Disease Severity in a Murine Model of Lyme Borreliosis Is Associated with the Genotype of the Infecting *Borrelia burgdorferi* Sensu Stricto Strain

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The pathogenicity of *Borrelia burgdorferi* sensu stricto clinical isolates representing 2 distinct ribosomal DNA spacer restriction fragment–length polymorphism genotypes (RSTs) was assessed in a murine model of Lyme disease. *B. burgdorferi* was recovered from 71.5% and 26.6% of specimens from mice infected with RST1 and RST3 isolates, respectively ($P < .0001$). The average ankle diameter and histologic scores for carditis and arthritis were significantly higher after 2 weeks of infection among mice infected with RST1 isolates than among those infected with RST3 isolates ($P < .001$). These clinical manifestations were associated with larger numbers of spirochetes in target tissues but not with the serum sensitivity of the individual isolates. Thus, the development and severity of disease in genetically identical susceptible hosts is determined mainly by the pathogenic properties of the infecting *B. burgdorferi* isolate. The RST1 genotype is genetically homogeneous and thus may represent a recently evolved clonal lineage that is highly pathogenic in humans and animals.

Lyme disease, which is caused by infection with the spirochete *Borrelia burgdorferi,* is the most common vectorborne disease in the United States. A total of 122,970 cases was reported to the Centers for Disease Control and Prevention between 1992 and 2000 [1]. Although the majority of patients show only transient erythema migrans at the site of the tick bite, some persons may develop more serious manifestations, such as carditis, neuroborreliosis, or arthritis, if appropriate antibiotic treatment is not administered at the time the cutaneous lesion appears [2]. Disease severity in humans and experimentally infected animals may depend on a number of factors. These include virulence of the infecting strain of *B. burgdorferi* sensu lato [3–5], the number of spirochetes in target tissues [6–9], coinfection with other tickborne pathogens [10–12], and inherent differences in individual host responses to infection [13, 14].

B. burgdorferi sensu stricto is the sole species causing Lyme disease in North America. Previously, we characterized *B. burgdorferi* sensu stricto isolates cultured from patients with Lyme disease in Westchester County, New York, by restriction fragment–length polymorphism (RFLP) analysis of the 16S–23S rDNA spacer. All isolates analyzed were grouped into 1 of 3 rDNA spacer RFLP genotypes (RSTs) [15–17]. RSTs are significantly correlated with other genotypic characteristics and can, therefore, serve as an accurate genetic marker of *B. burgdorferi* genotype [18]. A significantly higher percentage of patients with Lyme disease who were infected with RST1 strains had blood culture results positive for *B. burgdorferi,* compared with patients infected with RST2 or RST3 isolates [5]. The frequency of multiple erythema migrans lesions was also significantly greater among patients infected with RST1 strains, providing clinical evidence of an association between specific *B. burgdorferi* genotype and hematogenous dissemination in patients with early Lyme disease [5].

B. burgdorferi infection in laboratory mice has many clinicopathologic features in common with Lyme disease in humans [14]. Studies that used murine models of infection with *B. burgdorferi* or other pathogenic *Borrelia* species have suggested that strain differences in the spirochetes are one of the critical determinants of disease severity [4, 19–22]. Arthritis severity and spirochete burden in C3H or CB-17 SCID mice infected with the relapsing fever agent, *B. turicatae,* appear to be determined mainly by the serotype of the infecting strain [19]. The potential impact of genotypic variation of *B. burgdorferi* sensu stricto on pathogenicity was established for single RST1 and RST3 isolates in a previous study, in which spirochete dissemination and disease severity in mice varied significantly according to the specific RST [9]. The present study was intended to expand on these earlier observations by studying 10 additional *B. burgdorferi* clinical isolates representing 2 distinct RSTs. The aims of the present study were to assess the pathogenicity of *B. burgdorferi* clinical

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isolates in C3H/HeJ mice, to determine any potential association between severity of disease and genotype of the infecting *B. burgdorferi,* and to identify mechanisms that may contribute to strain variation in dissemination and pathogenicity.

Materials and Methods

B. burgdorferi *isolates.* Ten clinical isolates of *B. burgdorferi* at passage 2–5 were used in this study. These isolates were cultured from blood ($n = 2$) or skin ($n = 8$) specimens from patients who were given a diagnosis of Lyme disease in Westchester County, New York, between 1998 and 2000. On the basis of rDNA spacer RFLP analysis [15], these isolates were characterized as either RST1 (isolates BL203, BL268, B479, B491, and B515) or RST3 (isolates B331, B348, B408, B418, and B500).

Mice. Specific pathogen–free male and female 4-week-old C3H/HeJ mice were obtained from Jackson Laboratory. Mice were maintained in separate cages in the Department of Comparative Medicine at New York Medical College (Valhalla).

Infection of mice with B. burgdorferi. Mice were randomly divided into groups of 5. *B. burgdorferi* was cultured in BSK-H medium supplemented with 6% rabbit serum (Sigma Chemical) at 33°C for 5–7 days. Spirochetes were examined for motility by darkfield microscopy, and the number of microorganisms was determined by fluorescence microscopy by mixing 10-μL aliquots of culture material with 10 μL of an acridine orange solution (100 μg/mL). All cultures were adjusted to a final concentration of 10^5 spirochetes/mL in incomplete BSK-H medium (without rabbit serum). Each mouse in the experimental groups was inoculated intradermally with 0.1 mL of the culture material (10^4 spirochetes) in the shaven back. In addition, 5 mice were inoculated with 10^6 ($n = 2$) or 10^7 ($n = 3$) organisms of isolate B331. Mice from the control group were injected at the same site with an equal volume of incomplete BSK-H medium.

