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Generality of Post-Antimicrobial Treatment Persistence of *Borrelia burgdorferi* Strains N40 and B31 in Genetically Susceptible and Resistant Mouse Strains

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ABSTRACT A basic feature of infection caused by *Borrelia burgdorferi*, the etiological agent of Lyme borreliosis, is that persistent infection is the rule in its many hosts. The ability to persist and evade host immune clearance poses a challenge to effective antimicrobial treatment. A link between therapy failure and the presence of persister cells has started to emerge. There is growing experimental evidence that viable but noncultivable spirochetes persist following treatment with several different antimicrobial agents. The current study utilized the mouse model to evaluate if persistence occurs following antimicrobial treatment in disease-susceptible (C3H/HeJ [C3H]) and disease-resistant (C57BL/6 [B6]) mouse strains infected with *B. burgdorferi* strains N40 and B31 and to confirm the generality of this phenomenon, as well as to assess the persisters' clinical relevance. The status of infection was evaluated at 12 and 18 months after treatment. The results demonstrated that persistent spirochetes remain viable for up to 18 months following treatment, as well as being noncultivable. The phenomenon of persistence in disease-susceptible C3H mice is equally evident in disease-resistant B6 mice and not unique to any particular *B. burgdorferi* strain. The results also demonstrate that, following antimicrobial treatment, both strains of *B. burgdorferi*, N40 and B31, lose one or more plasmids. The study demonstrated that noncultivable spirochetes can persist in a host following antimicrobial treatment for a long time but did not demonstrate their clinical relevance in a mouse model of chronic infection. The clinical relevance of persistent spirochetes beyond 18 months following antimicrobial treatment requires further studies in other animal models.

KEYWORDS *Borrelia burgdorferi*, antimicrobial tolerance, mouse model, persistence

B*orrelia burgdorferi*, the etiological agent of Lyme borreliosis, has become an important public health problem, and borreliosis has become the most prevalent tick-borne disease in the United States (1, 2). It occurs across much of the Northern Hemisphere, causing considerable morbidity and, in some cases, mortality in humans, domestic animals, and occasionally wildlife. It is the most frequently diagnosed tick-borne disease in the United States. According to the Centers for Disease Control and Prevention (CDC), it is estimated that there are more than 300,000 human cases of Lyme borreliosis annually (3), which is expected to rise (4).

Untreated human Lyme borreliosis results in disease with a wide range of clinical symptoms and protean manifestation, depending on the stage of infection (5–9). Treatment of patients with diagnosed Lyme borreliosis with antimicrobial agents mainly resolves the objective clinical manifestations. Associated subjective symptoms often may persist for many weeks, or even months (10). However, a proportion of patients remain ill (11, 12), and delayed treatment is associated with negative clinical

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outcomes (13, 14). There are many reports that, despite resolution of the clinical signs of infection after treatment with antimicrobial agents, a minority of patients experience disabling subjective symptoms, a condition recognized by the term "post-Lyme disease syndrome" (PTLDS) (15–22).

Persistent Lyme borreliosis infection (without antimicrobials) has been experimentally demonstrated in various animal models by culture and PCR (23–30), as well as in reported confirmed spontaneous cases in humans based on culture (31–37) and PCR (38–41). A prolonged treatment regimen has been evaluated in patients with chronic Lyme disease, and treatment is often less effective (15, 42–44). The general findings of some of these studies were that a small population of persistent spirochetes that survived antimicrobial treatment are noncultivable but that *B. burgdorferi*-specific DNA (BbDNA) can be detected by PCR. The persistent spirochetes were primarily localized in collagenous tissues (17–21, 29, 45–54). There is also strong scientific evidence that uninfected ticks were able to acquire *B. burgdorferi* spirochetes that survived various antimicrobial treatments and transmit them to naive hosts after molting to the next stage (45–47, 50, 54).

The nature of the formation of persistent spirochetes is still unknown and is a matter of speculation. Several mechanisms have been proposed that involve suppression of the innate and adaptive immune systems, complement inhibition, induction of anti-inflammatory cytokines, formation of immune complexes, antigenic variation, and physical seclusion (27, 53, 55–60). Persistence of noncultivable and attenuated spirochetes has been demonstrated following antimicrobial treatment, and the phenomenon is likely explained by antimicrobial tolerance (61–63). *B. burgdorferi* has numerous linear and circular plasmids that are inclined to loss from a bacterial cell during long-term *in vitro* cultivation (64–66), which may correlate with loss of infectivity. It is likely that plasmid loss occurs during the course of infection and increases over time.

In our earlier studies, we detected populations of *B. burgdorferi* in tissues after antimicrobial therapy in mice (45–47, 50) and in rhesus macaques (53, 54) treated with ceftriaxone, doxycycline, or tigecycline at various intervals of infection, and tissues were tested at intervals after treatment. Specific BbDNA was consistently detected in tissues of mice as late as 12 months after treatment, but culture was consistently negative (46). Spirochetes could be visualized by immunohistochemistry in collagen-rich tissues of mice after treatment (46, 47). We have demonstrated that morphologically intact antimicrobial-tolerant persistent spirochetes could be acquired by larval ticks; that after a blood meal, the ticks molted into the next life stages, nymphs and adults, which remained BbDNA positive; that nymphs transmitted BbDNA to recipient immunocompromised mice with multiple tissues PCR positive, with no obvious inflammation observed; and that allografts from treated mice transplanted into recipient immunocompromised mice transferred BbDNA to the recipient mice (46). Transcriptional activity of BbDNA-positive tissues was detected for several target genes, suggesting their viability (47, 50). Furthermore, quantitative PCR (qPCR) indicated low levels of replication during the various stages (46, 47, 50). In the most recent study, the subpopulation of viable, antimicrobial-tolerant, but slowly dividing and persistent spirochetes of *B. burgdorferi* resurged in mice 12 months after treatment and redisseminated into multiple tissues. Isolation of these spirochetes has been unsuccessful due to their slow growth, low numbers, and probably changes in plasmid content. The noncultivable spirochetes in host tissues remained transcriptionally active, BbDNA was acquired by xenodiagnostic ticks, and spirochetal forms could be visualized within ticks and mouse tissues. A number of host cytokines were up- or downregulated in tissues of both saline- and antimicrobial-treated mice, indicating host response to the presence of such spirochetes, despite the lack of inflammation in tissues (47).

We hypothesize that a selected persistent *B. burgdorferi* population proliferates and incidentally generates an increasingly heterogeneous population of noncultivable, replicatively attenuated spirochetes. We propose that this may be due to a relatively large subpopulation of attenuated spirochetes as infection proceeds, which may lose one or more plasmids. Results obtained from this study demonstrated the generality of

TABLE 1 Analysis of *flaB-ospA* DNA in heart base, ventricular muscle, and tibiotarsus from *B. burgdorferi*-infected mice treated with ceftriaxone and saline solution and then necropsied 12 months after completion of treatment

Mouse strain	<i>Borrelia</i> strain	Treatment	No. of samples positive/no. tested		
			Heart base	Ventricular muscle	Tibiotarsus
C3H	N40	Ceftriaxone	3/5	1/5	5/5
C3H	B31	Ceftriaxone	2/5	0/5	5/5
B6	N40	Ceftriaxone	1/5	1/5	5/5
B6	B31	Ceftriaxone	1/5	0/5	5/5
C3H	N40	Saline	4/5	5/5	3/5
C3H	B31	Saline	5/5	5/5	3/5
B6	N40	Saline	4/5	5/5	2/5
B6	B31	Saline	1/3	3/3	2/3

spirochete persistence following antimicrobial treatment by demonstrating the phenomenon in genetically susceptible C3H/HeJ (C3H) and resistant C57BL/6 (B6) mouse strains infected with *B. burgdorferi* strain N40 (C3H/N40 and B6/N40) compared to infection with strain B31 (C3H/B31 and B6/B31) for up to 18 months after treatment.

