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# Antigenic Stability of *Borrelia burgdorferi* during Chronic Infections of Immunocompetent Mice

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**Mice were actively immunized by intradermal inoculation with $10^4$ cloned *Borrelia burgdorferi* bacteria and then cured of the *B. burgdorferi* infection with an antibiotic after 90 days. They were resistant to intradermal $10^2$- or $10^4$-bacterium challenge infection with either the original cloned *B. burgdorferi* or *B. burgdorferi* isolated from ear punch biopsies at 90 days of infection (prior to antibiotic treatment), including autologous *B. burgdorferi* isolates. In contrast, sham-infected (nonimmune) mice were susceptible to challenge infection with both early and late *B. burgdorferi* isolates. Since there was a potential for in vitro modification of the spirochetes during the 2-week culture period which would obscure results, an alternate means of challenge infection, using tissue transplants, was implemented. By using the same approach, mice were immunized by infection, treated with antibiotics, but challenged by subcutaneous transplantation of ear skin pieces biopsied and frozen prior to antibiotic treatment. Mice were infected for 15, 90, or 180 days before biopsy and antibiotic treatment and then transplant challenged with autologous infected tissue. Sham-immunized mice received infected tissue, and immune mice received uninfected tissue as controls. Mice infected for only 15 days, but not mice infected for 90 or 180 days, could be reinfected by autografts, whereas nonimmune mice became infected with tissues collected at each of these intervals and immune mice transplanted with normal skin were uninfected. These results indicate that immunity to *B. burgdorferi* is effective against the original inoculum, late isolates of the spirochete, or infected tissues collected at intervals of up to 180 days, suggesting that there is no significant antigenic change in *B. burgdorferi* during chronic infection.**

The issue of *Borrelia burgdorferi* persistence in immunologically intact hosts is among the most perplexing mysteries of Lyme disease pathogenesis. There is mounting evidence that *B. burgdorferi* infection can be persistent in humans, with isolation of the organism from patients months to years after the initial infection (3, 4, 20, 29, 32, 39, 46, 47). Experimental inoculation with *B. burgdorferi* results in persistent infections in laboratory mice (*Mus* sp.) (2, 9, 43), white-footed mice (*Peromyscus* sp.) (44, 45), rats (28), hamsters (16, 22, 41), gerbils (31), and dogs (1). The typical course of disease in laboratory mice following intradermal inoculation with low doses of the spirochete is early dissemination, with acute disease in multiple joints, bursae, tendons, ligaments, muscles, arteries, and the heart, followed by immune-mediated disease resolution but persistent infection for periods of greater than 1 year (2, 9–11, 43). During the chronic phase of the infection, there are intermittent bouts of active joint and heart disease, similar to events that occur in human Lyme disease patients who have not benefitted from antibiotic therapy (9).

The mechanisms of spirochete persistence in immunocompetent hosts are not known. *B. hermsii*, the agent of relapsing fever, is capable of genetic rearrangements and antigenic modulation as means of evading immune clearance, but there is no evidence of purposeful genetic or antigenic fluctuation in *B. burgdorferi* (6, 7). Animals and humans infected with *B. burgdorferi* mount an antibody response to the spirochete which readily kills the organism in vitro (13, 23, 24, 36, 41). Passively transferred immune serum protects naive animals against challenge infection with *B. burgdorferi* (8, 13, 40, 41). Remarkably, this protective antibody is effective at preventing infection only when administered shortly before or at the time of challenge inoculation, not after (8, 21). Furthermore, actively infected animals and humans develop these antibodies but often do not clear their own infections (8, 13). These findings suggest that *B. burgdorferi* is either antigenically modified or immunologically sequestered in its host.

In a recent experiment, the immunizing activity of serum collected from mice at 1 year of infection was compared among naive mice passively immunized and then challenged with the original (time zero) cloned inoculum or *B. burgdorferi* isolated at 1 year from the mouse of serum origin. No difference in protective activity could be found, suggesting that no obvious antigenic differences existed between the original inoculum and late *B. burgdorferi* isolates (8). These results cannot be viewed as conclusive and need to be verified. The present study sought to determine whether mice actively immunized by infection with *B. burgdorferi* and then cured of their *B. burgdorferi* infection with an antibiotic are resistant to reinfection by challenge with the original isolate or late isolates of *B. burgdorferi*. These studies provide additional evidence that *B. burgdorferi* elicits a protective immune response in infected hosts but the organism does not change antigenically during the course of infection.

## MATERIALS AND METHODS

**Mice.** Pathogen-free C3H/HeNCrlBr (C3H) mice were purchased from Charles River Laboratories, Portage, Mich., shipped in filtered containers, and maintained in isolator cages (Lab Products, Maywood, N.J.). They were given food (Agway, Syracuse, N.Y.) and water ad libitum. Mice were inoculated at 4 weeks of age, killed with carbon dioxide gas, and then exsanguinated by cardiocentesis.