Blood samples from each mouse were collected at 4, 7, 14, and 21 days after inoculation. Tibiotarsal joints were measured at the same time points with a digital metric caliper (SPI Digmax; Ralmike's Tool-A-Rama) through the thickest anteroposterior diameter of the ankle. All mice were killed by exposure to carbon dioxide at day 21 after inoculation. Samples of blood, ear, and various internal tissues (joint, heart, urinary bladder, and brain) were collected aseptically from each mouse for culture, polymerase chain reaction (PCR), and/or histopathologic analysis.

Culture of B. burgdorferi *from mouse samples.* Three to five drops of whole blood (~100 μL) were added directly into 5 mL of BSK-H medium. For culture of spirochetes from ear and other tissues, a 2-mm-diameter ear punch specimen or ~10 mg of internal tissue each was immersed separately in 70% ethanol for ~3 min, washed twice in PBS (pH 8.0) in 24-well culture plates, and incubated in 5 mL of BSK-H medium containing 50 μg/mL rifampin (Sigma). Cultures were maintained at 33°C for 4 weeks and examined for the presence of spirochetes every 7–10 days by darkfield microscopy.

Histopathologic analysis. One hind limb (ankle joint) and half of the heart from each mouse were immersion-fixed in 10% neutral buffered formalin, ethanol dehydrated, xylene cleared, paraffin embedded, and examined as described elsewhere [9]. Each histopathologic parameter was assessed and scored separately by 2 pathologists by means of a semiquantitative criteria-based scoring method. Scores of 0 (absent), 10 (mild), 20 (moderate), or 30 (severe) were assigned to each parameter, and the average scores of all of these parameters were used to reflect the overall severity of carditis and arthritis for each group of mice. The histopathologic sections were labeled in code, and the observers were blinded to the study conditions until after the histopathologic features were assessed.

Serologic analysis. Mouse serum or plasma samples were collected and stored at −20°C until use. The presence of *B. burgdorferi*–specific antibodies was determined by Western blotting with a commercial kit (MarDx). The dilution of serum or plasma was 1:160. A 1:3000 dilution of alkaline phosphatase–labeled goat anti–mouse IgM plus IgG plus IgA antibody (Kirkegaard & Perry Laboratories) was used to develop the blots by following the procedure recommended by the manufacturer.

Preparation of DNA from mouse samples. Mouse tissue (10–60 mg, on average: ~10 mg of bladder; ~20 mg of skin, ear, heart, or brain tissue; or ~60 mg of joint) was digested as described elsewhere [9]. DNA was prepared from a 100–200-μL aliquot of each digestion by using a commercial nucleic acid extraction kit (Isoquick; Orca Research). The purified DNA was resuspended initially in a total volume of 50–100 μL of sterile water. The yield and purity of DNA was estimated on the basis of the optical density at 260 nm and was subsequently adjusted to a final concentration of 50–100 μg/mL. DNA from 100 μL of plasma was extracted with the Qiagen Blood DNA Preparation Kit and eluted into 50 μL of sterile water. Two microliters of purified DNA was used in each PCR.

Real-time quantitative PCR. Simultaneous detection and quantification of *B. burgdorferi* DNA was done with the Lightcycler PCR system (Roche Diagnostics), as described elsewhere [9]. PCR was carried out in glass capillaries, in a final volume of 10 μL, containing 1× Lightcycler master mix (Roche), 3 m*M* $MgCl_2$, 1 μM each primer, and 2 μL of mouse DNA template or external standard template containing 10–10^5 copies of *B. burgdorferi recA,* as described elsewhere [9]. The fluorescent product was collected at 80°C at the last step of each cycle, to minimize signal from nonspecific products. The number of spirochetes was calculated by comparing the crossing points (C_t) of the samples with those of the standards with the Lightcycler software provided by the manufacturer. Melting curves were used to determine the specificity of the PCR products. Numbers of spirochetes in all tissue specimens were normalized to 200 ng of mouse DNA, which equals approximately the amount of DNA from 0.1 mg of tissue, to account for variation in mouse cell density in different tissues.

Complement-mediated serum sensitivity assay. Normal mouse serum and immune mouse serum were collected from C3H/HeJ mice before inoculation and at 8 weeks after inoculation with *B. burgdorferi* isolate B515, respectively. The immune mouse serum had antibodies against *B. burgdorferi* at a titer of ⩾1:2560. Serum samples were not subjected to heat inactivation, to maintain complement activity. Serum sensitivity was assayed in a double-blinded manner, as described elsewhere [23]. In brief, 75 μL of a cultured spirochete suspension was mixed with 25 μL of either normal mouse serum, immune mouse serum, or PBS buffer (pH 7.4) in 96-well plain-bottom microtiter plates. The plates were sealed and incubated at 33°C for ~20 h, and numbers of motile spirochetes in each

well were assessed by darkfield microscopy. The experiment was done in duplicate, and the percentage of killing was calculated by dividing the average number of spirochetes in aliquots of normal mouse serum or immune mouse serum mixtures by that in PBS mixtures.

DNA sequencing and phylogenetic analysis. A 941-bp fragment of 16S–23S rDNA spacer and an ~630-bp region of *ospC* were amplified by PCR, as described elsewhere [17, 24]. PCR products were purified by use of a Qiagen PCR purification kit and sequenced with an ABI 377 DNA sequencer (Applied Biosystems). Sequences determined in this study have been assigned GenBank accession numbers of AF467855–AF467866 (for 16S–23S rRNA intergenic spacer) and AF467867–AF467878 (for *ospC* sequences). DNA sequences from additional *B. burgdorferi* isolates were retrieved from GenBank and included in phylogenetic analysis. Sequences were aligned with the ClustalW program (available at http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html), and phylogenetic analysis was carried out with MEGA software (version 2.1 [25]).

Statistical analysis. Statistical analysis was done with GraphPad Prism software (version 3). Significant differences in the mean values of ankle-joint diameter, number of spirochetes in tissues, and scores of disease severity were determined by 2-tailed Student's t test; the 2-tailed χ^2 test was used to compare culture and serum sensitivity assay data.

