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Duration of Immunity to Reinfection with Tick-Transmitted *Borrelia burgdorferi* in Naturally Infected Mice

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The ability of naturally infected and cured mice to resist reinfection with tick-transmitted *Borrelia burgdorferi* was tested over a 1-year period. All of the mice were resistant to reinfection when they were challenged at 1.5 months after cure. The majority of animals were resistant to reinfection for up to 10.5 months after cure, but this resistance was lost at 1 year after cure. Both protected and unprotected animals showed a diverse array of antibodies on Western immunoblots. Protection was not associated with the killing of spirochetes in ticks, and naturally infected mice produced no antibodies to outer surface protein A (OSP A). The titers to whole *Borrelia* sonicate and OSP C, however, remained high throughout the 1-year study period. The levels of borreliacidal antibodies were highest in the 1.5 month-after-cure group. Natural immunity to reinfection with *B. burgdorferi* is limited in time, is complex, and may involve both humoral and cellular components.

Lyme disease is an extremely focal disease; it is hyperendemic to areas where populations of infected *Ixodes* ticks are found (15). Just a few counties in the Northeast account for the majority of cases reported each year in the United States (18). Even within counties where Lyme disease is hyperendemic, certain communities experience an extremely high risk of Lyme disease (1, 16), especially where ticks are present in abundance on residential properties (8, 17). The residents of communities hard hit by Lyme disease often ask the simple question of whether someone who has already had Lyme disease is protected from reinfection with the Lyme disease spirochete.

There are isolated reports in the literature of individuals who have suffered at least two distinct infections with *Borrelia burgdorferi*. Since the discovery of *B. burgdorferi* as the specific etiology of Lyme disease (4), at least nine patients have been reported to be reinfected based on clinical and serological evidence. In Germany, a patient was infected by tick bite in 1983 and suffered erythema migrans and Bannwarth's syndrome; she was treated and recovered. In 1985, she was bitten by another tick and suffered erythema migrans again, with a subsequent rise in antibody titer to *B. burgdorferi* (20). Pfister et al. (20) also reviewed four published cases that were consistent with reinfection. An additional case of reinfection with *B. burgdorferi* was reported in Germany in 1994 (13), and one unlucky individual in Austria was infected three times within 4 years (19). In the United States, two children who lived in Delaware were treated for Lyme arthritis, recovered, and subsequently developed erythema migrans (25).

Although these isolated instances of human reinfection suggest that Lyme disease patients are still at risk of reacquiring the infection after treatment, few controlled laboratory trials have addressed this question in animal models. In one report, mice infected by intradermal *B. burgdorferi* inoculation were immune to reinfection for up to 6 months when they were challenged by intradermal inoculation or with autographs from infected mice (2). To date, there has been no formal demonstration of previous infection and cure providing protection against subsequent tick-transmitted infection. Moreover, the longevity of immunity to spirochetes transmitted by the natural route of infection (tick bite) is unknown. Accordingly, we infected mice by tick bite, cured them, and challenged them by infected-tick bite over a 1-year period to determine the duration of immunity to naturally transmitted *B. burgdorferi* infection.

MATERIALS AND METHODS

Animals. Male outbred Imperial Cancer Research (ICR) mice, 3 weeks of age, were obtained from the special pathogen-free mouse colony maintained at the Centers for Disease Control and Prevention laboratory in Fort Collins, Colo.

Infected-tick colony. The *Ixodes scapularis* tick colony originated from field-collected females from Great Island, Mass. and Westchester County, N.Y. Uninfected $\geq F_2$ generation ticks were infected with the B31 strain of spirochete (from Shelter Island, N.Y.) by feeding on infected mice as larvae as previously described (21). Larvae were allowed to molt to nymphs at 21 to 22°C in saturated-humidity conditions. Nymphs were used in these experiments at >1 month postmolting. The infection rates in this colony were routinely >80%. Ticks were allowed to feed ad libitum on mice.