RESULTS

Pharmacokinetics. Average serum concentrations of ceftriaxone were 18.625 $\mu\text{g/ml}$ and 1.795 $\mu\text{g/ml}$ at 2 and 4 h, respectively, but no inhibition was detected at 8 h. As expected, intraperitoneally administered ceftriaxone accumulated rapidly in circulation and then decreased gradually during the following 2 h. The serum ceftriaxone concentrations at 2 and 4 h after administration were at least 300- and 30-fold greater than the *B. burgdorferi* minimal bactericidal concentration (MBC) (obtained in our previous mouse studies), respectively. No differences were observed in serum ceftriaxone concentrations between different mouse strains (C3H and B6).

Persistence of *B. burgdorferi* N40 and B31 following antimicrobial treatment in C3H and B6 mice. Mouse tissue samples—heart base, ventricular muscle, and tibiotarsal joint—were collected aseptically, immediately weighed, snap-frozen in liquid nitrogen, and stored at -80°C before total nucleic acid extraction. Quality control of qPCR was assessed by targeting the mouse *gapDH* gene. Spirochetal DNA was assessed by targeting *flaB* and *ospA*. Quantitative data were expressed as the number of DNA copies per milligram of tissue weight. Mouse *gapDH* qPCR was positive for all assessed samples (average quantification cycle [C_q] \pm standard deviation [SD], 19.36 ± 4.69), confirming successful extraction of total nucleic acid.

Based upon *flaB-ospA* DNA qPCR on assessed tissue samples, all C3H and B6 mice inoculated with either *B. burgdorferi* strain N40 or B31 and treated with saline were qPCR positive at 12 and 18 months after treatment in most tissues. The data in Table 1 and Table 2 summarize positive or negative *flaB-ospA* DNA qPCR results. Among ceftriaxone-treated C3H and B6 mice infected with N40 or B31, 100% and 74% tested positive on *flaB-ospA* DNA qPCR at tibiotarsal joints at 12 and 18 months after treat-

TABLE 2 Analysis of *flaB-ospA* DNA in heart base, ventricular muscle, and tibiotarsus from *B. burgdorferi*-infected mice treated with ceftriaxone and saline solution and then necropsied 18 months after completion of treatment

Mouse strain	<i>Borrelia</i> strain	Treatment	No. of samples positive/no. tested		
			Heart base	Ventricular muscle	Tibiotarsus
C3H	N40	Ceftriaxone	0/4	0/4	1/4
C3H	B31	Ceftriaxone	0/5	0/5	5/5
B6	N40	Ceftriaxone	0/5	0/5	4/5
B6	B31	Ceftriaxone	0/5	0/5	5/5
C3H	N40	Saline	3/4	4/4	4/4
C3H	B31	Saline	3/3	3/3	3/3
B6	N40	Saline	4/5	5/5	5/5
B6	B31	Saline	5/5	5/5	5/5

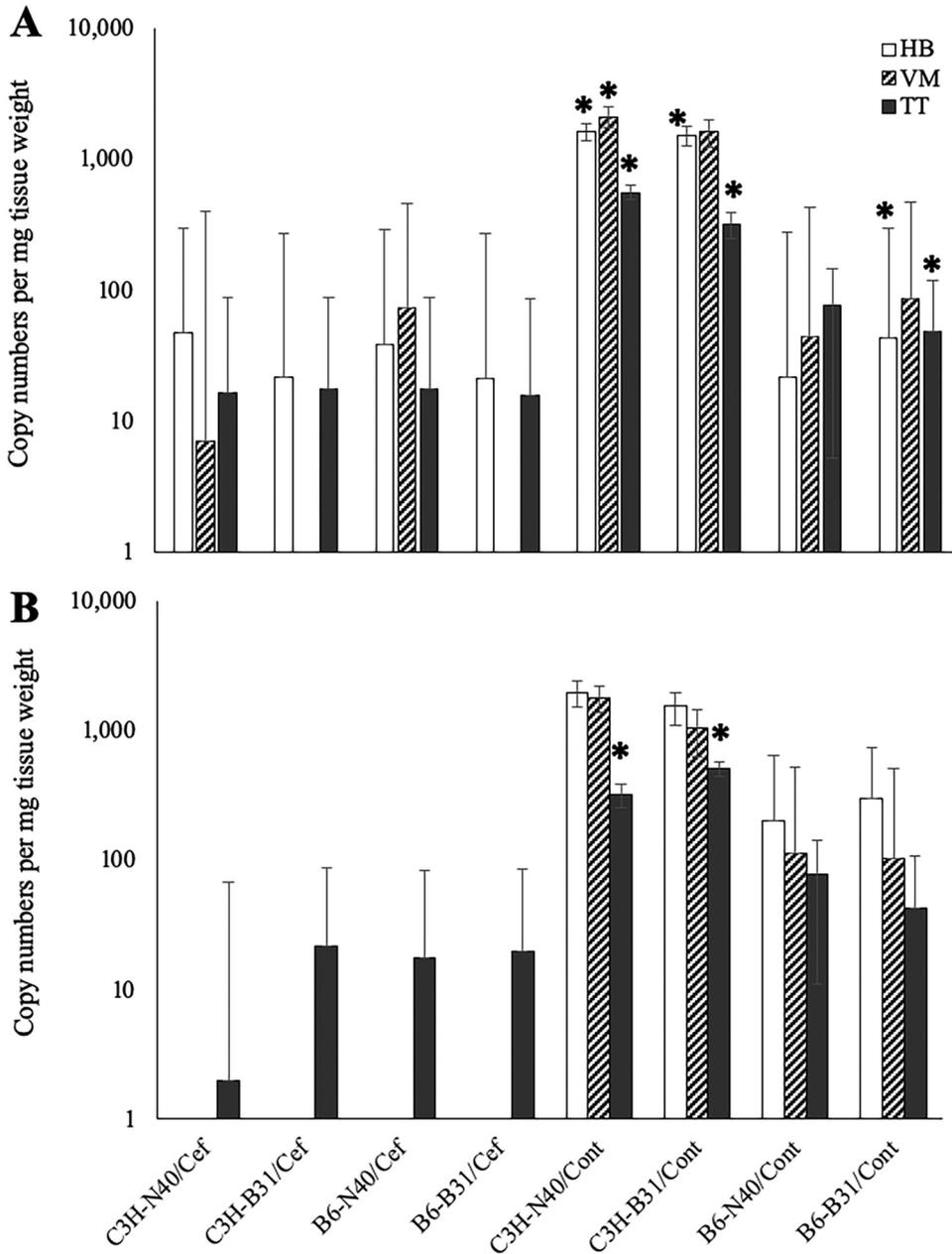


FIG 1 Copy numbers of *B. burgdorferi* DNA of *flaB-ospA* genes in heart base (HB), ventricular muscle (VM), and tibiotarsus (TT) of C3H and B6 mice infected with either strain N40 or B31 at 12 (A) and 18 (B) months after ceftriaxone and saline solution treatment. *, significantly higher copy number ($P < 0.05$). The error bars indicate SD.

ment, respectively. Sporadically positive heart base and ventricular muscle samples were detected in mice at 12 months after antimicrobial treatment (Table 1), but not in mice at 18 months (Table 2).

Quantification of each target gene in the assessed samples was accomplished by preparing plasmid standards in order to create absolute standard curves to determine the starting copy number. Mean DNA copy numbers of *flaB-ospA* genes in collected tissue samples among different mouse strains (C3H and B6) and different *B. burgdorferi* strains (N40 and B31) in ceftriaxone-treated groups at 12 months after treatment were nearly equivalent (mean \pm SD, $2.97 \times 10^1 \pm 1.72 \times 10^1$). Among saline-treated groups, significantly higher DNA copy numbers of *flaB-ospA* were found in C3H mice infected with N40 and B31 than in B6 mice infected with N40 and B31 ($P < 0.05$) (Fig. 1A).