Results

Culture of B. burgdorferi *from blood and tissue specimens.* C3H/HeJ mice were inoculated with 10^4 organisms of either *B. burgdorferi* RST1 or RST3 isolates (50 total; 5 animals were inoculated with each of 10 isolates). Three groups of 5 mice were injected with BSK-H medium and used as controls. Of the 298 blood and tissue specimens from RST1 isolate–infected mice, 213 samples (71.5%) were positive by culture, significantly greater than the culture rate from mice infected with RST3 isolates (26.6%; 86/323) ($P < .0001$). The culture positivity rate from blood and from all individual tissues except brain was significantly higher for mice infected with RST1 isolates than for mice infected with RST3 isolates (table 1; all $P < .001$).

Multiple blood and tissue samples from all mice ($n = 25$) infected with any of the 5 RST1 isolates yielded positive results by culture. In contrast, positive culture results were obtained from only 12 of the 15 mice infected with RST3 isolates B348, B408, and B500; specimens from mice ($n = 10$) inoculated with RST3 isolates B331 and B418 did not yield any positive culture results. In addition, all blood and tissue specimens from 5 additional mice for which the inoculum of B331 was increased

Table 1. Results of culture of *Borrelia burgdorferi* from blood, ear, skin, heart, bladder, and brain tissue specimens from mice infected with 16S–23S rDNA spacer restriction fragment–length polymorphism genotype (RST) 1 and RST3 (A and B) isolates.

Type *(n)*,inoculum, day after inoculation	No. of samples with positive cultures/no. tested (%)						
	Blood	Ear	Skin[a]	Heart	Bladder	Brain	Total
RST1 (5), 10^4							
Day 4	18/25 (72.0)	NT	25/25 (100)				43/50 (86.0)
Day 7	18/25 (72.0)	2/25 (8.0)	NT				20/50 (40.0)
Day 14	19/25 (76.0)	24/25 (96.0)	NT				43/50 (86.0)
Day 21	10/25 (40.0)	25/25 (100)	24/25 (96.0)	23/23 (100)	23/25 (92.0)	2/25 (8.0)	107/148 (72.3)
Total	65/100 (65.0)	51/75 (68.0)	49/50 (98.0)	23/23 (100)	23/25 (92.0)	2/25 (8.0)	213/298 (71.5)
RST3A[b] (2)							
10^4							
Day 4	0/10 (0)	NT	0/9 (0)				0/19 (0)
Day 7	0/10 (0)	0/9 (0)	NT				0/19 (0)
Day 14	0/9 (0)	0/10 (0)	NT				0/19 (0)
Day 21	0/9 (0)	0/10 (0)	0/10 (0)	0/9 (0)	0/10 (0)	0/9 (0)	0/57 (0)
10^6							
Day 4	0/2 (0)	NT	0/2 (0)				0/4 (0)
Day 14	0/2 (0)	0/2 (0)	0/2 (0)	0/2 (0)	0/2 (0)	NT	0/10 (0)
10^7							
Day 4	0/3 (0)	NT	0/3 (0)				0/6 (0)
Day 14	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	NT	0/15 (0)
RST3B (3), 10^4							
Day 4	6/15 (40.0)	NT	12/14 (85.7)				18/29 (62.1)
Day 7	5/15 (33.3)	0/15 (0)	NT				5/30 (16.7)
Day 14	5/13 (38.5)	11/15 (73.3)	NT				16/28 (57.1)
Day 21	2/14 (14.3)	11/14 (78.6)	12/15 (80.0)	11/15 (73.3)	10/14 (71.4)	1/15 (6.7)	47/87 (54.0)
Total	18/105 (17.0)	22/78 (28.2)	24/58 (41.4)	11/29 (37.9)	10/29 (34.5)	1/24 (4.2)	86/323 (26.6)

NOTE. *B. burgdorferi* isolates were designated as RST1 or RST3 on the basis of analysis of RSTs [15–17]. RST3 isolates were divided into RST3A and RST3B subgroups on the basis of cultivability from inoculated C3H/HeJ mice. Of 10 *B. burgdorferi* isolates examined, 2 were recovered from blood (BL203 and BL268), and the remainder were recovered from skin biopsy specimens from patients with erythema migrans. $P < .001$, for total positivity rate of culture from each type of specimen, except for brain tissue, mice infected with RST1 vs. RST3 isolates (2-tailed χ^2 test); contaminated specimens were excluded. NT, not tested.

[a] Skin biopsy specimen was taken from inoculation site of each mouse.

[b] Five mice were also inoculated with RST3A isolate B331 at either 10^6 or 10^7 spirochetes. Skin biopsy specimens were taken from these mice 24 and 48 h after inoculation, and all cultures were negative.

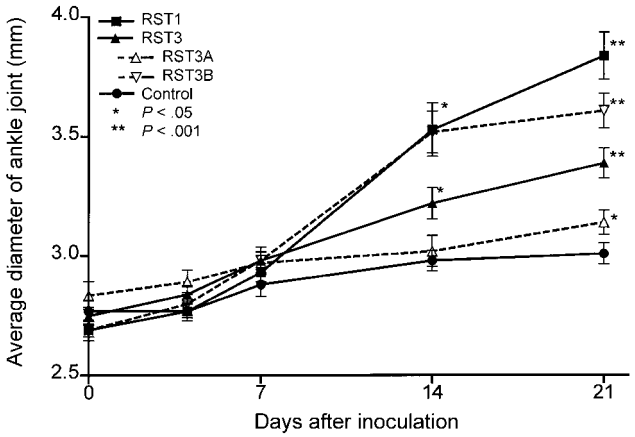

Figure 1. Measurement of ankle joints of mice infected with distinct rDNA spacer restriction fragment–length polymorphism genotypes (RSTs) of *Borrelia burgdorferi.* Data are average of results for mice infected with 5 RST1 isolates ($n = 25$), 2 RST3A isolates ($n = 10$), or 3 RST3B isolates ($n = 15$) and the control group ($n = 15$). *P* values are comparisons with the control group (2-tailed Student's *t* test).

from 10^4 to 10^6 ($n = 2$) or 10^7 ($n = 3$) were also negative by culture. This indicates that there are at least 2 distinct subgroups within the RST3 RFLP type. Therefore, data from mice infected with these 2 subgroups of RST3 isolates, referred to as "RST3A" (for noncultivable RST3 isolates) and "RST3B" (for cultivable RST3 isolates), are reported separately below.