Culture of *B. burgdorferi*. Ticks were examined for spirochetes at 10 to 12 days after repletion. Individual replete nymphal ticks were disinfected by submersion in 3% H_2O_2 and 70% ethanol for 3 min each. Ticks were ground in glass tissue homogenizers containing 0.25 ml of Barbour-Stoenner-Kelly (BSK) medium. The homogenate was poured into 6-ml snap-cap tubes containing BSK medium. A wedge of ear tissue was obtained from each live mouse, or mice were sacrificed and both ears, the entire urinary bladder, and the heart were obtained. Ear biopsies were soaked for 15 min in wescodyne and for 15 min in 70% ethanol. Internal organs were washed rapidly in 70% ethanol. Tissues were finely minced with scissors and placed into 6-ml snap-cap tubes containing BSK medium. All cultures were maintained at 33 to 34°C for 1 month and examined at weekly intervals for viable spirochetes under dark-field microscopy. Contaminated cultures were discarded.

Antibiotic treatment. All of the mice in this study were treated for 2 weeks with tetracycline by the availability of drinking water ad libitum containing 1 mg/ml. Water bottles were wrapped with tinfoil to diminish light exposure and replenished every 1 to 2 days.

ELISA. Assay plates were coated with 100 ng of purified recombinant protein or whole sonicate per well in 100 μl of 0.1 M carbonate buffer (pH 9.6), and plates were incubated overnight at 4°C. Plates were washed five times in Tris-buffered saline–0.5% Tween 20 (TBS-t). Wells were blocked with 300 μl of TBS-t–3% fetal bovine serum (blocking buffer) for 30 min at 37°C. Serum was diluted in blocking buffer as noted above, and 100 μl/well was incubated at 37°C for 1 h. Plates were washed five times with TBS-t. Secondary antibody, alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (IgG) plus IgM

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TABLE 1. Chronology of procedures in challenge experiment

Time[a]	Procedure
−2 mo	3-week-old mice exposed to infected (test) or uninfected (control) ticks
−1 mo	Ear biopsies cultured from all test mice
Day 0	Tetracycline treatment of all test and control mice started
2 wk	Tetracycline treatment terminated
4 wk	Ear biopsies cultured from all test mice
1.5 mo[b]	First cohort of test and control mice challenged with 10 B31 nymphs
3 mo	Second cohort challenged
6 mo	Third cohort challenged
10.5 mo	Fourth cohort challenged
12 mo	Fifth cohort challenged

[a] Day 0 was set as the first day of antibiotic treatment.
[b] Ear, heart, and bladder tissues were cultured from each mouse at 1 month after challenge feeding.

(Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.), was diluted 1:2,000 in TBS-t, added to assay wells at 100 μl per well, and incubated at 37°C for 90 min. Plates were washed five times with TBS-t, and 100 μl of *p*-nitrophenyl phosphate substrate was added to each well and incubated for 30 min at 37°C. The enzyme reaction was stopped with 100 μl of 5 N NaOH, and plates were read on an ELISA plate reader. A negative cutoff of 1 standard deviation (SD) above the mean optical density reading of the preimmunization serum sample from each mouse was determined. The data reported are the reciprocal endpoint titers of the doubling dilutions of each serum sample, beginning at 1:125. Recombinant outer surface protein A (OSP A) was expressed and purified as previously described (7). Similarly, OSP C from the B31 strain of *B. burgdorferi* was expressed in a pET 9c system with a T7 promoter.

Western blot assays. The serum antibody response and monoclonal antibodies of interest were assayed by Western blot as previously described (12). Samples were run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% polyacrylamide) preparatory gels at a concentration of 100 μg of protein per gel. Proteins were electrophoretically transferred to nitrocellulose filters, and filters were incubated in blocking solution overnight. Filters were loaded into slot blotters (Immunetics, Inc., Cambridge, Mass.) and assayed with the designated sample of serum antibody at a final dilution of 1:100. The detection system was a colorimetric assay based on alkaline phosphatase conjugated to anti-mouse IgG plus IgM (Kirkegaard & Perry Laboratories, Inc.).