***B. burgdorferi.*** Spirochetes were grown at 33°C in 8-ml glass screw-cap tubes containing 7 ml of BSK II medium (5) without antibiotic and examined with a dark-field microscope. Inocula were enumerated with a Petroff-Hauser bac-

terial counting chamber (Baxter Scientific Products, McGaw Park, Ill.). Mice were (i) infected intradermally with $10^4$ cloned *B. burgdorferi* N40 bacteria which had been cloned by limiting dilution in the fourth in vitro passage (9) in 0.1 ml of BSK II medium or (ii) sham inoculated with sterile BSK II medium. Tissues for culture were collected aseptically. Cardiac blood was cultured by placing 2 drops into a culture tube with medium. Spleens were triturated in 4 volumes of BSK II medium in glass tissue grinders, solids were allowed to settle, and then 0.5-ml supernatant samples were transferred to culture tubes with medium. Pieces of urinary bladder and ear tissues were placed directly into tubes with medium without triturition. Animals were considered infected if one or more tissues were culture positive after 2 weeks of incubation. Late isolates were obtained from ear biopsies collected under methoxyflurane (Metofane; Pitman-Moore, Mundelein, Ill.) anesthesia at 90 days of infection, prior to antibiotic treatment.

**Antibiotic treatment.** Mice were treated with 16 mg of ceftriaxone sodium (Rochephin; Hoffman-La Roche, Nutley, N.J.) per kg subcutaneously twice daily for 5 days (total dose, 4.0 mg per mouse). This dose and treatment regimen were determined to be effective in pilot studies and proven effective in the executed experiments with appropriate control groups.

**Transplant challenge.** Pilot studies revealed that ear punch biopsies from infected mice could be frozen for extended periods and then transplanted into naive mice, resulting in consistent infection of all mice. Since ear skin is a preferred site of *B. burgdorferi* persistence in mice (9), ear tissue could be predictably used as an accessible tissue for analysis. In the autograft experiment, mice were anesthetized with methoxyflurane on day 15, 90, or 180 after inoculation with *B. burgdorferi* or sham inoculation and the distal half of one ear pinna was excised from each mouse. Each pinna was cut into multiple 1.5-mm squares with a sterile scalpel. Two pieces from each mouse were cultured to verify infection, and eight pieces were immediately frozen in dry vials at −70°C. Mice were then treated with antibiotic for 5 days. During the 2-week antibiotic treatment and the posttreatment interval, cultures of ear biopsies were incubated and then examined for growth. Only ear pieces from donor mice with infection verified by culture were used for transplantation, and only mice with culture-verified infection were used as immune recipients. Ten days after completion of antibiotic treatment (day 30, 105, or 195 after initial inoculation), two ear pieces from immune mice were transplanted back into the autologous donor mice and two duplicate pieces from the same immune mice were transplanted into nonimmune (sham-inoculated) mice. In addition, ear pieces from sham-inoculated mice were transplanted into immune mice. Tissues were transplanted by anesthetizing mice with methoxyflurane, clipping the fur from the dorsal lumbar region, disinfecting the skin, and then making small stab incisions with a no. 11 Bard-Parker surgical blade (Becton-Dickinson, Franklin Lakes, N.J.). Two ear pieces were then inserted into the subcutis of each mouse with fine-tip forceps. The small wound made by this procedure did not require sutures and healed within a day. No adventitious infections of the surgical sites were noted.

**Histology.** Rear limbs and hearts were fixed in neutral buffered formalin, bones were demineralized, and tissues were embedded, sectioned, and examined for arthritis in knees and tibiotarsal joints and inflammation in hearts (carditis), as previously described (2, 9–11).

**Statistical analysis.** Rates of infection or disease among treatment groups were tested for independence by chi-square analysis. Antibody titers were compared among treatment groups by using Student's unpaired *t* test.

**Enzyme-linked immunosorbent assay (ELISA).** Titers of immunoglobulin G (IgG) to *B. burgdorferi* were determined for selected sera as previously described (9), by using whole *B. burgdorferi* antigen, serial twofold dilutions of sera, and peroxidase-conjugated goat anti-mouse IgG (Tago Inc., Burlingame, Calif.). Titers were expressed as reciprocal dilutions.

## RESULTS

Two types of experiments were performed to detect antigenic differences between the original *B. burgdorferi* inoculum and *B. burgdorferi* obtained from chronically infected mice. In the first experiments, actively immune mice (infected and then cured of *B. burgdorferi* infection with an antibiotic at 90 days of infection) were challenged with original and late isolates of *B. burgdorferi* cultured from mice prior to antibiotic treatment. The other experiment examined mice challenged by transplantation of infected ear punch tissue to circumvent the variables potentially inherent with in vitro culture of *B. burgdorferi*. Both types of experiment revealed no significant antigenic differences between the original inoculum and *B. burgdorferi* from chronically infected mice.

**Immunity to challenge with original and late *B. burgdorferi* isolates.** Serum collected from mice after 1 year of infection has been shown to be equally effective in passive immunization against original and late isolates of *B. burgdorferi* (8). In the current experiment, I sought to extend this finding by challenging actively immune mice with different doses of original and late isolates of *B. burgdorferi* to determine whether late isolates have an infecting advantage in immune hosts. Mice were inoculated intradermally with $10^4$ cloned (original) *B. burgdorferi* bacteria or sham inoculated with an equivalent volume of sterile BSK II medium. At 90 days after inoculation, mice were anesthetized, ear punch biopsies were collected and cultured, and then all mice (both infected and sham inoculated) were treated with an antibiotic for 5 days. Four 90-day *B. burgdorferi* isolates (late isolates L1 to L4) from these mice were randomly selected for study. The 90-day interval was chosen because it is an interval at which immune-mediated disease regression is complete in mice (9, 11), ELISA IgG antibody titers are high, Western blots (immunoblots) reveal reactivity to numerous antigens, and protective antibody concentrations in serum are declining (relative to peak levels at day 30) (8). On day 105, 10 days after completion of antibiotic treatment, mice were randomly divided into groups of four (Table 1) as follows: sham-inoculated (nonimmune) mice were challenged intradermally with $10^2$ or $10^4$ original, L1, L2, L3, or L4 *B. burgdorferi* bacteria; infected, sterilized (immune) mice were challenged with the same isolates at $10^1$, $10^2$, $10^3$, or $10^4$ spirochetes per mouse; and a control immune group of two mice were sham challenged with medium. In addition, the individual immune mice from which L1 to L4 *B. burgdorferi* bacteria were isolated were challenged with $10^4$ bacteria of their autologous late *B. burgdorferi* isolates. Mice were killed on day 120 (15 days after challenge). Blood and spleens were cultured, and joints and hearts were examined for histopathology ($10^4$-bacterium dose groups only).