Clinical course of infection and measurement of ankle joints. Twenty-one (84%) of 25 and 11 (73%) of 15 mice infected with the 5 RST1 and 3 RST3B *B. burgdorferi* isolates, respectively, developed clinically apparent arthritis ($P > .05$). This was heralded by the appearance of clinical swelling and redness of the ankles appearing at about day 12 after inoculation and lasting until the mice were killed. No joint swelling was observed in any control mice or in those mice inoculated with *B. burgdorferi* RST3A isolates B331 and B418.

To obtain more-precise data on joint swelling, the ankle-joint diameters of each mouse were measured with a digital caliper on days 4, 7, 14, and 21. No significant differences in the average values were observed among the 3 groups of mice at day 4 or day 7. However, the average ankle-joint diameters were significantly greater for mice infected with RST1 isolates than for those infected with RST3 isolates and for control animals at days 14 and 21 after infection (figure 1). The average ankle-joint diameter was comparable for mice infected with RST1 and RST3B isolates on days 14 and 21 after infection ($P > .05$).

Severity of disease assessed by histopathologic analysis. The severity of arthritis was scored on the basis of the presence and number of lymphocytes in the joints, synovial proliferation, and skeletal muscle inflammation. The cardiac score was based on the presence and number of lymphocytes in the aortic-pulmonary root, in the valves, and in the myocardium. The average scores of carditis and arthritis for mice inoculated with each isolate are shown in figure 2. Mice infected with 3 RST1 isolates (B479, B491, and B515) developed severe carditis (average score, 30) and arthritis (average score, 30), whereas moderate disease was detected in mice infected with the 2 remaining RST1 isolates BL203 and BL268 (average scores, 16 and 10 for carditis and 15 and 13 for arthritis, respectively). In contrast, analysis of the RST3 isolate–infected mice showed relatively mild histopathologic outcomes. Moderate-to-severe carditis developed only in mice infected with RST3B isolates B348 and B500 (average score, 29 and 25, respectively), and severe arthritis was observed only in isolate B500–infected mice (average score, 29). Mice infected with RST3A isolate B418 had mild carditis (average score, 9.5). No significant pathologic abnormality was observed in any mouse inoculated with RST3A isolate B331.

No significant difference was observed in the average scores of carditis between mice infected with RST1 and RST3B isolates ($P > .05$). The overall average score of arthritis for mice infected with RST1 isolates was significantly higher than that for mice infected with RST3B isolates ($P = .035$). Mice infected with either RST1 or RST3B isolates showed significantly higher average scores for carditis and arthritis than did those infected

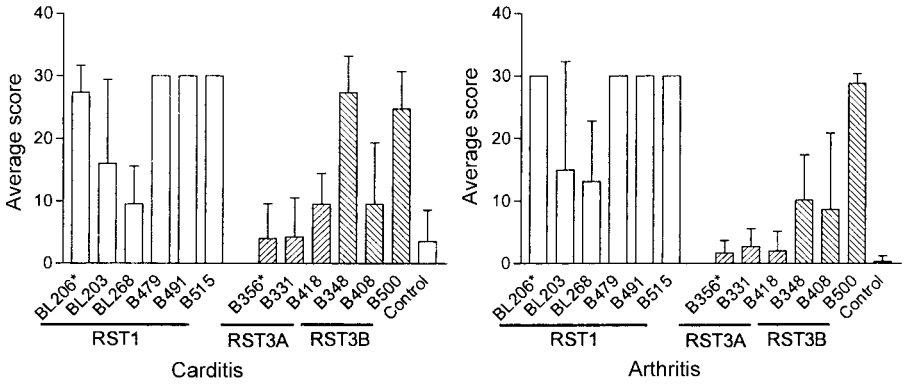

Figure 2. Cardiac and joint histopathologic analysis in mice infected with different genotypes of *Borrelia burgdorferi.* See Materials and Methods for a description of the scoring system. *Histologic data for these isolates were from a previous study [9].

