Detection of Attenuated, Noninfectious Spirochetes in *Borrelia burgdorferi*–Infected Mice after Antibiotic Treatment

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Xenodiagnosis by ticks was used to determine whether spirochetes persist in mice after 1 month of antibiotic therapy for vectorborne *Borrelia burgdorferi* infection. Immunofluorescence and polymerase chain reaction (PCR) were used to show that spirochetes could be found in *Ixodes scapularis* ticks feeding on 4 of 10 antibiotic-treated mice up to 3 months after therapy. These spirochetes could not be transmitted to naive mice, and some lacked genes on plasmids correlating with infectivity. By 6 months, antibiotic-treated mice no longer tested positive by xenodiagnosis, and cortisone immunosuppression did not alter this result. Nine months after treatment, low levels of spirochete DNA could be detected by real-time PCR in a subset of antibiotic-treated mice. In contrast to sham-treated mice, antibiotic-treated mice did not have culture or histopathologic evidence of persistent infection. These results provide evidence that noninfectious spirochetes can persist for a limited duration after antibiotics but are not associated with disease in mice.

Lyme disease is a multisystem disorder caused by infection with the tick-transmitted spirochete *Borrelia burgdorferi* [1, 2]. After establishing infection in the skin, spirochetes disseminate via the bloodstream to other sites, including the joints, heart, and nervous system, where disease manifestations can be seen. Disease can remit without specific therapy in both humans and animal models of Lyme disease, even though spirochetes persist in the host [1, 3]. At this phase of infection, spirochetes can be observed in the extracellular matrix of tissues without an associated inflammatory response [3].

Antibiotics are effective at establishing clinical cure in the majority of patients treated for Lyme disease, although some patients may have persistent symptoms that are unresponsive to subsequent antimicrobial treatment [4–6]. Culture and/or DNA evidence that active infection is the cause of persistent symptoms after antibiotic therapy for Lyme disease has been lacking—despite extensive analysis of samples from well-characterized patients [6]. Experimental models of Lyme borreliosis that used hamsters [7–9], gerbils [10, 11], and mice [12, 13] have confirmed that antibiotics can be curative, as assessed by culture and histopathology of tissues, although species vary with respect to optimum choice of antibiotics.

Most laboratory animal studies of antibiotic efficacy in Lyme borreliosis have introduced infection by inoculation of animals with in vitro–cultured spirochetes, which differ phenotypically from those deposited by ticks [14]. As a vectorborne agent, *B. burgdorferi* must adapt to the different environments of the tick vector and reservoir host. Adaptation is associated with selective gene expression, as well as recombination events that may facilitate spirochete infection, dissemination, and persistence within the new host [15–19]. Spirochetes begin to exhibit such changes within the feeding tick, and infecting spirochetes are partially host-adapted as they enter the mammal [16, 20, 21]. Vectorborne infection may therefore provide a particular survival advantage to infecting spirochetes that is not available to cultured spirochetes inoculated into laboratory animals. In this regard, a dog study that used field-collected ticks from an area of endemicity for Lyme disease provided DNA evidence that vectorborne spirochetes may not always be eradicated with antibiotic therapy [22]. Despite treatment of infected dogs with a 1-month course of ceftriaxone, doxycycline, or azithromycin, spirochete DNA was amplified from random skin biopsies obtained from some animals, independent of antibiotic used. Skin samples were collected monthly after treatment, and, although spirochete DNA was detected in some antibiotic-treated dogs as late as 12 months after therapy, in no case was any sample positive by culture. Moreover, viable spirochetes were not cultured from 25 tissue samples collected from each antibiotic-treated dog at the end of the >1-year experimental period, so

Received 31 May 2002; revised 15 July 2002; electronically published 23 October 2002.

Presented in part: Annual meeting of the American College of Rheumatology, San Francisco, 10–15 November 2001; Gordon Research Conference on the Biology of Spirochetes, Ventura, California, 27 January–1 February 2002.

All experiments were done in accordance with animal care and use guidelines of the Yale University Animal Care and Use Committee. No human subjects were involved.

Financial support: Centers for Disease Control and Prevention (cooperative agreement U50/CCU114685); National Institutes of Health (AR-42637, AR-47058 to L.K.B., and AI-26815 to E.H. and S.W.B.); US Department of Agriculture (cooperative agreement 58-1265-5023); G. Harold and Leila Y. Mathers Charitable Foundation (to D.F.).