Borreliacidal assay. A simple borreliacidal-antibody test was adapted from a previously described assay (5). Mouse sera were diluted 1:2 in HEPES buffer (10 mM with 1 mM $MgCl_2$ and 150 mM $NaCl_2$ [pH 7.4]) to obtain a final volume of 20 μl. *B. burgdorferi* (strain B31) spirochetes grown in BSK-H (Sigma Chemical Co., St. Louis, Mo.) were washed three times in HEPES buffer, resuspended to a final concentration of 6×10^6 cells/ml, added in equal volumes to diluted sera, and incubated at 33°C for 15 to 18 h. After incubation, the number of viable cells was counted by using phase-contrast microscopy and a Petroff-Hauser counting chamber. Viability was counted as motility, retention of a characteristic spirochete shape, and absence of membrane blebbing. The percent survival was determined by comparing the number of viable cells remaining in each sample per ml with that of the *Borrelia* control containing 20 μl each of HEPES and *B. burgdorferi* cells.

Chronology of experiment. The time course of the experiment is outlined in Table 1. Briefly, 3-week-old mice were placed into 10 cohorts of seven animals each; 5 cohorts were exposed to 10 B31-infected nymphal *I. scapularis* ticks per mouse, and 5 cohorts (controls) were exposed to 10 uninfected nymphal *I. scapularis* ticks. All ticks were allowed to feed to repletion. At 1 month after tick feeding, ear biopsies were taken to confirm that all of the mice exposed to infected nymphs had acquired *B. burgdorferi* infection. All 34 animals tested were infected. At 2 months after tick feeding, tetracycline treatment of all 10 cohorts was initiated. The first day of antibiotic treatment was designated day 0 of the overall experiment. Antibiotic treatment continued for 2 weeks; 2 weeks after the termination of treatment, the five cohorts previously exposed to B31-infected nymphs underwent ear punch biopsies. All test animals were ear punch negative at this point. Individual cohorts of matched test and control mice were challenged by exposure to 10 B31-infected ticks per mouse at 1.5, 3, 6, 10.5, and 12 months after day 0. At 1 month after challenge, animals were sacrificed and the remaining ear tissue, heart, and urinary bladder of each animal were cultured in BSK medium. The eventual number of mice in each cohort varied (from four to seven) due to the death of some mice during the 15-month course of the experiment. Replete challenge nymphs were also cultured in BSK medium.

TABLE 2. Duration of immunity to reinfection with tick-transmitted *B. burgdorferi*

Time of challenge (mo)	No. of mice culture positive/no. of mice examined	
	Test mice	Control mice
1.5	0/7	6/6[a]
3	1/6	5/5[a]
6	1/5	7/7[a]
10.5	2/6	6/6[a]
12	5/6	4/4
Total	9/30	28/28[a]

[a] $P < 0.05$ (by chi-square test) between results for test and control groups.

RESULTS

Mice were challenged with *B. burgdorferi*-infected nymphal ticks during a 1-year period after cure (Table 1). On the initial challenge, at 1.5 months after cure, all seven test mice were resistant to reinfection and all control mice were susceptible (Table 2). The proportion of mice susceptible to reinfection remained ≤1/3 ($P < 0.05$) through 10.5 months postcure. At 12 months, however, five of six test mice became infected. Thus, significant immunity to reinfection lasted for <1 year in these experiments.

Replete nymphal challenge ticks were cultured to detect the presence of viable *B. burgdorferi* spirochetes at 10 to 12 days after feeding. The majority of replete nymphs derived from both test and control cohorts were infected (Table 3). No significant difference was noted in the proportions of test- and control-derived ticks at any time point. The overall proportions of spirochete-infected ticks derived from test (90%) and control (88%) mice were virtually identical.