All nonimmune mice became infected when challenged with either $10^2$ or $10^4$ original, L1, L2, L3, or L4 *B. burgdorferi* bacteria (Table 1). In contrast, none of the

TABLE 1. Resistance of actively immunized and nonimmune mice to intradermal challenge inoculation with $10^4$ original or late (L1 to L4) *B. burgdorferi* organisms

| Immune group[a] | Challenge inoculum[b] | Rate of infection[c] | ELISA IgG titer (geometric mean ± SD [range]) |
|---|---|---|---|
| Nonimmune | Original | 4/4 | 12,178 ± 5,120 (10,240–20,480) |
| | L1 | 4/4 | 14,482 ± 5,913 (10,240–20,480) |
| | L2 | 4/4 | 14,482 ± 5,913 (10,240–20,480) |
| | L3 | 4/4 | 7,241 ± 2,956 (5,120–10,240) |
| | L4 | 4/4 | 7,241 ± 7,680 (5,120–20,480) |
| Immune | Original | 0/4 | 28,963 ± 11,824 (20,480–40,960) |
| | L1 | 0/4 | 8,611 ± 7,241 (5,120–20,480) |
| | L2 | 0/4 | 12,177 ± 37,296 (2,560–81,920) |
| | L3 | 0/3 | 97,431 ± 61,432 (40,980–163,840) |
| | L4 | 0/2 | 57,926 ± 28,963 (40,960–81,920) |
| Immune | Sham | 0/2 | 327,680 ± 0 |
| Immune-autologous donor mice[d] | | | |
| 1 | L1 | 0/1 | 20,480 |
| 2 | L2 | 0/1 | 5,120 |
| 3 | L3 | 1/1 | 1,280 |
| 4 | L4 | 0/1 | Not done |

[a] Mice were actively immunized by intradermal inoculation with $10^4$ cloned (original) *B. burgdorferi* organisms (immune) or sham inoculated with BSK II medium (nonimmune) and then treated with an antibiotic for 5 days, commencing on day 90 of infection.

[b] Mice were challenged intradermally with different doses of original *B. burgdorferi* or with one of four late isolates (L1 to L4) obtained by culture of ear punches prior to antibiotic treatment or sham challenged with BSK II medium on day 105 after the initial inoculation.

[c] Number of infected mice/number tested by culture and histologic analysis on day 15 after challenge. Only data from the group challenged with the highest dose ($10^4$ bacteria) are shown.

[d] Immune-autologous mice were individuals from which L1, L2, L3, and L4 isolates were cultured that were then challenged with their own autologous isolates.

immune mice became infected at any dose ($10^1$, $10^2$, $10^3$, or $10^4$ bacteria) of either original, L1, L2, L3, or L4 isolates of *B. burgdorferi*. Of the four mice that were challenged with their autologous late isolates, one was culture positive and had active arthritis and carditis. Joints and hearts of all of the mice challenged with $10^4$ organisms were examined microscopically. Carditis, the most consistent indicator of *B. burgdorferi*-induced disease in mice (2, 9–11), was present in all 20 mice of the nonimmune groups, whereas arthritis was variably expressed, involving approximately half of the mice. None of 20 immune mice, except the single animal noted above, had either carditis or arthritis. Neither of the two mice in the immune, sham-challenged group was infected or had carditis or arthritis.

Because one of the four immune mice became infected and developed both carditis and arthritis following challenge with its autologous L3 isolate, the serological responses of immune and nonimmune mice at 15 days following challenge were examined and compared with that of the single infected immune mouse (Table 1). For unknown reasons, the single infected immune mouse had a remarkably lower ELISA IgG titer at 15 days after challenge inoculation than all of the other mice, which could explain its atypical susceptibility to reinfection. Immune mice challenged with the original inoculum developed higher antibody titers than the corresponding nonimmune mice ($P \leq 0.05$), and immune mice challenged with L3 and L4 isolates developed higher antibody titers than the corresponding nonimmune mice ($P \leq 0.01$ and 0.05, respectively). Notably higher IgG titers were present among sham-challenged immune mice than all other groups ($P \leq 0.001$).

**Immunity to challenge with autologous late isolates.** Since the first experiment revealed a single culture- and disease-positive immune mouse among four mice challenged with its own autologous late *B. burgdorferi* isolate, this experiment sought to confirm or negate the significance of this finding. By a similar experimental design, mice were inoculated intradermally with $10^4$ (original) *B. burgdorferi* bacteria or sham inoculated with sterile medium. At 90 days, ear punch biopsies were cultured and antibiotic treatment of all mice for 5 days was commenced. On day 105, 18 immune mice were challenged intradermally with $10^4$ bacteria of their autologous late isolates, 18 nonimmune mice were challenged with each of the 18 late isolates, and 10 immune mice were sham challenged (Table 2). Mice were killed on day 120 (15 days after challenge), and resistance to infection was assessed by culture of blood, spleen tissue, and urinary bladder tissue. All of the immune mice challenged with their autologous late isolates resisted infection. All of the nonimmune mice challenged with the same 18 late isolates became infected, verifying the infectivity of all of the late isolates.