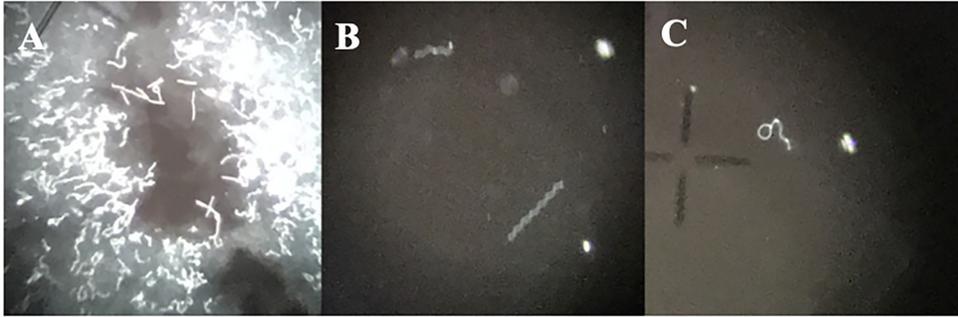


FIG 2 Dark-field images of *B. burgdorferi* spirochetes cultured from mouse tissues necropsied 18 months after completion of treatment. Magnification, $\times 400$. (A) Note the numerous spirochetes from ear culture of a saline solution-treated C3H mouse infected with strain N40. (B) Ear culture of a ceftriaxone-treated B6 mouse infected with strain B31. (C) Front joint of a ceftriaxone-treated B6 mouse infected with strain N40.

Significantly higher copy numbers of *flaB-ospA* were found in tissue samples from saline-treated mice than in those from the ceftriaxone-treated mice ($P < 0.05$) (Fig. 1A). Thus, these results suggested persistence of spirochetes at 12 months following antimicrobial treatment in both C3H and B6 mice infected with both *B. burgdorferi* strains, N40 and B31, although all the mice remained culture negative. The qPCR results also show persistence of BbDNA after antimicrobial treatment at 12 months, remaining in the mice even after 18 months. Persistent BbDNA was detected exclusively in tibiotarsal joints of all treatment groups, with mean copy numbers of *flaB-ospA* per milligram of tissue that were nearly equivalent (mean \pm SD, $1.52 \times 10^1 \pm 9.74 \times 10^0$) (Fig. 2). The qPCR results for saline-treated groups at 18 months were similar to the results for the same treatment groups at 12 months. *flaB-ospA* gene copies among the saline-treated groups at 18 months were lower in B6 mice infected with N40 and B31 than in C3H mice infected with N40 and B31, but not significantly. Mean *flaB-ospA* copy numbers in tibiotarsal joints at 18 months were significantly higher ($P < 0.05$) in saline-treated C3H mice infected with N40 and B31 than in the same groups of antimicrobial-treated mice (Fig. 1B).

Analysis of *B. burgdorferi flaB* and 16S rRNA gene transcriptional activity revealed that most of the saline-treated C3H and B6 mice infected with the N40 or B31 strain were positive. The transcriptional activities of *flaB* in samples from antimicrobial-treated mice (C3H and B6) necropsied at 12 and 18 months after treatment were 52% and 35%, respectively, while the 16S rRNA gene was constitutively detected in all samples. The results indicate viability among the persisting spirochetes.

Confirmation of spirochete noncultivability at 12 and 18 months following antimicrobial treatment. The infection status of mice at 12 months after antimicrobial treatment was assessed by culture of the inoculation site and the urinary bladder. For culture, we used a modified Barbour-Stoenner-Kelly II (BSK-II) medium. The sensitivity of BSK-II medium for detection of viable spirochetes was verified by serial 10-fold dilutions of *B. burgdorferi* N40 and B31 cultures, as described previously (46). The culture results revealed that all the saline-treated mice were positive, with significant numbers of spirochetes detected (Table 3). When mice treated with ceftriaxone were evaluated 12 months after completion of treatment, none of the tissues were culture positive. In a single culture, two spirochetes were observed, but we were unable to subculture them (Table 3).

For the first time point of this study (12 months), a limited number of sample sites (urinary bladder and inoculation site) were cultured. These are not optimal sites for culture after antimicrobial treatment, since persisting spirochetes tend to be localized in heart base and joint tissues. To determine if resurgent spirochetes regain cultivability 18 months after treatment, multiple sample types were collected, such as joint, ear, quadriceps muscle, spleen, urinary bladder, and inoculation site tissues. Heart tissue was not available for culture as it was for PCR and histology. To facilitate cultivation, several

TABLE 3 Culture results for individual mice treated with ceftriaxone and saline solution commencing 4 weeks after inoculation and then subjected to necropsy 12 months after completion of treatment

Mouse strain	<i>Borrelia</i> strain	Treatment	No. of samples positive/no. tested	
			Inoculation site	Urinary bladder
C3H	N40	Ceftriaxone	0/5	0/5
C3H	B31	Ceftriaxone	0/4	0/4
B6	N40	Ceftriaxone	0/5	1/5 ^a
B6	B31	Ceftriaxone	0/5	0/5
C3H	N40	Saline	5/5	5/5
C3H	B31	Saline	5/5	5/5
B6	N40	Saline	5/5	5/5
B6	B31	Saline	3/3	3/3

^aTwo spirochetes were observed, but we were unable to subculture them.

media have been introduced, such as BSK-II medium, modified Kelly-Pettenkofer (MKP) medium, BSK-II plus agarose, and BSK-II plus carbohydrates. The inoculated cultures were incubated for 50 days at 33°C and examined weekly. The culture tubes were gently but thoroughly stirred, and a drop of culture was examined by dark-field microscopy. The culture results revealed that all the saline-treated mice were positive, with significant numbers of motile spirochetes detected (Table 4 and Fig. 2A) in the majority of tissue samples cultured. When mice treated with ceftriaxone were evaluated, one ear tissue sample from a B6 mouse infected with B31 (Fig. 2B) and one front joint tissue sample from a B6 mouse infected with N40 (Fig. 2C) were culture positive (Table 4). Tissues were cultured in MKP and BSK-II plus carbohydrates media, respectively. After 4 weeks of incubation, spirochetes in culture medium from ceftriaxone-treated mice appeared nonmotile with irregular curves, so we were unable to subculture them. These results confirm our previous findings that antimicrobial-treated spirochetes are not cultivable. We hypothesized that noncultivability is probably a result of spirochetes being genetically impaired after treatment with antimicrobials.

Persistence of *B. burgdorferi* DNA in xenodiagnostic ticks that fed upon antimicrobial-treated mice. Several days prior to necropsy, each mouse was infested with approximately 40 larval *Ixodes scapularis* ticks, derived from a single population of laboratory-reared pathogen-free larvae. The ticks were allowed to feed to repletion. The engorged larvae were collected and allowed to molt into nymphs and harden, thereby allowing postfeeding amplification of spirochete populations and minimization of inhibitory products (blood) for PCR. Samples of 5 saline-treated and 10 ceftriaxone-treated randomly selected nymphal ticks from each tick cohort from each mouse were collected. The ticks were tested for the presence of *B. burgdorferi* *flaB-ospA* DNA by qPCR. The cohorts of nymphs from each mouse treated with saline solution at 12 and

TABLE 4 Culture results for individual mice treated with ceftriaxone and saline solution commencing 4 weeks after inoculation and then subjected to necropsy 18 months after completion of treatment

Mouse strain	<i>Borrelia</i> strain	Treatment	No. of samples positive/no. tested ^a					
			IS	Ear	UB	QM	FJ	Sp
C3H	N40	Ceftriaxone	0/4	0/4	0/4	0/4	0/4	0/4
C3H	B31	Ceftriaxone	0/5	0/5	0/5	0/5	0/5	0/5
B6	N40	Ceftriaxone	0/5	0/5	0/5	0/5	1/5 ^b	0/5
B6	B31	Ceftriaxone	0/5	1/5 ^b	0/5	0/5	0/5	0/5
C3H	N40	Saline	4/4	4/4	4/4	4/4	4/4	3/4
C3H	B31	Saline	3/3	1/3	2/3	2/3	3/3	1/3
B6	N40	Saline	5/5	4/5	1/5	3/5	3/5	2/5
B6	B31	Saline	3/3	3/3	3/3	3/3	3/3	3/3

^aIS, inoculation site; UB, urinary bladder; QM, quadriceps muscle; FJ, front joint; Sp, spleen.