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The Journal of Infectious Diseases 2002; 186:1430–7

0022-1899/2002/18610-0007$15.00

that persistence of DNA could not be equated with ongoing infection.

In this study, we used the murine model of Lyme borreliosis to clarify whether infectious spirochetes could persist after antibiotic treatment of disseminated infection. In addition to using laboratory-reared ticks to introduce infection with a single strain of *B. burgdorferi,* we also used xenodiagnosis with ticks as a parameter for detecting spirochetes that may remain after antibiotics. At the end of the experimental period, mouse tissues were analyzed for spirochete DNA, as well as by culture and histopathology, for evidence of unresolved infection.

Materials and Methods

Infection of mice. Four-week-old female C3H/HeJ (C3H) mice (Jackson Laboratories) were housed according to Yale University animal care and use guidelines. All ticks used in this study were derived from a specific pathogen–free, laboratory-reared colony of *Ixodes scapularis* ticks maintained at Yale University. Larval ticks were initially infected with spirochetes by feeding on mice previously inoculated with low-passage *B. burgdorferi* strain N40, which had been cloned by limiting dilution [3]. After molting into nymphs, N40-infected ticks were placed in the ear canals of mildly anesthetized mice (5 nymphs/mouse), allowed to feed to repletion, and collected after natural detachment into a water bath [23]. Transmission of infection was assessed by serologic analysis 14 days after tick feeding.

Antibiotic treatment of mice. At 30 days of infection, mice were treated with 16 mg/kg ceftriaxone in 500 mL total volume of 0.9% normal saline (10 mice) or sham-treated with 0.9% normal saline (4 mice) administered subcutaneously twice daily for 5 days, then daily for 25 days. A separate group of 10 mice received 50 mg/kg doxycycline in 500 mL of double-distilled H_2O administered twice daily by gavage for 30 days. Mice were weighed weekly, and antibiotic dosages were adjusted for age-related weight gain. Blood was obtained from 5 mice at 15 min, 30 min, and 1 h (ceftriaxone group) and 30 min, 1 h, and 2 h (doxycycline group) after antibiotic dosing to assess serum antibiotic levels. Because of the volume of serum required to perform the analysis, these mice were killed. Antibiotic levels in triplicate 10-mL samples of mouse serum were determined by an agar plate–based bacterial growth inhibition assay that used *Staphylococcus aureus* (ATCC 25923), as described elsewhere [12].

Xenodiagnosis of B. burgdorferi *infection.* At 1, 3, 6, and 9 months after the last antibiotic dose, uninfected nymphs were allowed to feed to repletion on individual mice. Engorged nymphs were retrieved and housed for 8 days in a 22°C humidified environmental chamber before evaluation for spirochetes. Midgut contents were expressed from individual ticks onto slides for direct immunofluorescence analysis (DFA), and the remaining tick body was processed for polymerase chain reaction (PCR), as described below. As negative control specimens, uninfected nymphs were fed on 3 uninfected mice and analyzed in similar fashion. These mice were followed longitudinally with infected mice and were assessed for seroconversion to *B. burgdorferi* 2 weeks after each tick feeding. At the 3-, 6-, and 9-month time points, ~100 larvae were fed on 1 mouse/group; after molting, nymphs were then fed on uninfected C3H mice (10 nymphs/mouse) to assess spirochete transmissibility.

Cortisone immunosuppression of mice. Mice were transiently immunosuppressed by intraperitoneal injection of 3 mg of cortisone acetate in 100 mL of double-distilled H_2O daily for 5 days immediately before the 9-month xenodiagnosis [24].

Immunofluorescence of spirochetes (DFA). DFA of tick midgut contents was done, as described elsewhere [25], with use of fluorescein isothiocyanate–conjugated anti–*B. burgdorferi* IgG (Kirkegaard & Perry Laboratories). Forty visual fields were examined per slide in blinded fashion before a specimen was considered to be negative.

