Comparison of Disseminated and Nondisseminated Strains of *Borrelia burgdorferi* Sensu Stricto in Mice Naturally Infected by Tick Bite

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Clinical isolates of *Borrelia burgdorferi* sensu stricto have been categorized into disseminated and nondisseminated groups based on distinct ribosomal spacer restriction fragment length polymorphism genotypes (RSTs). In order to determine whether transmission by tick bite would alter the dissemination dynamics and disease produced by distinct genotypes, disseminated isolates (RST1), nondisseminated isolates (RST3), and a standard laboratory strain (B-31) were established in a murine cycle utilizing infections transmitted by ticks. B-31 spirochetes circulated in the blood of inbred C3H/HeJ mice longer than in the blood of outbred mice. The majority of C3H mice exposed to RST1-infected ticks contained cultivable spirochetes in their blood for up to 17 days; in contrast, mice exposed to RST3 isolates demonstrated a precipitous decline in infection after day 7 postexposure. A quantitative PCR (q-PCR) assay demonstrated that the densities of spirochetes in blood were similar for the RST1 and RST3 isolates, except during the 2nd week postexposure, when the RST1 isolates displayed a markedly higher density in blood. Spirochete load in the heart and bladder of infected mice was measured by q-PCR at 8 weeks postexposure; the numbers of spirochetes in these tissues were similar for mice infected with either disseminated or nondisseminated strains. Similarly, histopathology samples of heart, bladder, and joint tissue obtained at 8 weeks postexposure did not reveal greater pathology in mice infected with the disseminated isolates. We conclude that although the spirochetaemia induced by tick-transmitted disseminated isolates was more intense and of longer duration than that induced by nondisseminated isolates, the resultant pathologies produced by these strains were ultimately similar.

Lyme disease, caused by the spirochete *Borrelia burgdorferi*, is the most common vector-borne disease in the United States (3). Lyme disease spirochetes (*B. burgdorferi*) are transmitted by *Ixodes scapularis* and *Ixodes pacificus* ticks in the United States and *Ixodes ricinus* and *Ixodes persulcatus* ticks in Europe and Asia (7). Lyme disease usually begins with a characteristic, expanding skin lesion referred to as erythema migrans (EM), which may be accompanied by flu-like symptoms. Some patients may develop more serious sequelae such as arthritis, myocarditis, and lesions of the peripheral and central nervous system (4, 23). Although *B. burgdorferi* sensu lato is a diverse group of bacteria representing up to 11 named genospecies, only three pathogenic genospecies (*B. burgdorferi* sensu stricto, *Borrelia afzelii*, and *Borrelia garinii*) are commonly known to cause human disease (10, 16). All three agents of Lyme disease are found in Europe and are associated with distinct clinical manifestations (24, 25); in contrast, *B. burgdorferi* sensu stricto is the sole agent associated with human cases of Lyme disease reported from North America (14).

*B. burgdorferi* sensu stricto isolates cultured from Lyme disease patients in Westchester County, N.Y., have been categorized into three distinct ribosomal spacer restriction fragment length polymorphism genotypes (RSTs) based on analysis of the 16S-23S ribosomal DNA (rDNA) spacer region (8, 11, 12). Wormser et al. (29) found that a significantly higher number of Lyme disease patients infected with RST1 strains had *B. burgdorferi*-positive blood cultures and a higher frequency of multiple EM than did patients infected with either RST2 or RST3 strains. Thus, RST1 isolates were judged to be disseminating and RST3 isolates nondisseminating.

A murine model utilizing C3H/HeJ mice was established to study the kinetics of hematogenous dissemination and disease severity of infection with disseminating and nondisseminating genotypes (26, 27). Wang et al. (26) used C3H/HeJ mice to study the dissemination and pathogenicity for single RST1 and RST3 isolates. The RST1 isolate demonstrated remarkably higher densities in plasma, heart, ear, and joint tissue than the RST3 isolate. In addition, severe arthritis and aortitis were detected only in mice infected with the RST1 strain. Wang et al. (27) subsequently expanded these studies to include five isolates from the RST1 group and five isolates from the RST3 group. In both experimental studies by Wang et al. (26, 27), mice were infected via intradermal inoculation of cultured spirochetes. It has been well established that the antigenic profile and subsequent host reaction to cultured spirochetes inoculated artificially via needle differ substantially from those of spirochetes delivered naturally via tick bite (5, 18, 20, 31). Accordingly, the goal of the present study was to compare the disseminations of RST1 and RST3 group spirochetes delivered to mice via tick bite. Spirochete densities were determined in the blood and specific target organs, and the resultant pathology was determined by standard microscopy.