Serum samples were obtained from all of the mice in the experiment prior to initial tick exposure and at 1 month after cure. In addition, serum samples were obtained from each cohort immediately prior to challenge tick exposure, except for the cohort exposed at 10.5 months after cure. Characterization of the antibody responses of individual mice by Western immunoblotting revealed that naturally infected mice developed antibodies against a diverse array of *B. burgdorferi* antigens (Fig. 1). Reactivities against 20 to 25 distinct proteins were visible on Western immunoblots; the intensity and diversity of the immune response did not decrease from 1 to 12 months after cure. In addition, no specific activity in protected animals that was lacking in unprotected animals could be demonstrated. The reciprocal endpoint titers of individual mice were determined by ELISA with whole *B. burgdorferi* sonicate (Fig. 2) or recombinant OSP C (Fig. 3). The antibody response

TABLE 3. Proportion of challenge ticks infected with *B. burgdorferi*[a]

Time of challenge (mo)	No. of ticks positive/no. of ticks examined (%)	
	Test	Control
1.5	21/30 (70)	27/33 (82)
3	39/44 (89)	31/32 (97)
6	37/42 (88)	37/46 (80)
10.5	44/46 (96)	18/19 (95)
12	48/48 (100)	27/29 (93)
Total	189/210 (90)	140/159 (88)

[a] Nymphs were cultured 10 to 12 days after repletion.

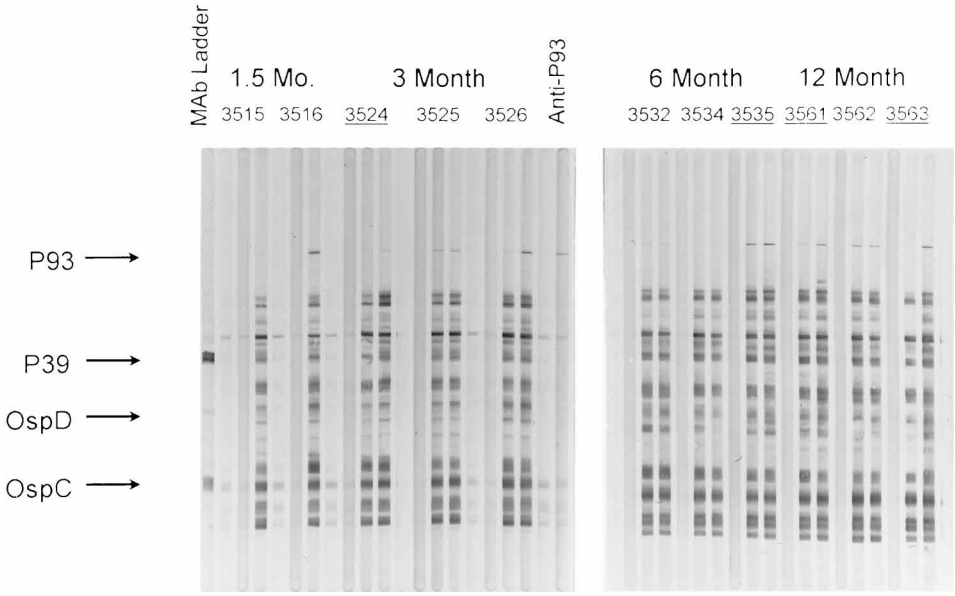

FIG. 1. Serum antibody responses of mice assayed by Western immunoblotting. Three serum samples from each mouse were included. For each mouse challenged at 3, 6, or 12 months (≥3524), the first lane contained serum obtained before exposure to ticks, the second lane contained serum obtained at 1 month after cure, and the third lane contained serum obtained immediately before tick reinfection challenge. For the two mice exposed at 1.5 months (3515 and 3516), the first and second lanes contained sera obtained before exposure to ticks and the third lane contained serum obtained at 1 month after cure. Underlined mouse numbers indicate mice that were not protected from reinfection. The immunoblot on the left has 21 lanes; lanes 1 and 21 are monoclonal antibody (MAb) markers; lanes 8, 12, 16, and 20 are spacer lanes. The immunoblot on the right has 20 lanes; lanes 1 and 20 are spacer lanes.

remained high throughout the 12-month period after cure. In fact, some of the highest titers to both whole sonicate and OSP C were present in the 12-month group. Moreover, unprotected animals had high titers to both whole sonicate and OSP C. Sera from a total of four mice were selected at random from among cured mice at 1 month after cure and tested for antibody to OSP A; no antibody to OSP A by ELISA was present at the lowest dilution tested (1:250).