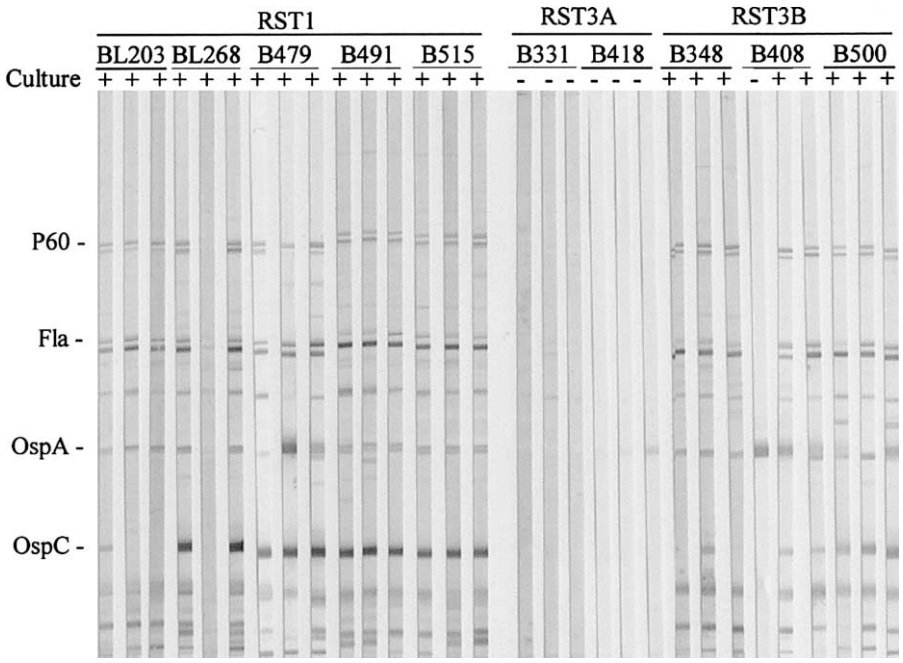

Figure 3. Detection of *Borrelia burgdorferi*–specific antibodies by Western blotting. Serum or plasma specimens were collected from individual mice 3 weeks after infection. Three representative immunoblot strips for mice infected with each isolate are shown. Identities of major bands on blots are indicated at left. A different lot of strips was used for isolates B491 and B515, which resulted in minor shift in molecular mass of certain bands on blot. +, Positive; −, negative.

with RST3A isolates (for carditis, $P = .0009$, RST1 vs. RST3A, and $P = .004$, RST3B vs. RST3A; for arthritis, $P = .0004$, RST1 vs. RST3A, and $P = .005$, RST3B vs. RST3A).

Serologic analysis. Representative Western blots of mouse serum samples collected on day 21 are shown in figure 3. Mice infected with 5 RST1 and 3 RST3B isolates showed strong antibody responses against *B. burgdorferi* proteins. However, serum samples from mice inoculated with RST3A isolates B331 and B418 showed only weak reactivity against several *B. burgdorferi* proteins. A significant correlation between the positivity of culture (positive or negative) and serologic tests (positive or negative, as defined by manufacturer's recommendations) was observed in mice inoculated with 10^4 organisms ($r = 0.95$; 95% confidence interval [CI], 0.91–0.97; $P < .0001$). Although no positive culture result was achieved, 3 mice inoculated with 10^7 organisms of isolate B331 had serologic responses similar to those of mice infected with the RST1 isolates (data not shown).

Quantification of spirochete burden in tissues and association with severity of disease. Spirochete loads in tissue specimens of the infected mice were monitored by quantitative PCR over the course of infection. Numbers of spirochetes in day 7 plasma and day 21 tissue specimens are shown in figure 4. The peak spirochete load detected in blood on day 7 was $1.8 \times 10^4 \pm 3.8 \times 10^3$ spirochetes/mL of plasma (mean ± SE) for mice infected with RST1 and $1 \times 10^4 \pm 3.0 \times 10^3$ spirochetes/mL of plasma for mice infected with RST3B isolates ($P > .05$); each was significantly higher than that detected in mice inoculated with RST3A isolates ($3.2 \times 10^3 \pm 1.8 \times 10^3$ spirochetes/mL of plasma; $P = .002$ and $P = .02$, respectively). No significant difference was found in the mean number of spirochetes in ear biopsy samples between RST1- and RST3B-infected mice (380 ± 63 vs. 286 ± 69; $P > .05$). However, spirochete burdens in skin, heart, and joint tissues of mice infected with RST1 isolates were 2–3-fold higher than those of animals infected with RST3B isolates (mean ± SE of skin tissue for RST1 vs. RST3B isolates, 196 ± 28 vs. 100 ± 19 spirochetes/200 ng of mouse DNA, $P = .035$; heart, 409 ± 54 vs. 161 ± 34, $P = .001$; joint, 342 ± 38 vs. 198 ± 37, $P = .02$). Spirochete DNA was detectable in plasma samples from 4 of 5 mice infected with RST3A isolate B418; no spirochete DNA was detected in any B331-infected mice. Similarly, spirochete loads were much lower or undetectable in tissues from mice infected with RST3A isolates (all $P < .001$, mice infected with RST1 isolates vs. those infected with RST3B isolates).

To assess the potential association between the severity of disease and spirochete burden in tissues, the numbers of spirochetes in joint and heart tissues determined by quantitative PCR were evaluated relative to the average severity score of carditis and arthritis as assessed by histopathologic analysis. A significant correlation between the severity of disease and the number of spirochetes in joint and heart tissues was observed (for joint: $r = 0.76$; 95% CI, 0.60–0.86; $P < .0001$; for heart: $r = 0.64$; 95% CI, 0.43–0.78; $P < .0001$).