PCR of B. burgdorferi *DNA.* DNA was isolated from individual ethanol-fixed nymphs or pooled larvae by means of the Isoquick DNA isolation kit (ORCA Research) and was resuspended in 20 μL of double-distilled H_2O. Primers used for amplification were as follows: *osp*A (GenBank accession no. M57248, product amplicon coordinates 80–781): forward, 5′-AAAACAGCGTTTCAGTAGATTTGCCTGGTG-3′, and reverse, 5′-CAACTGCTGACCCCTCTAATTTGGTGCC-3′; BBE21.1 (GenBank accession no. AE000785, product amplicon coordinates 14663–14921): forward, 5′-AGAATTATGTCGGTGGCGTTGT-3′, and reverse, 5′-ATTAAAGCCGCCTTTTCCTTGGT-3′; and p37-47 (GenBank accession no. AE000794, product amplicon coordinates 1309–1457): forward, 5′-TTCTGATGGCACTGAGCAAACCA-3′, and reverse, 5′-AACCCTTTACACTTTCTTCGATTGCGCT-3′. The primer set for p37–47 has 100% homology to sequences in both *B. burgdorferi* strains B31 and N40, and the gene has been localized to lp28-1 in both strains [26, 27]. The primer set for BBE21.1 amplifies a unique region in lp25 of *B. burgdorferi* strain B31 downstream of BBE21 (amplicon coordinates 13403–14530) [28]. BBE21 is located on a similar-size plasmid within *B. burgdorferi* strain N40 [29]. We have been able to amplify by PCR the region corresponding to GenBank accession number AE000785, product amplicon coordinates 14195–14921, indicating that BBE21 and BBE21.1 reside on the same plasmid in N40 (authors' unpublished data).

DNA (4% vol/vol) was amplified in a 50-μL reaction volume containing 25 m*M* primers, 2.5 m*M* $MgCl_2$ (0.75 m*M* $MgCl_2$ in the case of *osp*A only), 2 m*M* dNTPs, and 0.3 U of Taq polymerase in Qiagen buffer (Qiagen). Robocycler (Stratagene) settings for BBE21.1 and p37–47 were 35 cycles of 94°C denaturation for 1 min, 60°C annealing for 1 min, and 72°C extension for 1 min, followed by a final extension at 72°C for 3 min. Robocycler settings for *osp*A amplification were 95°C denaturation for 1.5 min, 56°C annealing for 1 min, and 73°C extension for 1.25 min, followed by a 4-min extension at 73°C. Products were separated by agarose gel electrophoresis and verified by Southern blot by use of electrochemiluminescence according to the manufacturer's recommendation (Amersham Pharmacia Biotech). Probes for the Southern blot were as follows: *osp*A, amplicon coordinates 215–434; BBE21.1, amplicon coordinates 14715–14784; and p37-47, amplicon coordinates 1380–1439. Negative (no DNA) and positive (*B. burgdorferi* N40 DNA) control subjects were included in all assays.

Real-time PCR for the *B. burgdorferi* flagellin gene (GenBank accession no. X14841) and for the *B. burgdorferi* 16S rRNA target gene (GenBank accession no. AF139516) was done on DNA isolated from mouse tissues (ear skin, urinary bladder [30], tibiotarsal joint [31], heart, and spleen [32]), as described elsewhere. Primers BB16S-559F (5′-TGCTAAGTGTGATGCCTGAAAG-

AA-3′) and BB16S-686R (5′-AATCTTTGCCTTCCTCCAACTTATAA-3′) were synthesized to amplify a 128-bp fragment of 16S rRNA. The internal oligonucleotide probe BB16S-620p (5′-TTGTCCGAATCCCTACCACTAAATACTGGCAA-3′) was labeled at the 5′ end with the reporter dye 6-carboxy-fluorescein and at the 3′ end with the quencher dye 6-carboxy-tetramethylrhodamine. Primers amplifying a 107-bp fragment of the flagellin gene and an internal oligonucleotide probe were synthesized and labeled as described elsewhere [32]. DNA samples were coded, and an observer blinded to the experimental protocol performed the PCR. For analysis, results were normalized to the starting copy number of the eukaryotic actin gene for each sample [32].

B. burgdorferi–*specific IgG titers.* End-point titers of IgG antibody to *B. burgdorferi* were determined by ELISA with *B. burgdorferi* N40 spirochete lysate, as described elsewhere [33].