TABLE 2. Resistance of actively immunized and nonimmune mice to intradermal challenge inoculation with autologous late isolates of *B. burgdorferi*

| Immune group[a] | Challenge inoculum[b] | Rate of infection[c] |
|---|---|---|
| Nonimmune | Late isolates 1–18 | 18/18 |
| Immune autologous | Late isolates 1–18 | 0/18 |
| Immune | Sham | 0/10 |

[a] Mice were actively immunized by intradermal inoculation with $10^4$ cloned *B. burgdorferi* organisms (immune) or sham inoculated with BSK II medium (nonimmune) and then treated with an antibiotic for 5 days, commencing on day 90 of infection.

[b] Mice were challenged intradermally with 1 of 18 isolates obtained by culture of ear punches prior to antibiotic treatment or sham challenged with BSK II medium on day 105 after the initial inoculation. Immune-autologous mice were challenged with their own autologous late isolates.

[c] Number of infected mice/number tested by culture on day 15 after challenge.

TABLE 3. Resistance of actively immune and nonimmune mice to challenge with tissue transplants from mice infected with *B. burgdorferi* for 15, 90, or 180 days or from sham-inoculated mice

| Transplant donor,[a] time (days) postinfection | Challenge transplant recipient,[b] time (days) postinfection | Culture[c] | Disease[d] |
|---|---|---|---|
| Infected, 15 | Immune, 30 | 6/7 | 1/7 |
| Sham inoculated, 15 | Immune, 30 | 0/4 | 0/4 |
| Infected, 15 | Nonimmune, 30 | 5/5 | 5/5 |
| Infected, 90 | Immune, 105 | 0/10 | 0/10 |
| Sham inoculated, 90 | Immune, 105 | 0/5 | 0/5 |
| Infected, 90 | Nonimmune, 105 | 3/5 | 3/5 |
| Infected, 180 | Immune, 195 | 0/5 | 0/5 |
| Sham inoculated, 180 | Immune, 195 | 0/5 | 0/5 |
| Infected, 180 | Nonimmune, 195 | 3/3 | ND |

[a] Ear pieces were collected from mice at 15, 90, or 180 days after intradermal inoculation with cloned *B. burgdorferi* or sham inoculation with BSK II medium. Infection was verified by culture of two pieces of ear tissue from each mouse, while other pieces were frozen at −70°C.

[b] Recipient mice, which were the same mice as the transplant donors (immunized by infection with *B. burgdorferi* or sham inoculated), were treated with an antibiotic for 5 days, commencing on day 15, 90, or 180. Mice were challenged subcutaneously with two freezer-stored ear pieces 10 days after completion of antibiotic treatment. Immune mice challenged with infected ear pieces received autografts. Nonimmune mice challenged with infected ear pieces received duplicate samples from tissues used to challenge immune mice.

[c] Number of infected mice/number tested by culture on day 15 after challenge.

[d] Number of diseased mice/number examined for carditis and arthritis on day 15 after challenge. ND, not done.

None of the cultures from immune, sham-challenged mice were positive, verifying the effectiveness of the antibiotic treatment.

**Immunity to challenge with early and late ear punch autografts.** Since it could be argued that potential *B. burgdorferi* antigenic changes may revert during the required 2-week in vitro culture period, the next experiment sought to verify challenge immunity to infection by transplantation of infected autografts that were not subjected to the culture variable. In this experiment (Table 3), mice were inoculated intradermally with $10^4$ *B. burgdorferi* organisms or sham inoculated with sterile medium. On day 15, 90, or 180, groups of 15 infected and 5 sham-inoculated mice were anesthetized, and ear pinnae were biopsied, cultured, and frozen (see Materials and Methods). All of the mice were then subjected to antibiotic treatment for 5 days. Ten days after completion of the antibiotic treatment (day 30, 105, or 195 after inoculation, depending on the group), ear pieces from up to 10 infected mice (numbers depended upon culture verification) were transplanted back into the autologous (now immune) donor mice; pieces from up to five infected mice (numbers depended upon culture verification) were transplanted into up to five age-matched, nonimmune (sham-inoculated) mice. In addition, ear pieces from five nonimmune (sham-inoculated) mice were transplanted into five immune mice. Thus, the experimental design examined active immunity to challenge infection by tissue transplantation from mice infected for 15, 90, or 180 days. Fifteen days after transplantation, mice were killed; blood, spleen tissue, bladder tissue, and transplanted tissue (collected at the site of subcutaneous transplantation) were cultured, and joints and hearts were examined microscopically for disease. The final numbers of mice in each experimental group (Table 3) varied because only tissues from mice with infections verified by culture and only recipient mice with verified transplanted tissue (visible in the subcutis) at the time of necropsy were utilized. Other mice were discarded from the protocol.