Serum sensitivity assay. Complement-mediated serum sen-

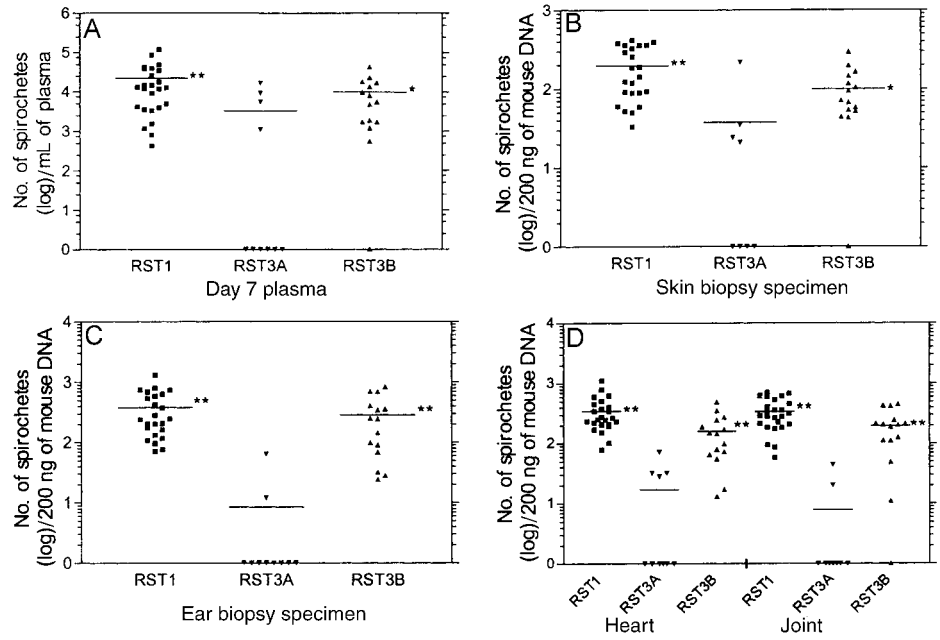

Figure 4. Quantification of spirochetes in plasma and tissue samples of C3H/HeJ mice infected with distinct genotypes of *Borrelia burgdorferi.* All specimens that contained no detectable *B. burgdorferi* DNA were assigned a value of 1 for comparison. $*P < .05$ and $**P < .001$, vs. mice infected with RST3A isolates (Student's *t* test). Significant differences in the mean no. of spirochetes were also found in skin biopsy ($P = .04$), joint ($P = .02$), and heart tissues ($P = .002$) between mice infected with RST1 and RST3B isolates.

sitivity was assessed for 9 clinical isolates used in this study and for 2 clinical isolates of *B. burgdorferi* from a previous study [9]. All isolates showed intermediate sensitivity to normal mouse serum; 13%–76% of spirochetes were killed after 20 h of incubation with normal mouse serum (table 2). However, no significant correlation was noted between the serum sensitivity and genotype of any isolate. More than 90% of spirochetes of all RST1 and RST3 isolates were killed after incubation with 25% immune serum from C3H/HeJ mice infected with an RST1 isolate B515.

Phylogenetic analysis. To further clarify the genetic relationship among the isolates under study, a portion of the 16S–23S rDNA spacer and *ospC* were amplified by PCR, and the products were sequenced. The nucleotide identity among all of these *B. burgdorferi* isolates was 95%–100% for the 16S–23S rDNA spacer and 82%–100% for *ospC.* Sequences from all RST1 isolates were virtually identical to each other at both loci and clustered as a monophyletic group in phylogenetic trees on the basis of both 16S–23S rDNA spacer and *ospC* sequences (figures 5 and 6). In contrast, sequences of the 16S–23S rDNA spacer and *ospC* from RST3 isolates were more divergent and constituted several subclusters in either phylogenetic tree. The topologies of both trees were identical, except for the RST3B isolate B408. This isolate clustered with RST3 isolates N40, B348, and B418 on the basis of the 16S–23S rDNA spacer sequences but with RST3 isolates B331 and B500 on the basis of *ospC* sequences. It is noteworthy that no correlation was observed between the pathogenicity of an RST3 isolate in mice and its *ospC* genotype.

Discussion

Despite intensive investigation and availability of the *B. burgdorferi* genome sequence, the pathogenesis of Lyme disease is

Table 2. In vitro sensitivity of *Borrelia burgdorferi* sensu stricto against normal mouse serum and immune mouse serum from C3H/HeJ mice.

Isolate	Type	Cultivability from inoculated mice[a]	Killing (%)[b] by	
			25% NMS	25% IMS
BL206[c]	RST1	+	66.9	99.4
BL203	RST1	+	14.9	99.1
BL268	RST1	+	49.8	99.3
B479	RST1	+	75.5	98.5
B491	RST1	+	15.2	97.8
B515	RST1	+	29.1	96.9
B348	RST3B	+	74.7	98.9
B408	RST3B	+	56.7	94.2
B500	RST3B	+	75.1	98.9
B356[c]	RST3A	−	12.6	94.7
B331	RST3A	−	59.1	88.4

[a] Cultivability from any tissue specimen of C3H/HeJ mice inoculated with individual isolate. +, Culture positive; −, culture negative.

[b] The percentage of killing was calculated by dividing the average no. of live spirochetes in mixtures with 25% normal mouse serum (NMS) or immune mouse serum (IMS) with that in PBS control for each isolate. Data are average of duplicate experiments.

[c] The pathogenicity of isolate BL206 and B356 in C3H mice was reported elsewhere [9].

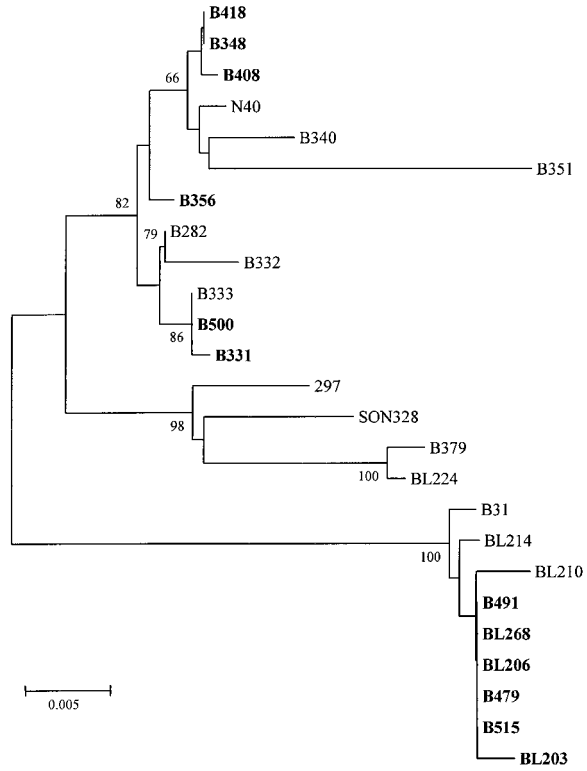

Figure 5. Phylogenetic tree based on 16S–23S rDNA spacer sequences of *Borrelia burgdorferi.* Tree was constructed by the neighbor-joining method with the MEGA program [25]. *Bar,* 0.5% divergence. Nos. at branch nodes are the results from bootstrap analysis. *B. burgdorferi* isolates used in the present study are shown in bold.

not well elucidated [27]. Several studies have shown that the genetic composition of the host determines the severity of disease in laboratory animals [6, 13, 14] and possibly contributes to antibiotic treatment–resistant arthritis in humans [28, 29]. On the other hand, the presence of *B. burgdorferi* in target tissues and consequent interactions with the host play a role in promoting host inflammation and affect the clinical presentation of disease [8, 30–32]. In Europe, 3 species of *B. burgdorferi* sensu lato *(B. burgdorferi* sensu stricto, *B. garinii,* and *B. afzelii)* are pathogenic in humans, and each of these species has been associated with distinct clinical symptoms of Lyme disease [4, 33]. The present study addressed the role of subtypes of North American *B. burgdorferi* sensu stricto in the development of clinical disease in genetically susceptible C3H/HeJ mice.