^bSeveral spirochetes were observed, but we were unable to subculture them.

TABLE 5 Summary results of xenodiagnosis testing for mice treated with ceftriaxone and saline solution 4 weeks after infection and then infested with larval ticks 12 and 18 months after completion of treatment

Mouse strain	<i>Borrelia</i> strain	Treatment	No. of mice positive/ no. tested (xenoDx ^a)	
			12 mo	18 mo
C3H	N40	Ceftriaxone	3/5	1/4
C3H	B31	Ceftriaxone	2/5	0/0
B6	N40	Ceftriaxone	2/5	2/5
B6	B31	Ceftriaxone	2/5	0/0
C3H	N40	Saline	5/5	4/4
C3H	B31	Saline	5/5	3/3
B6	N40	Saline	5/5	5/5
B6	B31	Saline	3/3	5/5

^axenoDx, xenodiagnosis.

18 months were all *flaB-ospA* PCR positive (Table 5). The mean copy numbers of *flaB-ospA* DNA ranged from 389 to 13,100 (mean \pm SD, 3,120 \pm 3,790) and from 97 to 11,950 (mean \pm SD, 2,340 \pm 2,820) copies per tick at 12 months and 18 months, respectively. In contrast, 9 of 20 and 3 of 19 ceftriaxone-treated mice had 1 *flaB-ospA* PCR-positive tick each among the 10 ticks tested from each mouse at 12 and 18 months, respectively. The mean copy numbers of *flaB-ospA* DNA ranged from 238 to 1,090 (mean \pm SD, 596 \pm 249) and from 69 to 714 (mean \pm SD, 473 \pm 352) copies per tick at 12 months and 18 months, respectively (Table 5).

Histopathology of mouse tissues following antimicrobial treatment. Here, we report on the general features of histopathology. Legs were examined for evidence of arthritis and synovitis and hearts for neutrophil and macrophage infiltration. At 12 months after treatment, inflammatory scores were greatest and were more robust for myocarditis in control C3H/N40 and C3H/B31 mice than in the antimicrobial treatment groups (Fig. 3). Tibiotarsal arthritis was mild, characterized predominantly by synovial hyperplasia and hypertrophy with joint or tendon sheath effusion in both antimicrobial-treated and saline-treated mice at 12 months. There were statistically significant differences (Kruskal-Wallis test; $P = 0.0227$) between groups based on the myocarditis scores. The antimicrobial-treated C3H/B31 mice had statistically significantly lower scores than the saline-treated C3H/B31 mice (Dunn's multiple-comparison test; $P = 0.043$). No statistically significant differences (Kruskal-Wallis test; $P = 0.1187$) were observed between groups based on tibiotarsal arthritis scores (Table 6). At 18 months, inflammation scores were greatest for myocarditis in the saline-treated groups: C3H/N40, C3H/B31, and B6/N40 mice (Fig. 3). Chronic periarteritis and tenosynovitis were observed in a few saline-treated C3H/B31 and B6/N40 mice, respectively, a pathohistological finding frequently observed in chronically borrelia-infected mice (Fig. 4). Osteoarthritis was very mild and was considered a background, age-related finding. There were statistically significant differences (Kruskal-Wallis test; $P = 0.0019$) between groups based on the myocarditis scores. The C3H/N40 and C3H/B31 saline-treated groups had statistically significantly higher scores than the C3H/B31 antimicrobial-treated group (Dunn's multiple-comparison test; $P = 0.046$ and $P = 0.007$). No statistically significant differences (Kruskal-Wallis test; $P = 0.1431$) were observed between groups based on the tibiotarsal arthritis scores. Statistical analyses were performed using GraphPad Prism v. 7.03 (Table 6).

***B. burgdorferi* plasmid content in mouse tissues.** In our previous work, we conducted experiments utilizing primarily strain N40, so the designed qPCR assays were based on its genome sequence. For analysis of plasmid presence in tissue samples from C3H and B6 mice infected with *B. burgdorferi* strain B31, fewer genes were targeted. A comparison of the plasmid profiles, sizes, and GC contents of strains N40 and B31 appeared to show major structural differences, so only four designed qPCR assays targeting *bptA*, *arp*, *ospA*, and *erp23* of strain N40 are homologous to those of strain B31.

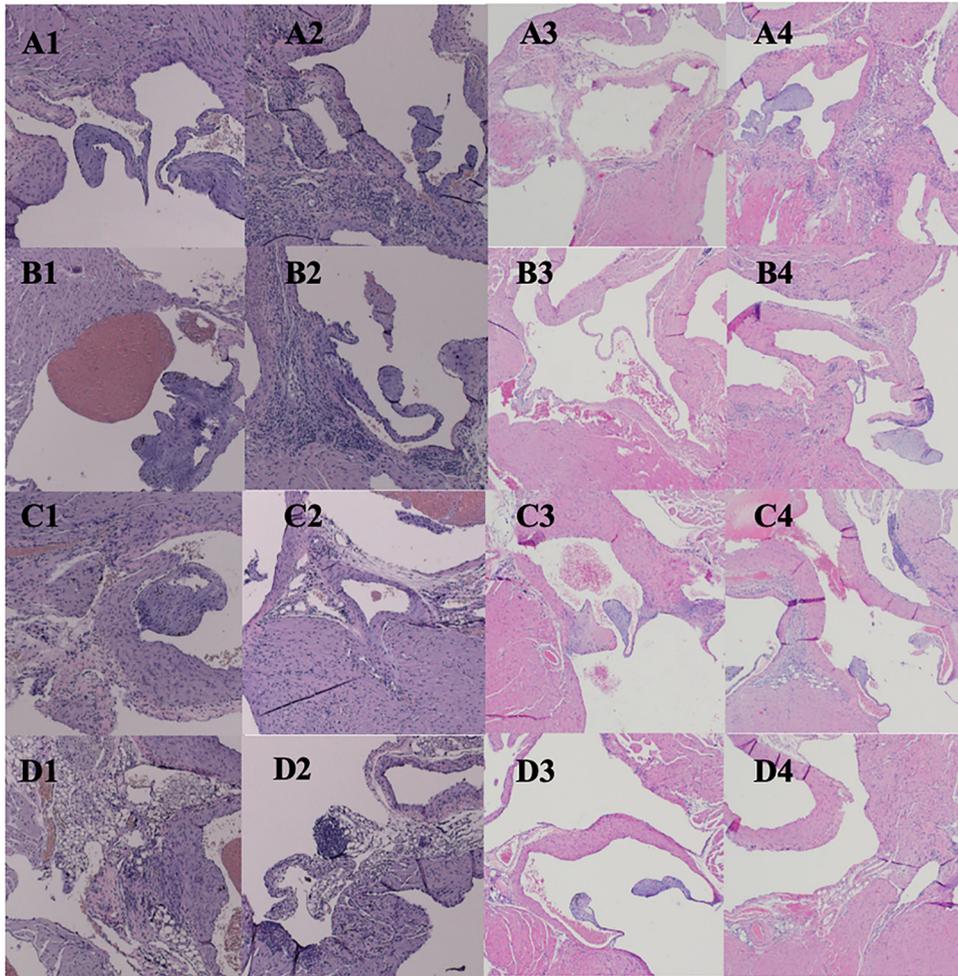


FIG 3 Comparisons of carditis severity between ceftriaxone-treated and saline-treated C3H and B6 mice infected with either *B. burgdorferi* strain N40 or B31 necropsied at 12 and 18 months after treatment. (A1 to A4) C3H/N40. (B1 to B4) C3H/B31. (C1 to C4) B6/N40. (D1 to D4) B6/B31. (Column 1) Ceftriaxone treated/12 months. (Column 2) Saline treated/12 months. (Column 3) Ceftriaxone treated/18 months. (Column 4) Saline treated/18 months.