Among the mice biopsied and treated at 15 days after initial infection, challenged by transplantation back into the autologous donor mouse resulted in reinfection of six of seven immune mice. Only one of these seven immune mice had carditis as well as arthritis, which included multiple joints. The other six immune mice had no active disease. In contrast, all five nonimmune mice challenged by transplantation of infected tissue developed infection and disease (five of five had carditis, and four of five had arthritis), and none of the four immune mice that received tissue transplants from sham-inoculated mice became infected or developed disease. Thus, in this earliest biopsy interval immune mice that received infected autografts had a significantly higher rate of reinfection but not disease compared with immune controls transplanted with uninfected tissue ($P \leq 0.01$). Immune mice that received infected autografts had significantly lower rates of disease than nonimmune mice that received infected transplants ($P \leq 0.001$). Among 10 mice biopsied and treated at 90 days after initial infection, transplant challenge into the autologous donor mice resulted in no evidence of infection or disease. In contrast, three of five nonimmune mice challenged by transplantation of infected tissue became infected and had both carditis and arthritis ($P \leq 0.01$). None of the five immune mice challenged by transplantation of uninfected tissue was infected or developed disease. Among five mice biopsied and treated at 180 days after initial infection, challenge by transplantation into the autologous donor mice resulted in no infection or disease, whereas transplantation of infected tissue into three nonimmune mice resulted in infection of all mice ($P \leq 0.01$). Immune mice challenged by transplantation of tissue from uninfected mice were not infected. Although transplanted tissues were usually culture positive when other tissue sites were also positive, in no case were transplants culture positive in animals with culture-negative status of other organs. Thus, spirochetes did not remain viable in tissue transplanted into immune mice. These results indicated that mice were immune to reinfection when challenged with autologous infected tissues at 90 or 180 days after infection, but mice cured of *B. burgdorferi* infection with an antibiotic at 15 days after infection were still susceptible to reinfection by this method.

## DISCUSSION

The results of the present study indicate that mice made actively immune by low-dose intradermal infection with *B. burgdorferi* and then cured of *B. burgdorferi* infection with an antibiotic become resistant to reinfection with the *B. burgdorferi* strain they were originally exposed to, as well as isolates of the organism obtained late in the course (90 days) of their own infection. To eliminate the possibility that in vivo antigenic modulation might be obscured by in vitro reversion during the culture period, we extended these findings with tissue transplants, thus eliminating the need for culture. Resistance to infected autografts was present in mice with tissues collected as late as 180 days of infection. These results complemented studies with sera collected from actively infected mice at intervals of up to 1 year after infection in which naive mice were passively protected equally well against challenge with either original or autologous late isolates of *B. burgdorferi* (8).

Results of the present study revealed that one of four immune mice challenged with their own autologous late *B. burgdorferi* isolates became reinfected and developed disease. This potentially significant finding prompted further analysis of this phenomenon, which indicated that the result appeared to be an anomaly rather than a more universal finding. Serological studies of this mouse revealed that it mounted a significantly weaker antibody response than all of the other infected mice in this study, which probably explained its susceptibility to reinfection. This animal's serum reacted against the same *B. burgdorferi* proteins as the sera of the other mice (data not shown). An additional experiment in which 18 mice were cured of *B. burgdorferi* infection with an antibiotic and then challenged with their own autologous 90-day isolates (similar to the single aberrant mouse mentioned above) and an experiment in which mice were challenge infected by autografting of infected skin both indicated that mice are resistant to reinfection with their own late isolates of *B. burgdorferi*.

The serological responses of immune and nonimmune mice to challenge infection with original and late isolates revealed several interesting findings. Immune mice challenged with the original inoculum and two of four late isolates developed significantly higher anti-*B. burgdorferi* IgG titers than did corresponding nonimmune mice challenged with the same isolates. This effect may be due to the transient immunosuppressive effect that workers in our laboratory have observed in mice during the early phase of infection with *B. burgdorferi* (15). Immune mice would presumably be over this effect, or at least further along toward recovery, than actively infected, nonimmunized mice. Immune, sham-challenged mice had higher antibody titers than both immune and nonimmune *B. burgdorferi*-challenged mice, suggesting that challenge inoculation of immune mice, even though they do not become infected, may have an immunosuppressive effect as well.

It appears very unlikely that *B. burgdorferi* undergoes any significant antigenic modulation which could explain its persistence in immunocompetent hosts. This is very much unlike the genetic and antigenic fluctuations which are integral to the immune evasion and survival of *B. hermsii*, the agent of louse-borne relapsing fever (6, 7). Genetic and antigenic variation certainly occurs among isolates of *B. burgdorferi* and isolates from different geographic areas (12, 26, 34), following extended in vitro passage (30, 42), or following in vitro incubation with immune sera (35, 36). Recombination between homologous outer surface protein (Osp)-encoding genes, with deletions and chimeric gene fusions, have been demonstrated with *B. burgdorferi*, with resultant variation of Osp expression (34). Growth of *B. burgdorferi* in the presence of anti-Osp antibody can induce antibody-resistant variants which lack specific Osp expression, have Osp gene mutations, or have lost entire Osp genes (35, 36). Prior to the cloning of *B. burgdorferi* N40, it was discovered that subpopulations within the infecting population contained a stop codon in the *ospB* gene, resulting in expression of a truncated *OspB* protein that lacked the protective epitope in the carboxyl terminus and conferred a survival advantage on OspB-immunized mice (18). Despite these findings, they do not seem to be relevant to, or at least necessary for, in vivo mechanisms of *B. burgdorferi* survival.