The borreliacidal-antibody levels in prebled serum samples as well as in serum samples at 1.5, 3, 6, and 12 months after cure were determined (Table 4). Spirochete survival after exposure to prebled serum was greater than that after exposure to immune serum (by two-factor analysis of variance, $P < 0.001$; $F = 51.1$; $df = 1$). Moreover, spirochete survival in sera from mice obtained at 1.5 months after cure, when all of the animals were protected from reinfection, was lower than that

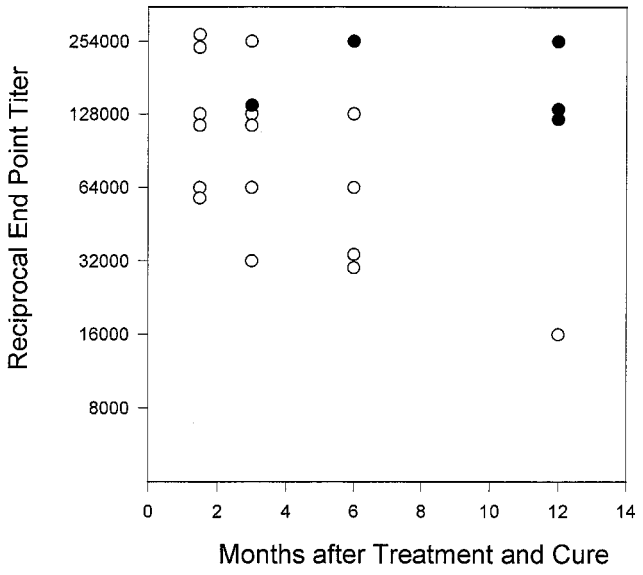

FIG. 2. Reciprocal endpoint ELISA titers of mice immediately before reinfection challenge. The antigen used was whole *B. burgdorferi* sonicate. Open circles, mice protected from reinfection; closed circles, mice susceptible to reinfection.

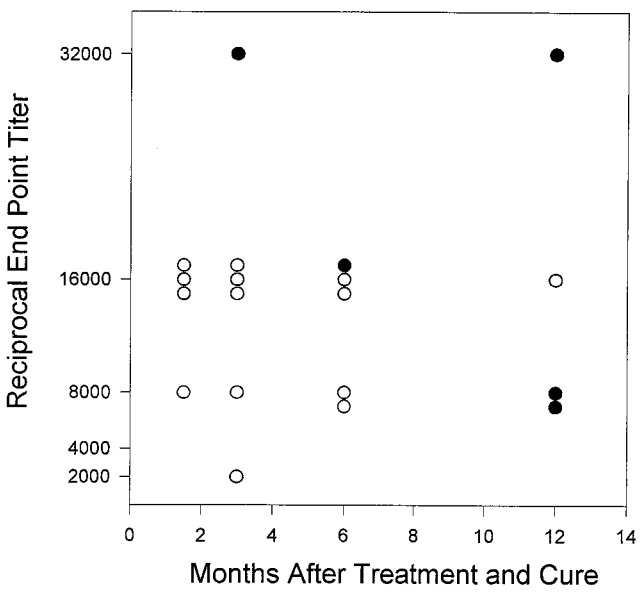

FIG. 3. Reciprocal endpoint ELISA titers of mice immediately before reinfection challenge. The antigen used was recombinant OSP C. Open circles, mice protected from reinfection; closed circles, mice susceptible to reinfection.