Among 12-month and 18-month samples, all the gene targets were detected in tissue samples from saline-treated C3H and B6 mice infected with either *B. burgdorferi* strain N40 or B31 (Tables 7 and 8). As controls for plasmid content we used cultured *B. burgdorferi* N40 and B31, confirming detection of all the targeted genes. The *B. burgdorferi* strain N40 genomes in tissue samples with high BbDNA copy numbers from 12 antimicrobial-treated mice were uniformly missing *bptA* (lp25), *erp22* (cp32-9), and *erp23* (cp34-2) and variably missing *arp* (lp28-5), *eppA* (cp9), and *ospC* (cp26), whereas *ospA* (lp54) was detected in all examined tissue samples (Table 7). All the assessed tissue samples from mice infected with strain B31 were uniformly missing *bptA* (lp25) and *arp* (lp28-1) and variably missing *erp23* (cp32-7). As in tissue samples from mice infected with strain N40, *ospA* (lp54) was detected in all the samples examined (Table 8). The data suggest loss of linear and circular plasmids in spirochetes persisting at 12 and 18 months after treatment with antimicrobials. No difference was observed between different *B. burgdorferi* strains (B31 and N40) or in genetically different strains of mice that are disease susceptible (C3H) and disease resistant (B6). Most notable was the uniform absence of lp25 in both *B. burgdorferi* strains N40 and B31. The results also demonstrated amplification of multiple gene targets, thereby verifying the specificity of residual-BbDNA results (Tables 7 and 8).

TABLE 6 Prevalence and severity of carditis and arthritis in different mouse strains infected with two different *B. burgdorferi* strains 12 and 18 months after treatment with ceftriaxone or sham treatment with saline solution

Mouse strain	<i>Borrelia</i> strain	Treatment/mo after treatment	Carditis prevalence ^a	Mean carditis score \pm SD ^b	Arthritis prevalence ^a	Mean arthritis score \pm SD ^c
C3H	N40	Ceftriaxone/12	2/5	0.40 \pm 0.55	4/5	0.88 \pm 0.45
C3H	B31	Ceftriaxone/12	0/4	0	0/4	0
B6	N40	Ceftriaxone/12	3/5	0.60 \pm 0.55	2/5	0.40 \pm 0.55
B6	B31	Ceftriaxone/12	3/5	0.60 \pm 0.55	0/5	0
C3H	N40	Ceftriaxone/18	2/4	0.50 \pm 0.58	2/4	0.50 \pm 0.58
C3H	B31	Ceftriaxone/18	0/5	0	0/5	0
B6	N40	Ceftriaxone/18	2/5	0.40 \pm 0.55	2/5	0.40 \pm 0.55
B6	B31	Ceftriaxone/18	2/5	0.40 \pm 0.55	0/5	0
C3H	N40	Saline/12	5/5	1.60 \pm 0.89	4/5	0.80 \pm 0.45
C3H	B31	Saline/12	5/5	2.00 \pm 1.00	4/5	0.80 \pm 0.45
B6	N40	Saline/12	3/5	0.60 \pm 0.55	4/5	0.80 \pm 0.45
B6	B31	Saline/12	3/3	1.33 \pm 0.58	2/3	0.67 \pm 0.58
C3H	N40	Saline/18	4/4	1.50 \pm 0.58	4/4	1.00 \pm 0.00
C3H	B31	Saline/18	3/3	2.00 \pm 0.00	1/3	0.33 \pm 0.58
B6	N40	Saline/18	5/5	1.20 \pm 0.45	3/5	0.60 \pm 0.55
B6	B31	Saline/18	4/5	0.67 \pm 0.58	1/5	0.33 \pm 0.58

^aNumber of hearts or joints with inflammation/total number of hearts or joints examined. One tibiotarsus from each mouse was examined.

^bThe severity of carditis was scored on a scale from 0 to 3.

^cThe severity of arthritis was scored on a scale from 0 to 1.

DISCUSSION

The most commonly prescribed antimicrobial agents for the treatment of human Lyme borreliosis, such as penicillin, amoxicillin, cefotaxime, cefuroxime, ceftriaxone, doxycycline, and erythromycin, have been shown to be effective against *B. burgdorferi* (67, 68). It is important to mention that treatment during early infection is more effective in clearing the pathogen than treatment during later infection in humans (69) and in the mouse model (46).

In this study, we used ceftriaxone, the half-life of which in mouse serum ranges from 1 h (70) to 1.35 h (71) or 1.41 h (72). Its antimicrobial activity and pharmacokinetic (PK)/pharmacodynamic (PD) properties display time-dependent killing (percent $T > MIC$) and moderate persistent effect, which were indications for multiple daily dosages (72–74). Time-dependent killing is considered a main parameter determining antimicrobial efficacy (73). The MICs and minimal bactericidal concentrations (MBCs) for ceftriaxone against both *B. burgdorferi* N40 and B31 were evaluated previously in our laboratory (46, 50) and were in accordance with those in similar published studies (75, 76). Studies with ceftriaxone in mice have been challenging because the pharmacodynamics ($T > MIC$) of ceftriaxone do not parallel those in humans (77). However, the ceftriaxone treatment regimen used (twice daily for 5 days, followed by once daily for 23 days) in our study transiently achieves more than adequate serum concentrations, >300-fold greater than the MBC. Cremieux et al. (78) demonstrated significant bacterial killing when ceftriaxone concentrations exceeded the MBC. The magnitude of bacterial regrowth after the effects of antimicrobials wane and before the next dose is admin-

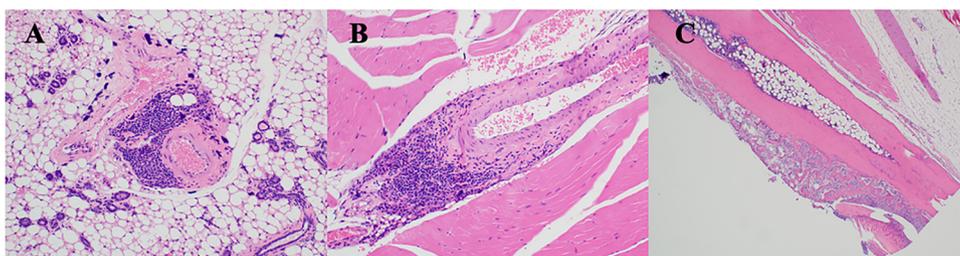


FIG 4 (A and B) Chronic periarteritis observed in a C3H mouse infected with *B. burgdorferi* B31 and then treated with saline solution and necropsied 18 months after treatment. (C) Tenosynovitis observed in a B6 mouse infected with *B. burgdorferi* N40 and then treated with saline solution and necropsied 18 months after treatment.

TABLE 7 Analysis of plasmid presence in heart base, ventricular muscle, and tibiotarsus of mice infected with *B. burgdorferi* strain N40 and then treated with ceftriaxone or sham treated with saline solution

Mouse strain	Treatment/time (h)	Sample type ^a	Plasmid presence ^b							
			Chrom <i>flaB</i>	lp25 <i>bptA</i>	lp28-5 <i>arp</i>	lp54 <i>ospA</i>	cp9 <i>eppA</i>	cp26 <i>ospC</i>	cp32-9 <i>erp22</i>	cp34-2 <i>erp23</i>
C3H	Saline/12	HB	+	+	+	+	+	+	+	+
B6	Saline/12	TT	+	+	+	+	+	+	+	+
C3H	Saline/18	HB	+	+	+	+	+	+	+	+
B6	Saline/18	VM	+	+	+	+	+	+	+	+
C3H	Ceftriaxone/12	HB	+	-	-	+	-	-	-	-
C3H	Ceftriaxone/12	TT	+	-	-	+	-	+	-	-
C3H	Ceftriaxone/12	TT	+	-	-	+	-	-	-	-
B6	Ceftriaxone/12	TT	+	-	+	+	-	-	-	-
B6	Ceftriaxone/12	HB	+	-	-	+	-	-	-	-
B6	Ceftriaxone/12	TT	+	-	-	+	-	-	-	-
C3H	Ceftriaxone/18	TT	+	-	-	+	-	-	-	-
C3H	Ceftriaxone/18	TT	+	-	-	+	-	-	-	-
C3H	Ceftriaxone/18	TT	+	-	+	+	+	+	-	-
B6	Ceftriaxone/18	TT	+	-	-	+	-	-	-	-
B6	Ceftriaxone/18	TT	+	-	-	+	-	-	-	-
B6	Ceftriaxone/18	TT	+	-	-	+	-	-	-	-

^aHB, heart base; VM, ventricular muscle; TT, tibiotarsus.^bChrom, chromosomal; +, present; -, absent.

istered depends on the doubling time of the bacteria (79), which is 5 to 12 h for *B. burgdorferi* (80, 81). Several publications reported clearance of bacteria with short doubling times (20 to 60 min) from infected mice after two daily doses (71), and even a single daily dose (70, 72), of ceftriaxone. Wu et al. (82) and Pavia and Wormser (83) reported *B. burgdorferi* clearance from infected mice after administering ceftriaxone twice or once a day for 5 days, respectively. These reports confirm that the ceftriaxone treatment regimen used in our study was adequate.