Apparent antigenic differences have been noted in *B. burgdorferi* organisms isolated from persistently infected white-footed and laboratory mice compared with the original inoculum (43, 45). In both studies, sera from persistently infected mice reacted differently on immunoblots against the original inoculum compared with late autologous isolates. Sera appeared to be more reactive against the original inoculum. These data should be interpreted with caution, as mice in both studies were originally inoculated with uncloned *B. burgdorferi*. Our laboratory recently demonstrated significant differences in protein expression among late *B. burgdorferi* isolates from mice originally infected with uncloned organisms, but these differences were not apparent among late isolates when mice were originally infected with cloned organisms (9). Likewise, we have shown significant differences in OspB in subpopulations of uncloned *B. burgdorferi* N40 (18). Studies examining antigenic changes in *B. burgdorferi* during persistent infections must be initiated with clonal populations of the organism, as was done in the present study.

Immunity to *B. burgdorferi* has a number of critical stages in the immunocompetent-mouse model, which presumably parallels Lyme borreliosis in other species. Following intradermal inoculation of mice with low numbers of *B. burgdorferi* bacteria, mice develop disseminated infections, resulting in inflammation of musculoskeletal tissues, the heart, and vessels. Disease in this early phase of infection is associated with the invasion and presence of relatively large numbers of visually discernible spirochetes (2, 9–11). At 4 to 12 weeks after infection, the disease undergoes resolution, with disappearance of most spirochetes from these diseased tissues (9). This event is immune mediated, because disease progresses rather than regresses in mice with severe combined immunodeficiency (11). Despite disease resolution and clearance of spirochetes from target tissues, infection persists in most, if not all, mice for periods of at least 1 year (9). During this period, mice mount a strong humoral immune response to *B. burgdorferi*. Antibody recognition of *B. burgdorferi* antigens is significantly influenced by inoculum size in experimental animals (8, 9, 33, 37). When mice are inoculated with low doses of spirochetes, only a few proteins are labelled on immunoblots, particularly 22-, 39-, and 41-kDa proteins, in the early stages of infection, followed by later response to the 31- and 34-kDa OspA and OspB proteins, among others (8, 9). Serum contains passive immunizing activity which peaks at 30 days and then gradually declines. Thus, the protective activity of serum may represent antibody to only a few major proteins, but this needs more intensive evaluation. Passively transferred serum from infected mice can effectively protect naive mice from challenge inoculation with *B. burgdorferi*, but only if administered prior to or at the time of inoculation, not after (8). Serum protective activity peaks at an interval corresponding to the time of arthritis and carditis regression, suggesting that the humoral response is responsible for this event. The role of T-cell immunity in these events is not known, but minimal antigen-specific T-cell responses can be demonstrated in vitro with splenocytes or lymph node cells from infected mice, and no delayed-type hypersensitivity reaction can be elicited in mice intradermally infected with low doses of spirochetes (15, 38). These responses can be elicited with high doses of *B. burgdorferi* (38), but this does not parallel the natural disease. Furthermore, adoptive transfer of T-enriched cells is ineffective at protecting naive mice against challenge infection or development of disease, in contrast to protection by small amounts of immune serum (15).

Borreliacidal activity exists in the serum of a persistently infected host but is ineffective at eliminating existing infection, including infection established in naive mice for only 24 h (8). This argues for an immediate adaptive response on the

part of the spirochete which is not immunologically driven. It has been speculated that spirochetes may localize intracellularly, including within endothelial cells, fibroblasts, and macrophages (19, 25, 27, 48). However, spirochetes that can be visualized within animal or human tissue appear to be primarily extracellular (2, 9–11, 14, 16, 17). We have recently shown that the most common site for detection and isolation of *B. burgdorferi* from mice at 1 year of infection is the skin, and spirochetes reside extracellularly in the dermal collagen without an apparent local host response (9). These findings suggest that the organism is in some way antigenically inert and not concealed in an immunologically privileged site.

For unexplained reasons, spirochetes appear to redisseminate periodically, causing recurrent transient joint and heart disease which correlates with the reappearance of spirochetes in these target tissues (9). Dissemination and disease recurrence occur during a stage of infection in which serum neutralizing activity steadily declines (8), but cause and effect have not been proven. Recurrent spirochetemia and disease could be explained on the basis of antigenic modulation, as in relapsing fever, but the current study seems to argue against this possibility with *B. burgdorferi*. Pressing issues in Lyme borreliosis are the sites of spirochete persistence and the mechanisms of immune evasion by the organism.