Culture and histopathology. Blood and tissues (ear skin, hearts, spleens, and urinary bladders) were cultured in modified BSK II medium, as described elsewhere [34]. Cultures were maintained for 6 weeks before a sample was considered to be negative. The heart base and joints from a single hindlimb of each mouse were processed for hematoxylin-eosin staining after formalyn fixation, as described elsewhere [23].

Results

Treatment of mice. Tick transmission of spirochetes to uninfected C3H mice was uniformly successful, with 100% of mice seroconverting to *B. burgdorferi* 2 weeks after tick placement. Mean *B. burgdorferi*–specific end-point titers were comparable among the infected mice at 30 days of infection, at which time mice were randomly assigned into 3 groups to receive a 30-day course of doxycycline, ceftriaxone, or saline treatment. A previous study of C3H mice infected with cultured *B. burgdorferi* strain N40 showed that ceftriaxone administered at 16 mg/kg twice daily for 5 days can cure infection and prevent disease relapses measured over a 90-day observation period, but doxycycline administered at 13 mg/kg orally twice daily for 14 days was ineffective [12]. In this study, we administered that dose of ceftriaxone twice daily for 5 days and once daily for an additional 25 days. We also increased the dose of doxycycline to 50 mg/kg and extended the treatment period to 30 days, because this treatment regimen has proven efficacy for other chronic infectious disease models in mice [35]. As measured by bioassay, the peak serum concentrations over a 1-h time period for ceftriaxone and a 2-h time period for doxycycline were comparable with those of the previous study comparing antibiotic efficacy in mice (ceftriaxone: 15 min, 38 mg/mL; 30 min, 38 mg/mL; and 60 min, 26 mg/mL; doxycycline: 30 min, 4.5 mg/mL; 60 min, 3.6 mg/mL; and 120 min, 1.1 mg/mL). Doxycycline levels remained elevated for at least 1 h, exceeding the duration of antibiotic elevation when mice were administered a lower dose that did not lead to negative culture results.

Spirochete persistence assessed by xenodiagnosis. A xenodiagnostic procedure with laboratory-reared *I. scapularis* ticks was used to assess whether spirochetes could persist after antibiotic treatment of mice. Uninfected nymphs were able to acquire spirochetes from all infected mice 1 and 3 months after completion of sham (saline) treatment (table 1). Spirochetes were visualized by DFA in the majority of ticks that fed on these mice (figure 1*A*), although, at the 3-month tick feeding, spirochetes were observed to be less abundant. We also observed rare spirochetes in a small proportion of ticks that fed on mice treated with either ceftriaxone (figure 1*B*) or doxycycline (figure 1*C*). The same antibiotic-treated mice with positive xenodiagnoses at the 1-month tick feeding interval also had spirochetes detectable by this method at the 3-month feeding, at which time 2 of 5 mice in each group tested positive by this method. *osp*A DNA could be amplified from DFA-positive ticks, confirming that the spirochetes visualized were *B. burgdorferi.* Xenodiagnostic nymphs that were fed on uninfected mice did not harbor spirochetes, as assessed by PCR (table 1) and DFA, and mice serving as their hosts did not seroconvert to *B. burgdorferi* (data not shown). Because of the small number of ticks retrieved after xenodiagnosis, we did not attempt to culture spirochetes from ticks.

We next sought to determine whether spirochetes observed in ticks continued to be infectious to mammals. In earlier studies, we had been unable to transmit infection from antibiotic-treated mice to naive SCID mice by transplantation of ear punch specimens, a method that successfully passes infectious, host-adapted spirochetes from mammal to mammal [36]. We therefore attempted to transfer infection by ticks. Because ticks feed only once per developmental stage (larva, nymph, or adult), and adult ticks do not feed on mice [37, 38], we fed larvae on saline- and antibiotic-treated mice and then allowed engorged larvae to molt into nymphs. Engorged larvae that had fed on either saline-treated or ceftriaxone-treated mice tested positive by PCR for *osp*A DNA; those feeding on uninfected mice or doxycycline-treated mice tested negative (table 2). After molting from larvae, nymphs from the saline group, but not from the ceftriaxone group, remained positive for *osp*A DNA. Nymphs from each mouse treatment group were then allowed to feed on groups of 5 uninfected mice (10 nymphs/mouse). Two weeks after tick feeding, only mice serving as hosts for nymphs derived from the saline-treated mouse group seroconverted to *B. burgdorferi,* in-

Table 1. Polymerase chain reaction amplification of *osp*A gene of xenodiagnostic ticks.