Over the past several years, there have been numerous well-supported reports of *Borrelia* detection after completion of antimicrobial therapy (46, 50, 53, 60, 84, 85). The prevalent findings of all these studies were detection of *B. burgdorferi* by PCR, but not by culture. In BbDNA-positive tissues from antimicrobial-treated mice, morphologically intact spirochetes were observed by immunohistochemistry. Ticks could acquire the spirochetes and transmit them to recipient mice. Transcriptional activity of mouse tissues treated with antimicrobials was detected for numerous targeted *B. burgdorferi* genes (86).

TABLE 8 Analysis of plasmid presence in heart base, ventricular muscle, and tibiotarsus of mice infected with *B. burgdorferi* strain B31 and then treated with ceftriaxone or sham treated with saline solution

Mouse strain	Treatment/time (h)	Sample type ^a	Plasmid presence ^b				
			Chrom <i>flaB</i>	lp25 <i>bptA</i>	lp28-1 <i>arp</i>	lp54 <i>ospA</i>	cp32-7 <i>erp23</i>
C3H	Saline/12	VM	+	+	+	+	+
B6	Saline/12	VM	+	+	+	+	+
C3H	Saline/18	HB	+	+	+	+	+
B6	Saline/18	HB	+	+	+	+	+
C3H	Ceftriaxone/12	TT	+	-	-	+	-
C3H	Ceftriaxone/12	HB	+	-	-	+	-
C3H	Ceftriaxone/12	TT	+	-	-	+	-
B6	Ceftriaxone/12	TT	+	-	-	+	+
B6	Ceftriaxone/12	HB	+	-	-	+	-
B6	Ceftriaxone/12	TT	+	-	-	+	-
C3H	Ceftriaxone/18	TT	+	-	-	+	-
C3H	Ceftriaxone/18	TT	+	-	-	+	-
C3H	Ceftriaxone/18	TT	+	-	-	+	-
B6	Ceftriaxone/18	TT	+	-	-	+	-
B6	Ceftriaxone/18	TT	+	-	-	+	-
B6	Ceftriaxone/18	TT	+	-	-	+	-

^aHB, heart base; VM, ventricular muscle; TT, tibiotarsus.^bChrom, chromosomal; +, present; -, absent.

In the current study, we evaluated persistence at 12 and 18 months after ceftriaxone treatment in two strains of mice that are disease susceptible (C3H) and disease resistant (B6). We recognize that inbred mice do not represent genetically heterogeneous humans, but the use of two genetically and phenotypically disparate mouse strains confirmed the generality of persistence following antimicrobial treatment. Disease severity and tissue spirochete burdens vary among mouse strains (87), although the 50% infective doses (ID_{50s}) among the mice are identical (88). C3H/HeJ mice are susceptible to infection by Gram-negative bacteria as a result of spontaneous mutation in the *Tlr4* gene (89). It has been demonstrated that C3H mice develop more severe arthritis and carditis following *B. burgdorferi* infection than B6 mice (90). B6 mice harbor tissue spirochete burdens equivalent to those of C3H mice, become persistently infected, and have normal macrophage function (89).

Our results have shown that, indeed, persistence in fully immunocompetent B6 mice is similar to that in disease-susceptible C3H mice, which confirms the generality of persistence in a mouse model not being affected by the genetic and immunological makeup of the mammalian host. These results confirm our previous finding (47) that a subpopulation of viable, antimicrobial-tolerant, but slowly dividing spirochetes of *B. burgdorferi* persists in mice 12 months after treatment and is redisseminated into multiple tissues. Importantly, the copy numbers of spirochetal DNA in tissue samples from antimicrobial-treated disease-resistant B6 mice were nearly equivalent to those in disease-susceptible C3H mice. However, significantly higher DNA copy numbers of spirochetal DNA were determined in saline-treated groups of C3H mice infected with either N40 or B31 than in antimicrobial-treated mice (C3H and B6). This finding is in concordance with the results published by Armstrong et al. (91) showing that C3H mice develop more severe disease than B6 mice, with greater spirochete numbers and later clearance.

Despite the fact that tissues of treated mice remained BbDNA PCR positive at 12 months with significant copy numbers of the targeted genes, culture was consistently negative. Actually, in a single cultured urinary bladder, we observed two spirochetes for up to 3 weeks of incubation. On the other hand, all saline-treated mice were culture positive. Our studies (46, 47, 50) and those of others (45, 49, 92) in mice, dogs (29, 52), and nonhuman primates (53, 54) have all reached similar conclusions: spirochetes persist in tissues after antimicrobial treatment but, paradoxically, cannot be cultured. It has been suggested that the persisting remnants of *B. burgdorferi* in the tissues of infected mice after antimicrobial treatment are DNA or DNA-containing structures rather than live bacteria (77, 85, 93). In their *in vitro* study, Iyer et al. (94) could not successfully subculture spirochetes after exposure to ceftriaxone. However, BbDNA was detected by PCR for up to 56 days in aliquots from both ceftriaxone-treated and untreated cultures. Pavia and Wormser (83) demonstrated that *B. burgdorferi* could not be cultured from experimentally infected mice after ceftriaxone treatment with only 5 daily doses. The treatment regimen used in the study was judged based on the absence of a positive culture.

It has been stated that "unless proven otherwise, culture should be regarded as the gold standard to address viability of *B. burgdorferi*" (95, 96). Culture may indeed be a gold standard when it is positive, but it is often not. It is apparent that not all isolates or strains can be easily cultured, and this is especially apparent during long-term infection. It is becoming increasingly clear that in *B. burgdorferi*-infected and antimicrobial-treated animals, spirochetes are noncultivable but viable, as has been demonstrated by xenodiagnoses (46, 47, 50, 54), immunohistology (46, 47, 84), and transcriptional activity of mRNA (47, 50, 54). Here, we demonstrated that *B. burgdorferi* spirochetes that survived antimicrobial treatment in disease-susceptible C3H mice, as well as in disease-resistant B6 mice, could be acquired by larval ticks. BbDNA was detected in nymphs after molting. In addition, transcription of the chromosomal *B. burgdorferi* 16S rRNA and *flaB* genes was detected in treated mice, indicating that spirochetes are metabolically active and alive 12 and 18 months after treatment. The data obtained in this study indicate that in a significant number of culture-negative

tissue samples following antimicrobial treatment, complementary methods in diagnostic microbiology should be considered. The clinical significance and the prognostic value of these findings have to be more deeply investigated. It was shown that dead bacterial DNA can be detected for up to 4 to 5 months after antimicrobial treatment (97), as extracellular DNA is very prone to degradation (98–100). Lazarus et al. (101) demonstrated that BbDNA could not be detected in skin tissues of mice that received killed spirochetes 8 h after injection. Bacterial mRNAs, on the other hand, have very short half-lives, as they are degraded by exonucleases very quickly. The half-life ranges from a few minutes to several hours, depending on the bacterial strain (102–104). These findings support the notion that the noncultivability of antimicrobial-tolerant and persistent spirochetes does not negate their viability.