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## REFERENCES

1. **Appel, M. J. G., S. Allan, R. H. Jacobson, T. L. Lauderdale, Y. F. Chang, S. J. Shin, J. W. Thomford, R. J. Todhunter, and B. A. Summers.** 1993. Experimental Lyme disease in dogs produces arthritis and persistent infection. J. Infect. Dis. **167:** 651–664.
2. **Armstrong, A. L., S. W. Barthold, D. H. Persing, and D. S. Beck.** 1992. Carditis in Lyme disease susceptible and resistant strains of laboratory mice infected with *Borrelia burgdorferi*. Am. J. Trop. Med. Hyg. **47:**249–258.
3. **Asbrink, E., and A. Hovmark.** 1985. Successful cultivation of spirochetes from skin lesions of patients with erythema chronica migrans afzelius and acrodermatitis chronica atrophicans. Acta Pathol. Microbiol. Immunol. Scand. **93:**161–163.
4. **Asbrink, E., A. Hovmark, and B. Hederstedt.** 1984. The spirochetal etiology of acrodermatitis chronica atrophicans Herxheimer. Acta Dermato-Venereol. **64:**506–512.
5. **Barbour, A. G.** 1984. Isolation and cultivation of Lyme disease spirochetes. Yale J. Biol. Med. **57:**521–525.
6. **Barbour, A. G.** 1991. Molecular biology of antigenic variation in Lyme borreliosis and relapsing fever: a comparative analysis. Scand. J. Infect. Dis. Suppl. **77:**88–93.
7. **Barbour, A. G., C. J. Carter, N. Burman, C. S. Freitag, C. F. Garon, and S. Bergstrom.** 1991. Tandem insertion sequence-like elements define the expression sites for variable antigen genes of *Borrelia hermsii*. Infect. Immun. **59:**390–397.
8. **Barthold, S. W., and L. K. Bockenstedt.** 1993. Passive immunizing activity of sera from mice infected with *Borrelia burgdorferi*. Infect. Immun. **61:**4696–4702.
9. **Barthold, S. W., M. S. de Souza, J. L. Janotka, A. L. Smith, and D. H. Persing.** 1993. Chronic Lyme borreliosis in the laboratory mouse. Am. J. Pathol. **143:**959–971.
10. **Barthold, S. W., D. H. Persing, A. L. Armstrong, and R. A. Peeples.** 1991. Kinetics of *Borrelia burgdorferi* dissemination and evolution of disease after intradermal inoculation of mice. Am. J. Pathol. **139:**263–272.
11. **Barthold, S. W., C. L. Sidman, and A. L. Smith.** 1992. Lyme borreliosis in genetically resistant and susceptible mice with severe combined immunodeficiency. Am. J. Trop. Med. Hyg. **47:**605–613.
12. **Boerlin, P., O. Peter, A.-G. Bretz, D. Postic, G. Baranton, and J.-C. Piffaretti.** 1992. Population genetic analysis of *Borrelia burgdorferi* isolates by multilocus enzyme electrophoresis. Infect. Immun. **60:**1677–1683.
13. **Callister, S. M., R. F. Schell, K. L. Case, S. D. Lovrich, and S. P. Day.** 1993. Characterization of the borreliacidal antibody response to *Borrelia burgdorferi* in humans: a serodiagnostic test. J. Infect. Dis. **167:**158–164.
14. **Defosse, D. L., P. H. Duray, and R. C. Johnson.** 1992. The NIH-3 immunodeficient mouse is a model for Lyme borreliosis myositis and carditis. Am. J. Pathol. **141:**3–9.
15. **de Souza, M. S., A. L. Smith, D. S. Beck, G. A. Terwilliger, E. Fikrig, and S. W. Barthold.** 1993. Long-term study of cell-mediated responses to *Borrelia burgdorferi* in the laboratory mouse. Infect. Immun. **61:**1814–1822.
16. **Duray, P. H., and R. C. Johnson.** 1986. The histopathology of experimentally infected hamsters with the Lyme disease spirochete, *Borrelia burgdorferi*. Proc. Soc. Exp. Biol. Med. **181:** 263–269.
17. **Duray, P. H., and A. C. Steere.** 1988. Clinical pathologic correlations of Lyme disease by stage. Ann. N.Y. Acad. Sci. **539:**65–79.
18. **Fikrig, E., H. Tao, F. S. Kantor, S. W. Barthold, and R. A. Flavell.** 1993. *Borrelia burgdorferi* lacking the carboxyl terminus of outer surface protein B evades immune destruction. Proc. Natl. Acad. Sci. USA **90:**4092–4096.
19. **Georgilis, K., M. Peacocke, and M. S. Klempner.** 1992. Fibroblasts protect the Lyme disease spirochete, *Borrelia burgdorferi*, from ceftriaxone in vitro. J. Infect. Dis. **166:**440–444.
20. **Hovmark, A., E. Asbrink, and I. Olsson.** 1986. The spirochetal etiology of lymphadenosis benigna cutis solitaria. Acta Dermato-Venereol. **66:**479–484.
21. **Johnson, R. C., C. Kodner, and M. Russell.** 1986. Passive immunization of hamsters against experimental infection with the Lyme disease spirochete. Infect. Immun. **53:**713–714.
22. **Johnson, R. C., C. Kodner, M. Russell, and P. H. Duray.** 1988. Experimental infection of the hamster with *Borrelia burgdorferi*. Ann. N.Y. Acad. Sci. **539:**258–263.
23. **Kochi, S. K., and R. C. Johnson.** 1987. Role of immunoglobulin G in killing of *Borrelia burgdorferi* by the classical complement pathway. Infect. Immun. **56:**314–321.
24. **Kochi, S. K., R. C. Johnson, and A. P. Dalmasso.** 1991. Complement-mediated killing of the Lyme disease spirochete *Borrelia burgdorferi*. J. Immunol. **146:**3964–3970.
25. **Ma, Y., A. Surrock, and J. J. Weis.** 1991. Intracellular localization of *Borrelia burgdorferi* within human endothelial cells. Infect. Immun. **59:**671–678.
26. **Marconi, R. T., and C. F. Garon.** 1992. Phylogenetic analysis of the genus *Borrelia*: a comparison of North American and European isolates of *Borrelia burgdorferi*. J. Bacteriol. **174:**241–244.
27. **Montgomery, R. R., M. H. Nathanson, and S. E. Malawista.** 1993. The fate of *Borrelia burgdorferi*, the agent for Lyme disease, in mouse macrophages—destruction, survival, recovery. J. Immunol. **150:**909–915.
28. **Moody, K. D., S. W. Barthold, G. A. Terwilliger, D. S. Beck, G. M. Hansen, and R. O. Jacoby.** 1990. Experimental chronic Lyme borreliosis in Lewis rats. Am. J. Trop. Med. Hyg. **42:**165–174.
29. **Nadelman, R. B., C. S. Pavia, L. A. Magnarelli, and G. P. Wormser.** 1990. Isolation of *Borrelia burgdorferi* from the blood of seven patients with Lyme disease. Am. J. Med. **88:**21–26.
30. **Norris, S. J., C. J. Carter, J. K. Howell, and A. G. Barbour.** 1992. Low-passage-associated patterns of *Borrelia burgdorferi* B31: characterization and molecular cloning of OspD, a surface-exposed, plasmid-encoded lipoprotein. Infect. Immun. **60:**4666–4672.
31. **Preac-Mursic, V., E. Patsouris, B. Wilske, S. Reinhardt, B. Gross, and P. Mehraein.** 1990. Persistence of *Borrelia burgdor-*