TABLE 4. Survival of *B. burgdorferi* in a borreliacidal assay with sera collected from mice prior to infectious-tick challenge

Time after cure (mo)	No. of sera tested	Survival (%)[a]
Prebleed	19	74.1 ± 29.9
1.5	6	16.1 ± 19.4
3	6	34.3 ± 16.3
6	3	54.6 ± 8.1
12	4	41.3 ± 8.3

[a] Data are means ± SDs.

in sera collected later (by two-factor analysis of variance, $P < 0.001$; $F = 9.72$; $df = 3$). Overall, spirochete survival after exposure to sera from protected animals (mean percent survival ± SD, 30.8% ± 21.3%) was lower than that after exposure to sera from unprotected animals (mean percent survival ± SD, 42.3% ± 9.8), but the difference was not significant.

DISCUSSION

From a public health perspective, the present study adds emphasis to the message that residents of regions where Lyme disease is hyperendemic must continue to avoid *I. scapularis* bites. An extrapolation from our mouse data to humans suggests that even those who have been infected with *B. burgdorferi*, treated, and cured may be fully susceptible to reinfection during the next transmission season. Avoiding infected ticks includes limiting contact with heavily forested areas, tucking pant legs into socks upon entry into infested habitats, judiciously using repellents, inspecting daily for attached ticks during the transmission season, and promptly removing those that are found (22). The primary months of risk are May through July, when nymphal *I. scapularis* populations are at their peak (23). The present study also suggests that persons with prior histories of Lyme disease and continued exposure should be candidates to receive test vaccine preparations. These persons are not protected from reacquiring Lyme disease by their prior infections. If the vaccine is safe and effective, persons with prior Lyme disease should be prime candidates, since they have been proven to be at high risk.

The mechanism of immunity to reinfection observed in naturally infected mice is unknown. One mechanism of immunity to naturally transmitted infection is clearly present in animals with high OSP A titers. Such OSP A-directed immunity is due to the killing of *B. burgdorferi* within the tick before spirochetes have a chance to be transmitted to the host (9). This killing must take place before spirochetes have a chance to convert their surface proteins from OSP A to OSP C and migrate to the salivary glands (6, 27). Clearly, the immunity expressed by mice in the present study was via a mechanism other than OSP A antibody, since naturally infected and cured mice had no detectable titers of antibody to OSP A.

The immune response of naturally infected mice in this experiment resembled that in previous experiments with naturally infected rodents, namely, a diverse response lacking antibodies to OSP A and including a strong response to OSP C (10, 12, 24). Since mice produced abundant antibodies to OSP C and these antibodies have previously been shown to be protective against natural infection (11), antibodies to OSP C were likely candidates to afford protection in naturally infected animals. In the present experiments, however, OSP C titers remained high (even 1 year after cure) and could not be associated with the loss of protection in the 1-year-after-cure group. Indeed, some of the unprotected animals had the highest OSP C titers and the titers to whole *Borrelia* sonicate and OSP C were still vigorous even 1 year after treatment.

Although all of the mice exposed to antibiotics in the present study were presumed to be cured based on a negative ear biopsy, the possibility that antibiotic treatment decreases the spirochete load in the host to the point where organisms are difficult to culture but are still present exists. This phenomenon has previously been described for dogs (28) and mice (14). If this was the case in our experiments, the infections seen in challenged mice may have been due to relapses after treatment rather than to true reinfection. We are engaged in long-term studies to see whether mice that are infected by tick bite and cured via the tetracycline regimen described in this study relapse with infection for up to 1 year after treatment.

The humoral arm of the immune response may be involved in the protection invoked in natural *B. burgdorferi* infection. Low-dose intradermal inoculation of mice with the spirochete produces a response that lacks antibodies to OSP A (3, 26); these antibodies protect against subsequent intradermal challenge when they are passively transferred to naive hosts. Moreover, borreliacidal antibodies were present in the mice in our study, particularly at the earliest time point (1.5 months) after cure. However, the possibility that the cellular immune response is also involved in protection against tick-transmitted *B. burgdorferi* in naturally infected and cured hosts exists. Experiments in which antibodies and various cellular components from naturally infected animals are separately and jointly transferred to test animals which are subsequently challenged by infected-tick bite will help to elucidate the protection afforded by the complex immune response to tick-transmitted *B. burgdorferi.*

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