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MATERIALS AND METHODS

Mice. All 8-week-old female C3H/HeJ mice were purchased from the National Cancer Institute (Bethesda, Md.) and housed under pathogen-free conditions. All 8-week-old female Imperial Cancer Research Fund (ICRF) outbred mice were derived from a special pathogen-free colony maintained at the Centers for Disease Control and Prevention (CDC), Fort Collins, Colo. The Animal Care and Use Committee of the CDC approved all tick feedings conducted on mice and animal treatment protocols.

Infection of mice with B. burgdorferi strain B-31. Outbred (n = 17) and C3H/HeJ (n = 21) mice were infected by feeding 10 laboratory-reared nymphal I. scapularis ticks infected with B. burgdorferi strain B-31 (infection rate, ≥90%) on individual mice as previously described (15). B. burgdorferi sensu stricto isolates. Six clinical isolates of B. burgdorferi sensu stricto were used in this study. These isolates were obtained from G. P. Wormser (Division of Infectious Diseases, Department of Medicine, New York Medical College) and originated from specimens obtained from patients diagnosed with Lyme disease in Westchester County, N.Y. (12, 29). Isolates were grown in Barbour-Stoenner-Kelly-H (BSK-H; Sigma-Aldrich Co., St. Louis, Mo.) medium from seed stock (passage 2), passed twice, and inoculated intradermally into mice (passage 4). Isolates were identified as either RST1 (isolates 142, 154, and 161) or RST3 (isolates 151, 169, and 173) as determined by Hinfl and MseI digestion profiles of the 16S-23S rDNA intergenic spacer (11, 12).

C3H/HeJ mice were randomly divided into six groups of five mice per group. B. burgdorferi was cultured in BSK-H medium at 33°C for 6 to 7 days. Cultures were examined by dark-field microscopy, and the number of motile spirochetes was enumerated. All cultures were adjusted to a final concentration of 10⁹ spirochetes/ml in BSK-H medium. Individual mice were inoculated intradermally with 132 to 250 μl of culture medium at the dorsal midline, between the scapulae. Twenty-one days after inoculation, all mice were ear biopsied and ear tissue was surface sterilized and cultured in BSK-H medium as previously described (22). All 30 mice were positive on culture by dark-field microscopy.

Four weeks after inoculation, one mouse from each group was infested with approximately 250 uninfected I. scapularis larvae from a spirochete-free colony to produce infected nymphs as described previously (15). Replete larvae were collected, held at 21°C and 95% relative humidity, exposed to a photoperiod of 16 h of light and 8 h of darkness, and allowed to molt to nymphs. Ten nymphs from each of six different mice were surface sterilized and cultured in BSK-H medium to determine infection rates in resultant nymphs as described previously (6). Cultures were read every 7 days for 4 weeks by dark-field microscopy. Two nymphal batches from each of the two RST types with the highest infection rates were used in subsequent experiments (strains 161 and 169 were omitted). Eighty C3H/HeJ mice were randomly divided into four groups and challenged with 10 I. scapularis nymphs representing each of the four strains: two RST1 strains (strains 142 and 154) and two RST3 strains (strains 151 and 173).

Collection and culture of blood samples from mice. Approximately 200 μl of whole blood was collected from mice by cheek punch on days 0, 4, 7, 10, 14, 17, 24, and 28 after the last tick fed to repletion and placed directly into BSK-H medium to detect infection with viable spirochetes. Significantly higher (74%) (chi-square 200.28; P < 0.001) and 17 (chi-square 200.001) than the proportion of outbred mice (42%) over the same time points (Fig. 1). At their initial appearance, spirochetes in C3H/HeJ mice were detected in blood at a higher proportion and persisted for a longer period of time than in outbred mice. The maximum proportion of detectable spirochetes occurred on day 4, with 100% of C3H/HeJ mice and 84% of outbred mice culture positive. The proportion of mice positive was significantly higher for C3H/HeJ mice on days 0 (chi-square = 17.61; P = 0.001), 14 (chi-square = 11.5; P = 0.001) and 17 (chi-square = 6.74; P = 0.01). Finally, ear and bladder tissue samples cultured from outbred and C3H/HeJ mice at 8 weeks postexposure were positive on culture for all mice tested. Due to their greater sensitivity to hematogenous dissemination of spirochetes, C3H/HeJ mice were used in all subsequent experiments.

