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

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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 *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% H2O2, and 70% ethanol for 3 min each. Ticks were ground in glass tissue homogenizers containing 0.25 ml of Barbour-Stoener-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 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-
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

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**TABLE 1. Chronology of procedures in challenge experiment**

<table>
<thead>
<tr>
<th>Time (mo)</th>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 mo</td>
<td>3-week-old mice exposed to infected (test) or uninfected (control) ticks</td>
</tr>
<tr>
<td>1 mo</td>
<td>Ear biopsies cultured from all test mice</td>
</tr>
<tr>
<td>0 mo</td>
<td>Tetracycline treatment of all test and control mice started</td>
</tr>
<tr>
<td>2 wk</td>
<td>Tetracycline treatment terminated</td>
</tr>
<tr>
<td>1 mo</td>
<td>Ear biopsies cultured from all test mice</td>
</tr>
<tr>
<td>1.5 mo</td>
<td>First cohort of test and control mice challenged with 10 B31 nymphs</td>
</tr>
<tr>
<td>3 mo</td>
<td>Second cohort challenged</td>
</tr>
<tr>
<td>6 mo</td>
<td>Third cohort challenged</td>
</tr>
<tr>
<td>10.5 mo</td>
<td>Fourth cohort challenged</td>
</tr>
<tr>
<td>12 mo</td>
<td>Fifth cohort challenged</td>
</tr>
</tbody>
</table>

*Day 0 was set as the first day of antibiotic treatment.

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

<table>
<thead>
<tr>
<th>Time of challenge (mo)</th>
<th>No. of mice culture positive/no. of mice examined</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>0/7 - 6/6</td>
</tr>
<tr>
<td>3</td>
<td>1/6 - 5/5</td>
</tr>
<tr>
<td>6</td>
<td>1/5 - 7/7</td>
</tr>
<tr>
<td>10.5</td>
<td>2/6 - 6/6</td>
</tr>
<tr>
<td>12</td>
<td>5/6 - 4/4</td>
</tr>
<tr>
<td>Total</td>
<td>9/30 - 28/28</td>
</tr>
</tbody>
</table>

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

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

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

*Nymphs were cultured 10 to 12 days after repletion.*
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, \( P < 0.001; F = 51.1; \text{df} = 1 \)). 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
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*.

**ACKNOWLEDGMENTS**

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**REFERENCES**


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