The results showed that RST1 *B. burgdorferi* clinical isolates were more invasive and produced more-severe disease in experimentally infected animals. *B. burgdorferi* was recovered from blood, ear, skin, and organ tissue specimens from all 25 animals infected with any of the 5 RST1 isolates. In contrast, *B. burgdorferi* could not be recovered from any of the 10 mice inoculated with RST3A isolates B331 and B418 and could be recovered from only 12 of 15 mice infected with RST3B isolates B348, B408, and B500. The average ankle-joint diameter was significantly larger for animals infected with RST1 isolates than for those infected with RST3A isolates after 2 weeks of inoculation ($P < .001$). Furthermore, more-severe carditis (average score, 24) and arthritis (average score, 24) were observed by histologic analysis in mice infected with RST1 isolates, compared with mice infected with RST3A isolates (average score for carditis, 7 [$P = .0009$]; average score for arthritis, 2 [$P = .0004$]). Although the average ankle-joint diameter and severity of carditis were comparable for mice infected with either RST1 or RST3B isolates, more-severe arthritis was noted in RST1 isolate–infected mice ($P = .034$). The data strongly suggest that certain genotypes of *B. burgdorferi* are more invasive and pathogenic than others. This confirms our previous observations on the association of specific subtypes of *B. burgdorferi* with hematogenous dissemination and severity of disease in patients

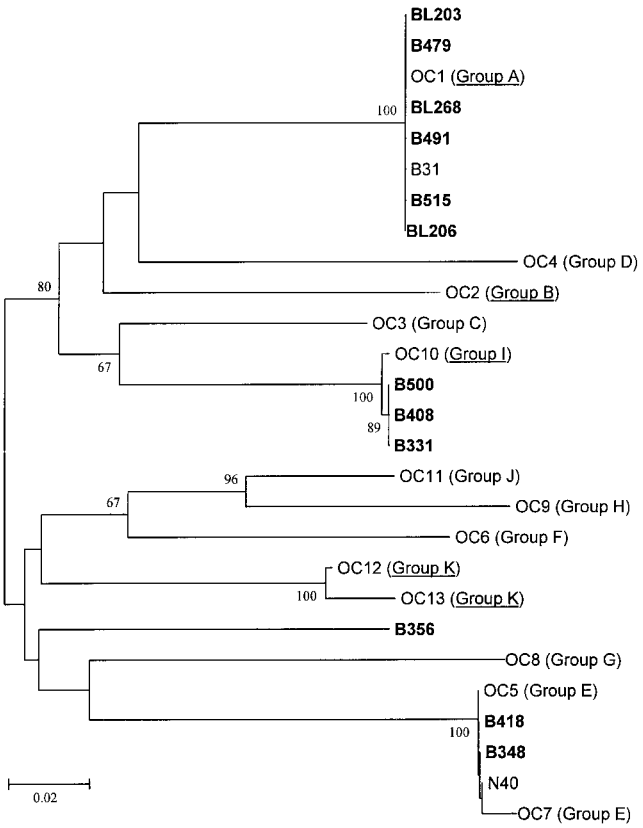

Figure 6. Phylogenetic tree based on *ospC* sequences of *Borrelia burgdorferi* sensu stricto. Tree was constructed by the neighbor-joining method with the MEGA program [25]. *Bar,* 2% divergence. Nos. at branch nodes are the results from bootstrap analysis. *B. burgdorferi* isolates used in this study are shown in bold. *B. burgdorferi ospC* (OC) alleles and their major OspC groups were defined by Seinost et al. [26]; 4 OspC groups that could cause invasive infection in humans (groups A, B, I, and K) are underlined.

[5] and expands on our prior findings among experimentally infected mice [9].

Comparative analysis of *B. burgdorferi* DNA in target tissues by quantitative PCR confirmed previous reports in which severity of Lyme arthritis was directly correlated with the number of spirochetes present in the joints of infected C3H/HeJ mice [6–8, 34]. A correlation between severity of carditis and spirochete burden in heart tissues of infected mice was also demonstrated in this study. It is known that spirochetes in target tissues can damage host cells [35, 36] and induce host inflammatory reactions [37, 38], thereby producing clinical disease.

All 6 RST1 *B. burgdorferi* isolates tested to date disseminate readily and cause invasive infection and clinical disease in C3H mice (5 isolates in this study and 1 isolate in a previous study [9]). These isolates exhibit a high degree of genetic homogeneity in 16S–23S rDNA spacer and *ospC* sequences. *ospC* has been described as being highly heterogeneous among *B. burgdorferi* sensu lato isolates [39–41], but genetic homogeneity has been reported for the *B. garinii* OspA serotype 4 strains, which are specifically associated with neuroborreliosis in Europe [42, 43]. In addition to the sequence analysis, all RST1 isolates displayed identical patterns, as determined by random-amplified polymorphic DNA (RAPD) analysis (data not shown), an approach with high discriminatory power among *B. burgdorferi* sensu lato isolates [43]. Taken together, the data suggest that RST1 *B. burgdorferi* sensu stricto isolates are a recently evolved clonal lineage that is genetically homogeneous and highly pathogenic to humans and C3H mice.