Culture of B. burgdorferi RST1 or RST3 isolates from blood of infected C3H/HeJ mice. In a preliminary experiment, outbred and C3H/HeJ mice were challenged by exposure to 10 B-31-infected nymphal I. scapularis ticks per animal. Strain B-31 is a representative of the RST1 (disseminating) group. Replete nymphs were collected, surface sterilized, and cultured in BSK-H medium, and they yielded an infection rate of 89%. Approximately 200 μl of whole blood was obtained from individual mice and placed directly into BSK-H medium at nine different time points over a 1-month period. The proportion of C3H/HeJ mice that were culture positive over the nine time points was significantly higher (74%) (chi-square = 30.28; P < 0.001) than the proportion of outbred mice (42%) over the same time points (Fig. 1). At their initial appearance, spirochetes in C3H/HeJ mice were detected in blood at a higher proportion and persisted for a longer period of time than in outbred mice. The maximum proportion of detectable spirochetes occurred on day 4, with 100% of C3H/HeJ mice and 84% of outbred mice culture positive. The proportion of mice positive was significantly higher for C3H/HeJ mice on days 0 (chi-square = 17.61; P = 0.001), 14 (chi-square = 11.5; P = 0.001) and 17 (chi-square = 6.74; P = 0.01). Finally, ear and bladder tissue samples cultured from outbred and C3H/HeJ mice at 8 weeks postexposure were positive on culture for all mice tested. Due to their greater sensitivity to hematogenous dissemination of spirochetes, C3H/HeJ mice were used in all subsequent experiments.

Culture of either B. burgdorferi RST1 or RST3 isolates from blood of infected C3H/HeJ mice. C3H/HeJ mice were challenged with 10 nymphal I. scapularis ticks infected with either B. burgdorferi

FIG. 1. Percentages of infected C3H/HeJ mice (number of culture-positive/near of mice examined) versus outbred mice over a 1-month period. The time points shown represent the days after the last tick fed to repletion. Mice were naturally infected by B-31-infected nymphal tick bite, and ~200 μl of whole blood was cultured in BSK-H medium to detect infection with viable spirochetes. Significant differences (P < 0.05) in infection rates among C3H and outbred mice occurred at days 0, 14, and 17 (†).
RST1 group spirochetes (strains 142 and 154) or RST3 (strains 151 and 175). An average of 6.2 replete nymphs was recovered from individual mice. These replete nymphs yielded an infection rate of 72% after being surface sterilized and cultured in BSK-H medium. Approximately 200 µl of whole blood was cultured in BSK-H medium at the previously mentioned time points (12 of 20 mice for each strain). Infection rates in blood of mice challenged with ticks infected with RST1 strains 142 and 154 were nearly identical (Fig. 2). In general, overlapping infection rates were also observed for mice infected with RST3 strains 151 and 173. The average infection rates for RST1 isolates 142 and 154 remained quite high at 94% through day 18, before a dramatic decline to 17% by day 21. In marked contrast, the proportion infected from RST3 isolates decreased rapidly after day 7 and was below detectable limits by day 14 with strain 173 and by day 17 with strain 151. Overall, a significantly higher proportion of mice exposed to RST1 strains were infected than were RST3-exposed mice on days 10 (chi-square = 16.39; P ≤ 0.001), 14 (chi-square = 30.14; P ≤ 0.001), and 17 (chi-square = 44.16; P ≤ 0.001).