Our previous studies in mice (46, 47, 50) and those of others (49, 92) are all based on *B. burgdorferi* strain N40. Although N40 probably represents *B. burgdorferi*, the generality of long-term spirochete persistence following antimicrobial treatment should be evaluated with other *B. burgdorferi* strains. Therefore, we used *B. burgdorferi* strain B31 and confirmed persistence at 12 months after antimicrobial treatment in both C3H and B6 mice. Our results indicate that disseminated spirochetes of two different *B. burgdorferi* strains can persist in mice at 12 and 18 months following antimicrobial treatment. Noncultivable spirochetes persisted in mice (105), dogs (29), and nonhuman primates (54) inoculated with alternate strains of *B. burgdorferi*, so we have confirmed that persistence is not unique to N40.

An additional long interval, holding mice up to 18 months, facilitated several observations regarding persistent antimicrobial-tolerant spirochetes. Within the observation period, there was molecular evidence of BbDNA in 79% of all assessed mice following antimicrobial treatment. Spirochetal DNA was exclusively detected in the tibiotarsal joint, but not in heart base and ventricular muscle. Copy numbers of BbDNA were not significantly different between treated C3H and B6 mice infected with either N40 or B31. Interestingly, BbDNA copy numbers in the tibiotarsal joints of all the antimicrobial-treated mice at 18 months were equivalent to those at 12 months, suggesting that antimicrobial-tolerant spirochetes were persistent exclusively in connective tissue. One of the proposed mechanisms of *B. burgdorferi* immune evasion and persistence is sequestration in collagen-rich connective tissue, which makes them less accessible to cells and molecules of the host's immune system (55, 106). It has been suggested that joint tissue is the niche of *B. burgdorferi* persistence in mice following antimicrobial treatment (49). There is scientific evidence that tissues with greater decorin expression levels, such as joint tissue, harbor the greatest spirochete loads during chronic infection (107). The findings of our study reinforce the notion of persistence of viable spirochetes following antimicrobial treatment and further implicate connective tissue as a privileged site that may contribute to treatment failure. Although spirochetes persisted for 12 months after antimicrobial treatment, overt disease was not present. Also, despite spirochete presence, postmortem gross signs of disease were not observed in the assessed tissues (heart and joint) of treated mice at 18 months. The resolution of arthritis and carditis in animal models of Lyme borreliosis has been shown to be mediated by the acquired humoral immune response of the host (108). Under these conditions, anatomically defined inflammation resolves, but infection persists, reinforcing the notion that inflammation does not inevitably correlate with the presence of spirochetes (108–110).

Spirochetal RNA was detected in the joint tissue of C3H and B6 mice infected with either N40 or B31 at 18 months following treatment, suggesting its viability. Interestingly, xenodiagnosis was positive in only 3 of 19 treated animals. We speculate that the inability of ticks to acquire spirochetes from all the treated mice could be ascribed to the clearance of spirochetes from heart tissue. Migration of spirochetes from joints to tick attachment sites, that is, usually the head and/or a dorsal part of the mouse body, is farther than from the heart. It was also suggested that the efficiency of spirochete acquisition from a host by a tick depends on the intensity and duration of infection, and it is significantly less during chronic infection (111). However, we recovered a few

slow-growing spirochetes by culture from joint tissues of two mice, utilizing several specially prepared media that were graciously provided by Monica E. Embers. We were unable to propagate the recovered spirochetes. Obviously, the media used were unable to support the prolonged growth of slow-growing spirochetes.

Several studies have shown that *B. burgdorferi* is inclined to plasmid loss (64, 66, 81, 112–114). In addition to a complete loss, there is a report of a partial plasmid loss of the *vls* gene cassette (115). Several plasmids were found to be associated with spirochete infectivity (65). Plasmid loss was reported to impact infectivity (66, 116), and spirochetes became more vulnerable to host-mediated clearance (117). Plasmid profile analysis of tissue samples from antimicrobial-treated C3H and B6 mice infected with either *B. burgdorferi* N40 or B31 revealed loss of several plasmids in persisting spirochetes at 12 and 18 months after antimicrobial treatment. A correlation between plasmid loss and persistence of antimicrobial-tolerant spirochetes is not clear and will require further investigation.

This study has shown that following antimicrobial treatment, both strains of *B. burgdorferi*, N40 and B31, produce a progressively diverse population of noncultivable and persistent spirochetes. The persistent spirochetes divide and grow slowly, which probably results in their tolerance for the effects of antimicrobial agents, as well as being noncultivable, confirming our previous findings (47). Antibacterial resistance has not been observed for *B. burgdorferi*, so the phenomenon of persistence of noncultivable spirochetes following antimicrobial treatment has been explained by antimicrobial tolerance (63). Unlike antimicrobial resistance, antimicrobial tolerance, for all classes of antimicrobials, fails to completely eliminate nondividing or slowly dividing subpopulations of a broad array of bacteria and fungi (62, 118). Persistence is difficult to study, as persisters are phenotypic variants of actively dividing cells produced in the population at low abundance during the late exponential phase of growth (118–121). The mechanisms of *B. burgdorferi* persistence, especially after antimicrobial treatment, is still to be determined. Several mechanisms have been proposed, such as gene modulation as a result of the stringent response (122), active immune suppression and evasion (55), metabolic alteration of guanosine pentaphosphate [(p)ppGpp] and probably the *dksA* gene (123, 124), and frequency of environmental changes (123). Further studies are required to establish the roles of those mechanisms in persistence.

The clinical relevance of microbial persistence has been demonstrated for *Burkholderia pseudomallei* (125), *Escherichia coli* (126), *Pseudomonas aeruginosa* (127), *Candida albicans* (128), *Streptococcus pneumoniae* (129), *Staphylococcus aureus* (126, 130), *Mycobacterium tuberculosis* (131, 132), *Legionella* spp. (133, 134), and *Salmonella enterica* (135). The persistent cells have transient tolerance for antimicrobial agents, and their ability to revert to a sensitive state, in which cells rapidly divide, makes them important in chronic infections (136). There are two major consequences of the presence of persister cells: (i) the continuous presence of viable cells during consecutive rounds of antimicrobial treatment contributes to the emergence of antimicrobial resistance and (ii) experimental evidence indicates that prolonged exposure to antimicrobial agents leads to the selection of highly persistent mutants (137, 138). The infectious life cycle relevance of attenuated *B. burgdorferi* spirochetes is probably inconsequential, while their clinical relevance in mice was a subject of this study. The study demonstrated that noncultivable spirochetes can persist in mice following antimicrobial treatment for up to 18 months but did not demonstrate their clinical relevance. The relatively short mouse life span (18 to 24 months) is a limiting factor in studying the persistence and resurgence of antimicrobial-tolerant *B. burgdorferi* beyond 18 months after treatment. The phenomenon of resurgence observed in mice at 12 months after antimicrobial treatment (47) resembles recurrent Lyme borreliosis observed in humans (139–141). Therefore, the clinical relevance of persistent spirochetes and their resurgence beyond 18 months following antimicrobial treatment compels further studies utilizing other animal models.