Mouse group	No. *osp*A-positive nymphs derived from mice/total no. (%), time after treatment		No. of mice positive by xenodiagnosis/total no.
	1 month	3 months	
Uninfected	0/12	0/6	0/3
Saline	21/29 (72)	10/15 (67)	4/4
Ceftriaxone	3/14 (21)[a]	5/14 (36)	2/5
Doxycycline	2/13 (15)[b]	3/24 (13)[c]	2/5

[a] $P = .0028$, vs. saline-treated mice (Fisher's exact test).
[b] $P = .0008$, vs. saline-treated mice (Fisher's exact test).
[c] $P = .0011$, vs. saline-treated mice (Fisher's exact test).

Table 2. Lack of transmission of *Borrelia burgdorferi* infection by infected ticks from antibiotic-treated mice.

Mouse group	*osp*A DNA in ticks by PCR		No. of naive mice seroconverting to *B. burgdorferi* after nymph feeding/total no.
	Pooled-larvae result	No. of nymphs testing positive/total no.	
Uninfected	Negative	0/10	0/5
Saline	Positive	10/10	5/5
Ceftriaxone	Positive	0/10	0/5
Doxycycline	Negative	0/10	0/5

NOTE. Larvae were fed on individual mice in each group 3 months after completion of antibiotic treatment. DNA was isolated from 10 pooled engorged larvae or individual nymphs from each mouse group before assaying for *osp*A DNA. Ten nymphs were fed on individual mice, which then were analyzed for spirochete acquisition. PCR, polymerase chain reaction.

dicating that spirochetes within these ticks were transmissible to new hosts.

Because spirochetes detected by ticks feeding on ceftriaxone-treated mice did not transmit infection, we examined the DNA of spirochete-containing ticks for the presence of 2 plasmids, lp25 and lp28-1, which have been reported to contain important virulence factors for *B. burgdorferi* [17, 28]. Both plasmids were present in a sampling of ticks that fed on saline-treated mice (figure 2, *lanes 7–12*) and in 2 of 3 ticks that fed on doxycycline-treated mice (figure 2, *lanes 1* and *3*). In contrast, we were unable to amplify the gene target BBE21.1 specific for lp25 in any of the ticks that fed on ceftriaxone-treated mice (figure 2, *lanes 4–6*) and in 1 of the ticks that fed on doxycycline-treated mice (figure 2, *lane 2*). The p37–47 gene found on lp28-1 could be faintly detected in 2 of 3 ticks from the ceftriaxone-treated group (figure 2, *lanes 4* and *5*), indicating the presence of the lp28-1 plasmid among the population of spirochetes within those ticks. All 3 targets amplified with similar efficiency in a serial dilution analysis with DNA from infected nymphs derived from saline-treated mice, indicating that differences in threshold for amplification cannot explain the noted absence of specific targets (data not shown).

Immunosuppression of mice enhances the ability to detect B. burgdorferi *by xenodiagnosis.* At 6 months after antibiotic therapy, we narrowed our analysis to the subgroup of antibiotic-treated mice that had previously tested positive by xenodiagnosis, along with 2 uninfected and 2 saline-treated mice for control specimens. At this time point, none of the ticks tested positive by PCR or DFA, even those that had fed on saline-treated mice (0/19 nymphs and 0/30 larvae). To reduce the impact of the host immune response on tick acquisition of spirochetes, we treated all mice with cortisone acetate [24] for 5 days immediately prior to the placement of larvae and nymphs, 9 months after the last antibiotic dose. Retrieved larvae and nymphs from the saline-treated group tested positive by PCR for *osp*A DNA, whereas ticks from antibiotic-treated mice remained negative (data not shown).

B. burgdorferi*–specific IgG titers fall after antibiotic treatment of mice.* End-point *B. burgdorferi*–specific IgG titers were determined periodically throughout the 11-month experimental period. Antibiotic therapy led to a rapid decline in antibody titers, but levels were still elevated at the time of sacrifice (figure 3). Mice testing positive by xenodiagnosis at 1 and 3 months after treatment could not be distinguished on the basis of IgG titers from those without evidence of spirochetes. Titers also declined in the saline-treated group, even before cortisone treatment, although levels remained significantly elevated in comparison with antibiotic-treated mice ($P = .029$; 2-tailed, unpaired Mann-Whitney U test).