Quantification of spirochetes in blood of C3H/HeJ mice. At days 0, 4, 7, 10, 14, 17, 21, 24, and 28, blood samples from two mice infected with RST1 strain 142 or RST3 strain 151 were analyzed by quantitative PCR (q-PCR) for B. burgdorferi DNA to determine spirochete load. RST1 strain 154 and RST3 strain 173 were not evaluated due to an insufficient number of ticks. TaqMan values were quantified as the number of spirochetes/0.1 ml of whole blood. During days 0 to 7 postexposure and on days 14 to 28, the numbers of detectable spirochetes for both strains were nearly identical (Fig. 3). However, the number of spirochetes for RST1 strain 142 was more than five times the number detected for RST3 strain 151 on day 10 postexposure.

Spirochete load in C3H/HeJ target organs 8 weeks after tick infestation. Eight weeks after ticks fed to repletion, spirochete load was assessed in bladder and heart tissue by q-PCR. There was no significant overall difference in the densities of spirochetes in bladder (Student’s t test; P = 0.154) and heart (Student’s t test; P = 0.124) tissue between the combined RST1 strains and the RST3 strains (Table 1). The lowest density of spirochetes observed in the bladder and heart tissue was observed in mice exposed to RST1 strain 154.

Pathology induced by RST1 and RST3 strains of B. burgdorferi in target organs of C3H/HeJ mice. Histopathology of the bladder, heart, and joint tissues was analyzed 8 weeks after tick infestation for 12 mice from each strain (n = 48). All mice analyzed were positive for B. burgdorferi by ear biopsy culture as determined by dark-field microscopy. In terms of the bladder, 21 of 24 mice receiving RST1 strains displayed lesions of the bladder; 23 of 24 mice receiving RST3 strains also demonstrated a nodular lymphoid cystitis of the submucosa, a lesion described previously with tick-transmitted infection in C3H mice (30). Although in descriptive terms the lesions were similar upon histopathological examination, there was a significant difference in the numbers of perivascular lymphoid nodules seen when we compared RST1 strain 154 (average of 1 ± 0.89) to either RST3 strain 151 (average of 2.4 ± 0.9 [Student’s t test]; P = 0.001) or strain 173 (average of 2.3 ± 0.1 [Student’s t test]; P = 0.005). No significant difference in perivascular lymphoid nodules within the submucosa was noted when RST1 strain 142 was compared to either RST3 strain. In terms of lesions within the heart, 24 of 24 (100% infected) mice receiving RST1 strains and 22 of 24 (91.7% infected) mice receiving RST3 strains demonstrated histopathologic lesions. Moreover, similar patterns of pathology induced by either RST1 or RST3 were noted: a perivasculitis of the great vessels and a disseminated myocarditis in the superior portion of the heart as described previously (30). There was no qualitative difference in the severity of the lesions induced by either RST1 or RST3.
strains. No joint pathology was noted in any animal receiving either the RST1 or RST3 strains 8 weeks after tick infestation, although four animals receiving RST1 strains developed lesions adjacent to the joint capsule, one mouse infected with strain 154 developed a mild myositis just superior to the femuro-tibial joint, and three mice infected with strain 142 demonstrated either a mild periostitis just inferior to the joint capsule or mild inflammation of the musculature just superior to the joint capsule.

DISCUSSION

In Europe, the distinct genospecies of B. burgdorferi sensu lato (B. burgdorferi sensu stricto, B. afzelii, and B. garinii) have been associated with distinct clinical manifestations (1, 24, 25). In general, isolates from patients suffering from the skin disease acrodermatitis chronica atrophicans have proven to be B. afzelii, isolates from patients suffering from neurological disease were B. garinii, and the few isolates from patients with arthritis were B. burgdorferi sensu stricto. In North America, all of the human isolates reported to date have been B. burgdorferi sensu stricto (14). Clearly, North America strains of B. burgdorferi sensu stricto can cause the full spectrum of skin, neurological, joint, and cardiac disease (4, 23) associated with Lyme disease in North America. However, there is great diversity within the genospecies B. burgdorferi sensu stricto in North America (13), and this variability may be associated with clinical outcome.