TABLE 9 Experimental design for eight treatment groups

Group	<i>B. burgdorferi</i> strain	Mouse strain	Treatment	No. of mice treated for:		Total no. of mice (<i>n</i>)
				12 mo	18 mo	
1	N40	C3H	Ceftriaxone	5	5	10
2	B31	C3H	Ceftriaxone	5	5	10
3	N40	B6	Ceftriaxone	5	5	10
4	B31	B6	Ceftriaxone	5	5	10
5	N40	C3H	Saline	5	5	10
6	B31	C3H	Saline	5	5	10
7	N40	B6	Saline	5	5	10
8	B31	B6	Saline	5	5	10

MATERIALS AND METHODS

Mouse infections. All experiments involving vertebrate animals were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California Davis (UCD). All the animals were purchased from The Jackson Laboratory, Bar Harbor, ME, and were cared for by staff from Teaching and Research Animal Care Services (TRACS) at UCD. The mice were maintained in an isolated room within filter top cages and were provided food and water *ad libitum*. The animals used in this study were C3H and B6 specific-pathogen-free 5-week-old female mice and were maintained in cohorts of 5 mice per cage under identical husbandry conditions in the same animal room. Euthanasia was performed by rapid carbon dioxide narcosis in accordance with guidelines of the American Veterinary Medical Association (AVMA) (142). C3H and B6 mice were randomly divided into two equal groups. One group of C3H and B6 mice were infected with *B. burgdorferi* N40 and another with *B. burgdorferi* B31 (Table 9). Each mouse was infected by syringe inoculation of 10^4 *B. burgdorferi* spirochetes at the mid-log phase in 0.1 ml of BSK-II medium intradermally at the dorsal thoracic midline.

***B. burgdorferi* culturing.** Two low-passage clonal strains of *B. burgdorferi sensu stricto* were used. *B. burgdorferi* strains N40 and B31 were cloned by 3-fold limiting dilution *in vitro* and passage in mice to prove infectivity and pathogenicity, as described previously (24). Frozen aliquots of clonal strains N40 and B31 were thawed and cultured in modified BSK-II medium (81) at 33°C. Spirochetes were assessed for viability and enumerated by dark-field microscopy using a Petroff-Hausser bacterial counting chamber (Hausser Scientific, Horsham, PA) immediately prior to use and diluted to appropriate concentrations in BSK-II medium. The mice were inoculated subdermally on the dorsal thoracic midline with 10^4 mid-log-phase spirochetes in 0.1 ml of BSK-II medium. The status of infection with cultivable *B. burgdorferi* was determined by culture of the urinary bladder and the subinoculation site (deep dermis) at 12 and 18 months after completion of antimicrobial treatment. Tissues were collected from mice aseptically at necropsy and then cultured in medium without antibiotic, as described previously (143).

To determine if persistent spirochetes regain cultivability at 18 months after treatment and to increase their detectability, multiple tissue sites were collected and cultured in four different media. In addition to the urinary bladder and inoculation site, we also collected front joint, ear, quadriceps muscle, and spleen tissues. Heart tissue was not available for culture as it was for PCR and histology. To facilitate cultivation, several media have been introduced, such as BSK-II medium, MKP medium, BSK-II plus agarose, and BSK-II plus carbohydrates (cordially provided by Monica E. Embers of Tulane National Primate Research Center, Covington, LA). However, these media differ in their potentials to support the growth of borrelia.

Xenodiagnosis. Laboratory-reared, pathogen-free *I. scapularis* larvae were provided by Melissa J. Caimano, Department of Molecular Biology and Biophysics, University of Connecticut Health, Farmington, CT. All the larvae were derived from single gravid ticks for each study. Around 40 larval ticks were placed on each mouse 1 week prior to necropsy, allowed to feed to repletion, collected, and then allowed to molt and harden into nymphs. Cohorts of ticks collected from each mouse were maintained separately, so that ticks within each cohort could be tested individually or in groups by qPCR. The nymphal ticks were frozen in liquid nitrogen and ground with a mortar and pestle, and DNA was extracted for PCR analysis.

Antimicrobial treatment and monitoring. Treatment with ceftriaxone was initiated during the early immune/dissemination stage at 4 weeks postinfection with *B. burgdorferi* in immunocompetent C3H and B6 mice to mimic the stages found in human disease. The experimental design for this experiment involved eight treatment groups, as depicted in Table 9. The treatment regimen used in the study was published previously (45, 46). A ceftriaxone dosage of 16 mg/kg of body weight in 500 μ l of 0.9% normal saline was administered intraperitoneally to the mice twice daily for 5 days, followed by a once-daily dose for 23 days. The serum concentrations of ceftriaxone in mice were measured on the day of treatment by bleeding the mice at 0, 2, 4, and 8 h after treatment. Serum samples were separated from whole blood and stored at -80°C until analysis. A previously described agar diffusion assay method (144) was utilized to determine the serum concentration of ceftriaxone.

Tissue processing for molecular analysis. Mouse tissue samples for molecular analysis were processed as previously described (47, 145), with slight modifications. Briefly, samples of the heart base, ventricular myocardium (the heart was bisected through the atria and ventricles, with one-half of the heart base and ventricles used for nucleic acid extraction and the other half processed for histology), and

the left tibiotarsal joint were collected from each mouse. The samples were collected aseptically, immediately weighed, snap-frozen in liquid nitrogen, and stored at -80°C before nucleic acid extraction. Total nucleic acid was extracted with a QIAcube HT system (Qiagen, Valencia, CA) according to the manufacturer's instructions for tissue or insects. The system enables semiautomated high-throughput nucleic acid purification, yielding high-quality pathogen nucleic acids free of contaminants and inhibitors. The copy number of each *B. burgdorferi* target gene was expressed per milligram of tissue weight.

Molecular quantitative analysis. Because low DNA/RNA copy numbers were expected in tissues from antimicrobial-treated mice, each sample was subjected to preamplification. We used the *B. burgdorferi* outer surface protein (*ospA*) and flagellin (*flaB*) primers and a mouse glyceraldehyde-3-phosphate dehydrogenase (*gapDH*) assay primer, standardized and optimized for real-time qPCR, as described previously (146). The preamplified products were diluted at a ratio of 1:10 and used as templates for qPCR analysis. To assess the transcriptional activity of the *B. burgdorferi* *flaB* and 16S rRNA genes, first-strand cDNA was synthesized from total RNA using a QuantiTect reverse transcription kit (Qiagen) in 50- μl reaction mixtures, as described previously (146). To increase the fidelity, efficiency, and yield of cDNA, Advantage 2 polymerase mix (Clontech Laboratories) was used, according to the manufacturer's instructions.

Two specific primers and one internal fluorescence-labeled probe were designed with Primer Express software (ThermoFisher Scientific), as described previously (147). The amplification efficiency (*E*) of all assays was calculated from the slope of a standard curve as described in detail previously (47). To determine the copy numbers of DNA target genes, plasmid standards were prepared in order to create absolute standard curves, as described previously (147). Based on the amplification efficiencies, the detection limits were <10 copies of DNA per reaction, and the analytical sensitivity for each target gene was in the range from <10 to 10^9 copies. All samples were assayed in duplicate with positive and negative controls by qPCR optimized assays and analyzed for the presence of mouse *gapDH* in order to determine the efficiency of the nucleic acid extraction and amplification and as an indicator of inhibition. Amplification, data acquisition, and data analysis were performed on a 7900HT Fast real-time PCR system (ThermoFisher Scientific). The thermal-cycling conditions were described previously (148).

Histology. Formalin-fixed rear legs (demineralized in 15% formic acid) and hearts were routinely processed for histology, embedded in paraffin, and sectioned at 5 μm . The sections were stained with hematoxylin and eosin. All tissues were evaluated by a board-certified veterinary anatomical pathologist. The legs were examined for evidence of arthritis and synovitis involving the knee and tibiotarsal joints, as characterized by examination of infiltration of neutrophils, synovial proliferation, and exudation of fibrin into joint or tendon sheath lumina, as well as neutrophil and macrophage infiltration of tissues at the base of the heart (myocarditis). All determinations were made in a blinded fashion.

Statistics. Statistical analysis of qPCR data between antimicrobial-treated and sham-treated mice at different time points was performed using an independent-samples *t* test or one-way analysis of variance, followed by multiple pairwise comparisons by Tukey's honestly significant difference (HSD) test (SPSS 16.0 for Mac; SPSS Inc., Chicago, IL). Differences were considered significant at a *P* value of ≤ 0.5 . To statistically evaluate the severity of inflammation in collected tissue between antimicrobial-treated and sham-treated mice, a Kruskal-Wallis test was performed using GraphPad Prism v. 7.03.

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We declare that no competing interests exist.

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