With the use of the *ospC* locus as a genetic marker, Seinost et al. [26] and Baranton et al. [44] reported that only 10 of 58 OspC sequence groups were associated with invasive infection in humans among the 3 pathogenic *B. burgdorferi* sensu lato species (4/22 OspC groups for *B. burgdorferi* sensu stricto, 2/14 for *B. afzelii,* and 4/22 for *B. garinii*). Interestingly, comparison of our data with those reported by Seinost et al. [26] revealed that all RST1 isolates analyzed in this study clustered into the same phylogenetic branch with OspC group A (or *ospC* allele 1), a *B. burgdorferi* genotype reported to be associated with disseminated infection in humans. However, RST3A and RST3B isolates showed crossover and mosaic-like distribution, and no correlation was found between dissemination and pathogenicity of an RST3 isolate in C3H mice and its location in the *ospC*-based phylogenetic tree. For instance, the nondisseminating RST3A isolate B331 grouped in the same subcluster as *ospC* allele 10 (group I), 1 of the 4 OspC groups associated with disseminated infection in humans [26], and RST3B isolates B408 and B500, which disseminated in mice in the current study. On the other hand, RST3B isolates B348 and N40, which cause disseminated infection in mice, clustered together with group E (*ospC* alleles 5 and 7), an OspC group in which isolates were not found to be invasive in humans. Therefore, it is unlikely that OspC functions as the sole determinant of pathogenicity, at least in C3H mice.

Three RST3A isolates (B331 and B418 in this study and B356 in a previous study [9]) caused no pathologic disorders or only mild disease in mice, despite the fact that they were recovered from the skin of patients with Lyme disease. More strikingly, no spirochetes could be recovered from any blood or tissue samples from mice inoculated with any of these isolates, although quantitative PCR indicated that some limited dissemination had occurred in several infected mice. In this study, only RST3A isolate B418 showed DNA loads in plasma and some tissues comparable to those observed in RST3B-infected mice. This isolate is genetically distinguishable from the other 2 RST3A isolates studied (B356 and B331; figures 5 and 6). In addition, it is closely related genotypically to isolate N40, a widely used strain pathogenic in mice, as indicated by *ospC* sequence (figure 6), RFLP, and RAPD analysis (data not shown). Inability to culture RST3A isolates from inoculated mice is unlikely to be due to inherent differences in cultivability in vitro, because the growth characteristics of these isolates in BSK-H medium were indistinguishable from those of RST1 and RST3B isolates. What might be responsible for the apparent differences in pathogenicity of these isolates in humans and animals? The isolates were deposited into the skin of individual patients directly from naturally infected *Ixodes scapularis* ticks. A number of *B. burgdorferi* genes are differentially expressed in ticks, compared with in vitro–cultivated organisms [45], and tick salivary glands contain compounds that can suppress host immunity [46–48]. Therefore, it is possible that some gene products expressed only in the tick and/or tick-induced host immunosuppression might facilitate the initiation of infection in humans. Second, it is known that there is a high incidence of mixed infection with RST3 and other genotypes of *B. burgdorferi* in field-collected *I. scapularis* ticks and in skin biopsy specimens from patients with Lyme disease [17] (authors' unpublished data). It is possible that coinfection with other genotypes of *B. burgdorferi* allows RST3A isolates to establish infection in mammalian hosts. Alternatively, these isolates might not be pathogenic at all, despite their recovery from patients with Lyme disease. The clinical disease in those patients (i.e., erythema migrans lesions) could have been caused by other coinfecting genotypes of *B. burgdorferi,* yet only the RST3A *B. burgdorferi* isolates were successfully cultivated from biopsy specimens, perhaps because of in vitro selection by culture, as reported elsewhere [17, 49].

Phylogenetic analysis demonstrates that RST3 isolates are genetically divergent (figures 5 and 6). It is, therefore, not surprising that the pathogenicity of *B. burgdorferi* RST3 clinical isolates varies substantially, even within the low-virulence subgroup RST3A. All blood and tissue samples from mice inoculated with 3 of the 6 RST3 *B. burgdorferi* clinical isolates yielded negative culture results in this and a previous study [9]. Only a few weak bands against *B. burgdorferi* proteins were detected by Western blotting in serum specimens of mice inoculated with 10^4 organisms. This suggests that the inoculated

spirochetes survived in mice for only a short time. Mice inoculated with 10^7 RST3A organisms (B331 or B356) showed antibody responses comparable to those of mice infected with RST1 isolates. Thus, seronegativity in these mice is unlikely to be due to the sequestering of antibodies in immune complexes, although such a possibility cannot be completely ruled out.

The mechanism by which RST3A spirochetes are killed in mice is unknown. No differences in serum sensitivity were found among RST1 and RST3A isolates. This indicates that complement-mediated killing is not involved in the clearance of RST3A isolates from infected mice, although the results from in vitro serum sensitivity assays may not accurately reflect the in vivo situation in infected mice. It is most likely that the inoculated *B. burgdorferi* were cleared by innate immune responses of the host, possibly at the initial injection site. This hypothesis is supported by the observation that no living spirochetes could be cultivated from skin biopsy specimens taken from the inoculation site by 24 h after injection. In addition, results identical to those obtained in the present study were observed in SCID C3H mice inoculated with *B. burgdorferi* RST3A isolate B356 (authors' unpublished data). These results suggest that host adaptive immunity is not responsible for clearing infection by RST3A isolates. The effectors of innate immunity are activated immediately after infection [50]. It is tempting to speculate that macrophage and/or dermal Langerhans cell–mediated innate immunity could play a key role in clearance of *B. burgdorferi* from the local injection site in the host. Experiments designed to elucidate the killing mechanisms of *B. burgdorferi* in vitro and in vivo are currently under way.

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