transmission in guinea pigs.

DISCUSSION

This paper uses the guinea pig model of tick immunity to study host-tick-spirochete interactions. While guinea pigs are not natural hosts for *I. scapularis*, larvae and nymphs readily attach and feed on these animals. Guinea pigs were chosen because of their ready development of tick immunity, which is difficult to demonstrate in mice. In the present work we have not investigated the mechanism of tick immunity; specifically we examined, and report for the first time, that tick immunity interferes with the capacity of *B. burgdorferi*-infected *I. scapularis* ticks to transmit borrelial infection to guinea pigs.

In our experiments, tick-induced infection determined by culture was restricted to the skin for up to eight weeks, even though anti-borrelial antibodies appeared in each infected animal. Sonnesyn and others reported that young guinea pigs inoculated by syringe with 10^7 – 10^8 strain 297 spirochetes became infected with recovery of organisms from visceral organs.¹⁸ We did not investigate the effect of size and age of the animals, and could not alter the inoculum size delivered by the ticks. In our experiments, tick-borne infection was restricted to the skin, unlike the case of needle-transmitted infection. This could be due to differences in *B. burg-*

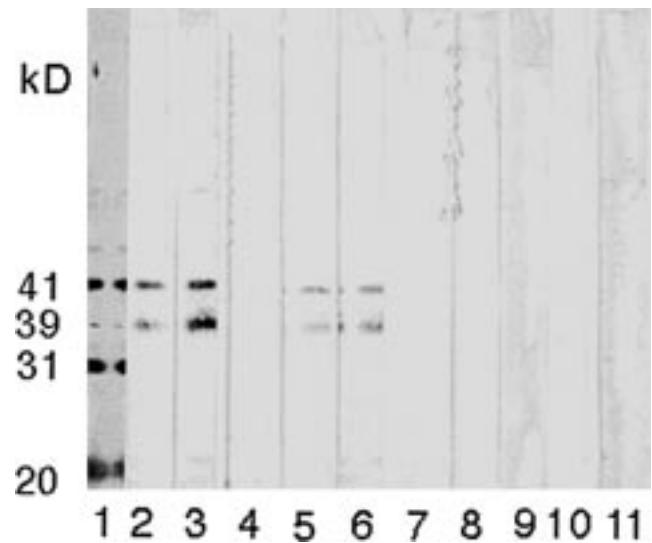


FIGURE 4. Immunoblots of extracts of N40 spirochetes probed with serum of tick-immune or naive guinea pigs obtained four weeks after challenge with *Borrelia burgdorferi*-infected ticks. Lane 1 was probed with monoclonal antibodies to outer surface protein (Osp) A, Osp B, and 41-kilodalton (kD) and polyclonal antibodies to recombinant Osp C and p39 antigens. Lanes 2–6 were probed with serum from naive guinea pigs challenged with infected ticks. Note the development of bands corresponding to p39 and flagellin in strips probed with sera from four of five naive guinea pigs challenged with infected nymphs. Lane 4 appears to be a naive animal challenged with infected ticks that failed to become infected. This animal was culture negative as well as seronegative. Lanes 7–11 were probed with sera from tick-immune guinea pigs. None showed antibodies to *B. burgdorferi*.

dorferi infectiousness when delivered by ticks since antigenic differences between spirochetes in culture and within ticks have been described.^{20, 21} More likely, the inoculum size delivered by the infected ticks was 5–6 logs less than that reported Sonnesyn and others, and this variable determined the extent of dissemination. Lack of adaptation of the spirochetes to the host species could also have contributed to the lack of dissemination. Host factors such as age and strain could also dictate the severity and extent of dissemination.¹⁸ We chose to challenge with five infected ticks to simulate the exposure of humans to ticks; infested field mice may harbor many times this number of ticks.

The capacity of tick immunity to interfere with pathogen transmission has been described in tick-immune cattle with *Babesia argentine* and *B. bigemina*²² and with *D. andersoni*-immune rabbits with *Franciscella tularensis*.²³ However, tick immunity may not necessarily interfere with transmission as in the case of *Theileria parva bovis* infection of cattle resistant to the tick *Rhipicephalus appendiculatus*.²⁴ Ticks may induce immunosuppression in the host during feeding^{25, 26} or may produce pathogen-enhancing factors that counteract tick immunity. The overall effect of these disparate forces determines whether transmission occurs. This is well demonstrated in the recent work of Wikel and others, who showed a marked reduction in transmission of borrelial infection from infected ticks to BALB/c mice that had previously been repeatedly infested by noninfected ticks.¹⁰ Even though there was no apparent tick immunity, as judged by no difference in mean weights of recovered ticks or the percentage feeding

to repletion, transmission was markedly reduced. In their second of two experiments, these investigators reported a significant reduction of postinfestation *B. burgdorferi* infection of the *I. scapularis* nymphs as determined by an Osp A capture ELISA. In the present work we showed no loss of *B. burgdorferi* postinfestation, when stained by a polyclonal anti-whole *B. burgdorferi* antibody. The mechanism of interference with transmission appears to be different in the two species studied.

What are the possible mechanisms by which *I. scapularis* immunity interferes with *B. burgdorferi* transmission? First, immunity, by reducing duration of tick attachment and size of blood meal, could decrease the opportunity for spirochetes to multiply, exit the midgut, traverse the hemolymph and then salivary glands before detachment takes place. *B. burgdorferi* requires 2–3 days of tick attachment before infection is transmitted.²⁷ These data assume that kinetics of *B. burgdorferi* transmission in a tick feeding on a tick-immune host is similar to that of one feeding on a naive one. Second, spirochetes could be directly affected by an immune process and die in the midgut. Examination of the gut of ticks feeding on an immune host shows damage and sloughing of epithelia.²⁸ Alternatively, spirochetes could exit the midgut and hemolymph but may not proceed further if immune serum recognizing salivary gland components prevented or blocked spirochete exit. Further studies are warranted to determine the mechanism of transmission blockade. From the data on duration of attachment, it may be seen that enough ticks remained attached at two days to permit transmission if time were the only factor. Clearly it is not.

The present work focused on the tick-borne pathogen *B. burgdorferi*, but *I. scapularis* may harbor other pathogens, including *Babesia microti* and the agent of human granulocytic ehrlichiosis, which have emerged as important human pathogens due to changing zoonosis of suburban areas.²⁹ Study of the effect of tick immunity in a variety of pathogen transmissions may be helpful in understanding the mechanism of how tick immunity interferes with infection. If *I. scapularis* immunity prevents *Babesia* or *Ehrlichia* transmission, it would favor the hypothesis that immunity worked at the salivary gland level, since both organisms are harbored in the salivary glands instead of the midgut of ticks.

We hypothesize that the absence or marked reduction of the proclivity to develop tick immunity in the natural host of *I. scapularis*, the white footed field mouse (*Peromyscus leucopus*), may have determined the natural selection of this host-parasite pair. Successive feeding of infected nymphs and naive larvae on the same host are necessary for the propagation of *B. burgdorferi*. Such repetitive feeding in a host capable of becoming sensitized early would seriously impair spirochetal propagation in nature. The present work gives impetus to the study of tick antigens and the mechanisms involved in tick immunity because if such immunity were safely and easily induced in humans, it is likely to have a protective effect upon transmission of several tick-borne diseases.

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