*feri* and histopathological alterations in experimentally infected animals. A comparison with histopathological findings in human disease. Infection **18:**332–341.

32. **Preac-Mursic, V., H. W. Pfister, B. Wilske, B. Bross, A. Baumann, and J. Prokop.** 1989. Survival of *Borrelia burgdorferi* in antibiotically treated patients with Lyme borreliosis. Infection **17:**355–359.
33. **Roehrig, J. T., J. Piesman, A. R. Hunt, M. G. Keen, C. M. Happ, and B. J. B. Johnson.** 1992. The hamster immune response to tick-transmitted *Borrelia burgdorferi* differs from the response to needle-inoculated, cultured organisms. J. Immunol. **149:**3648–3653.
34. **Rosa, P. A., T. G. Schwan, and D. Hogan.** 1992. Recombination between genes encoding major outer surface proteins A and B of *Borrelia burgdorferi*. Mol. Microbiol. **6:**3031–3040.
35. **Sadziene, A., P. A. Rosa, P. A. Thompson, D. M. Hogan, and A. G. Barbour.** 1992. Antibody-resistant mutants of *Borrelia burgdorferi*: in vitro selection and characterization. J. Exp. Med. **176:**799–809.
36. **Sadziene, A., P. A. Thompson, and A. G. Barbour.** 1993. In vitro inhibition of *Borrelia burgdorferi* growth by antibodies. J. Infect. Dis. **167:**165–172.
37. **Schaible, U. E., L. Gern, R. Wallich, M. D. Kramer, M. Prester, and M. M. Simon.** 1993. Distinct patterns of protective antibodies are generated against *Borrelia burgdorferi* in mice experimentally inoculated with high and low doses of antigen. Immunol. Lett. **36:**219–226.
38. **Schaible, U. E., M. D. Kramer, C. W. E. Justus, C. Museteanu, and M. M. Simon.** 1989. Demonstration of antigen-specific T cells and histopathological alterations in mice experimentally inoculated with *Borrelia burgdorferi*. Infect. Immun. **57:**41–47.
39. **Schmidli, J., T. Hunziker, P. Moesli, and U. B. Schaad.** 1988. Cultivation of *Borrelia burgdorferi* from joint fluid three months after treatment of facial palsy due to Lyme borreliosis. J. Infect. Dis. **158:**905–906.
40. **Schmitz, J. L., R. F. Schell, A. G. Hejka, and D. M. England.** 1990. Passive immunization prevents induction of Lyme arthritis in LSH hamsters. Infect. Immun. **58:**144–148.
41. **Schmitz, J. L., R. F. Schell, S. D. Lovrich, S. M. Callister, and J. E. Coe.** 1991. Characterization of the protective antibody response to *Borrelia burgdorferi* in experimentally infected LSH hamsters. Infect. Immun. **59:**1916–1921.
42. **Schwan, T. G., W. Burgdorfer, and C. F. Garon.** 1988. Changes in infectivity and plasmid profile of the Lyme disease spirochete, *Borrelia burgdorferi*, as a result of in vitro cultivation. Infect. Immun. **56:**1831–1836.
43. **Schwan, T. G., R. H. Karstens, M. E. Schrumpf, and W. J. Simpson.** 1991. Changes in antigenic reactivity of *Borrelia burgdorferi*, the Lyme disease spirochete, during persistent infection in mice. Can. J. Microbiol. **37:**450–454.
44. **Schwan, T. G., K. K. Kime, M. E. Schrumpf, J. E. Coe, and W. J. Simpson.** 1989. Antibody response in white-footed mice (*Peromyscus leucopus*) experimentally infected with the Lyme disease spirochete (*Borrelia burgdorferi*). Infect. Immun. **57:** 3445–3451.
45. **Schwan, T. G., and W. J. Simpson.** 1991. Factors influencing the antigenic reactivity of *Borrelia burgdorferi*, the Lyme disease spirochete. Scand. J. Infect. Dis. Suppl. **77:**94–101.
46. **Snydman, D. R., D. P. Schenkern, V. P. Bernardi, C. C. Lastavica, and K. K. Pariser.** 1986. *Borrelia burgdorferi* in joint fluid in chronic Lyme arthritis. Ann. Intern. Med. **104:**798–800.
47. **Stanek, G., J. Klein, R. Bittner, and D. Glogan.** 1990. Isolation of *Borrelia burgdorferi* from the myocardium of a patient with long-standing cardiomyopathy. N. Engl. J. Med. **322:**249–252.
48. **Szczepanski, A., and J. L. Benach.** 1991. Lyme borreliosis: host responses to *Borrelia burgdorferi*. Microbiol. Rev. **55:**21–34.