Outer surface protein C (OspC) is an extremely variable protein in B. burgdorferi: the variability of OspC has received intense scrutiny, and at least 21 major genetic groups or clones of OspC have been described among isolates of B. burgdorferi sensu stricto in North America (17, 21). Interestingly, 15 major OspC groups were isolated from patients with primary EM lesions, but only 4 of these major OspC groups (A, B, I, and K) have been linked to isolates obtained from the blood or spinal fluid of patients with presumably invasive or disseminated disease (21). Similarly, three major groups have been defined among clinical isolates of B. burgdorferi sensu stricto based on ribosomal spacer restriction fragment length polymorphism analysis of the 16S-23S rDNA spacer region; RST1 group isolates were found to be associated with disseminated disease, and RST3 isolates were associated with nondisseminated disease characterized by single EM lesions (11, 12, 29). Clearly, host genetic background (2, 9, 28), as well as the genetic background of the infecting B. burgdorferi strain, may play a role in defining clinical outcome of Lyme disease patients. In the present study, both the host genetic background and the genotype of B. burgdorferi sensu stricto influenced the ability of spirochetes to disseminate through blood in our animal model.

Unlike relapsing fever spirochetes, which can be easily seen in patients on smears of peripheral blood, Lyme disease spirochetes are present in such low densities in blood that they are rarely if ever visible on blood smears. This may be due to the fact that relapsing fever spirochetes contain an enzyme, glycerophosphodiester phosphodiesterase (GlpQ), and related enzymes that allow them to hydrolyze phospholipids, whereas B. burgdorferi lacks these enzymes and therefore cannot achieve high densities in blood (19). There may be other mechanisms by which B. burgdorferi can survive in blood at low levels and at least disseminate to internal tissues. In an initial study using cultured spirochetes inoculated into mice, RST1 genotype spirochetes represented by a single strain (BL206) were present in blood and internal tissues in much larger amounts (up to a 16-fold difference) than those of a single RST3 strain (B356) (26). A subsequent study using five RST1 and five RST3 strains showed between 1 and 2.5 logs of difference in the presence of RST1 and RST3 spirochetes in blood and tissue samples (27). The present study, utilizing tick-transmitted infection, demonstrated that both RST1 and RST3 strains were present in the blood of mice immediately after tick feeding, but that the duration of spirochetemia was longer with RST1 strains than with RST3 strains. In addition, based on q-PCR analysis, RST1 strain spirochetes were present in higher densities in blood than RST3 strains, but this difference was noticeable only briefly, during week 2 postexposure.

The disease severity of mice infected with RST1 and RST3 strains was determined in two previous studies, using ankle swelling and the histopathology of joints and heart tissue (26, 27). In those studies, although there was considerable variability from strain to strain, the overall severity of disease was greater in mice infected with RST1 strains than in those infected with RST3 strains. Wang et al. (27) also evaluated two subsequent RST isolates by needle inoculation and demonstrated that an RST3A subtype is nondisseminative while RST3B is disseminative. It would be interesting in future studies to more closely examine the differences between RST3A and RST3B. In the present study, the load of spirochetes in heart and bladder, as well as the severity of lesions observed by histopathology, did not show a clear difference between RST1 and RST3 strains. In fact, in terms of the bladder only, RST1 strain 154 demonstrated a significant decrease in the number of inflammatory lesions seen perivascularly, but this was not seen with RST1 strain 142. No difference was noted in the severity of myocarditis or joint lesions, although RST1 strains did demonstrate some propensity for inducing mild lesions outside the joint capsule in C3H mice. In our studies, both RST1 and RST3 strains disseminated when transmitted by ticks and induced pathology at target organs. Differences between the present study and former studies (26, 27) include the fact that in the former studies mice were needle inoculated with OspA-expressing spirochetes and examined at only 3 weeks postexposure, whereas the mice in our study were infected by tick bite and examined at 8 weeks postexposure. Moreover, the injection of organisms expressing OspA in previous studies (26, 27) may directly influence the dispersal pattern of organisms in mice. Furthermore, the studies by Wang et al. comparing RST1 and RST3 subtypes were evaluated by needle inoculating mice with 10,000 spirochetes, whereas our mice were infected via tick bite. Although the natural route of transmission was evaluated here, it should be noted that it is impossible to standardize the number of spirochetes delivered during natural tick infestation and this may alter the experimental outcome and impact the comparison performed here. Additionally, there is some evidence that tick saliva is important in altering the host immune response and may be an important factor in the establishment of early infection. The relationship between hematogenous spread of spirochetes and the subsequent severity of disease is a complex question that...
requires further examination. The pathogenesis of Lyme disease has yet to be fully elucidated.

REFERENCES