At the end of the experimental period, spirochetes were cultured from multiple tissues of the saline-treated mice, and 3 of 4 animals also had evidence of arthritis and/or carditis typical of *B. burgdorferi* infection (table 3). In addition, we were able to amplify *B. burgdorferi* 16S rRNA and flagellin gene sequences by real-time PCR from hearts, urinary bladders, and joints of all saline-treated mice (table 3). In contrast, among antibiotic-treated mice, spirochetes could not be cultured from any organ, and none of these mice had heart or joint inflammation. Although we were able to detect low levels of spirochete DNA in urinary bladders of the 2 ceftriaxone-treated mice that had previously tested positive by xenodiagnosis, values were at the lower detection limit of the assay and 100-fold less than those found in saline-treated mice. The number of tissues from ceftriaxone-treated mice in which spirochete DNA could be detected was significantly lower than that from saline-treated mice ($P < .0001$; Fisher's exact test). This was not the case for doxycycline-treated mice, in which spirochete DNA was detected in multiple tissues, especially the joints.

Discussion

This study of the murine model of Lyme borreliosis provides xenodiagnostic evidence that spirochetes can persist for limited duration after antibiotic treatment but do not survive in ticks so that they can infect new hosts. Laboratory-reared *I. scapularis* larvae and nymphs can acquire intact spirochetes from a subset of infected, inbred mice as long as 3 months after completion of antibiotics but not thereafter. Unidentified infection

Table 3. Analysis of mouse tissues for *Borrelia burgdorferi* in conjunction with antibiotic treatment.

Mouse group	PCR for 16S rRNA				Histopathology	
	Heart	Urinary bladder	Joint	Culture	Joints	Heart
Uninfected	0/3	0/3	0/3	0/3	ND	ND
Saline	4/4	4/4	4/4	4/4	1/4	3/4
Ceftriaxone	0/4	2/4	0/4	0/4	0/4	0/4
Doxycycline	1/4	2/5	4/5	0/5	0/4	0/4

NOTE. Data are no. of mice testing positive/total examined for each parameter. There was 100% concordance between samples testing positive for 16S rRNA and those testing positive for flagellin. There were statistically fewer *B. burgdorferi* DNA–positive sites in antibiotic-treated mice than in saline-treated mice: ceftriaxone vs. saline, $P = .0001$; doxycycline vs. saline, $P = .0031$; Fisher's exact test. PCR, polymerase chain reaction.

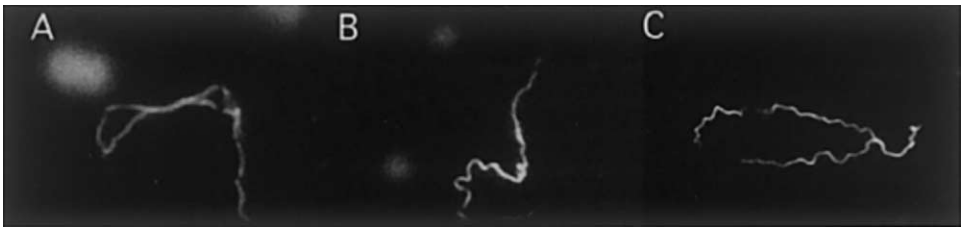

Figure 1. Results of direct immunofluorescence analysis of spirochetes within midguts of xenodiagnostic ticks. Representative spirochetes detected at 3-month xenodiagnosis were photographed with 100× objective after immunofluorescent staining. Results shown are from ticks fed on saline-treated (*A*), ceftriaxone-treated (*B*), and doxycycline-treated (*C*) mice.

of the ticks used for xenodiagnosis is unlikely to account for our results, because the population of larvae used for xenodiagnosis tested negative for *B. burgdorferi* infection by both DFA and PCR, and laboratory mice used for propagation of the larval colony did not seroconvert to this organism.

Vectorborne pathogens have evolved to rapidly adapt and persist in their various hosts. Our longitudinal in vivo study is the first to use the natural vector for *B. burgdorferi* both to transmit infection and to detect spirochetes that may persist after antibiotic treatment. This approach permitted documentation of residual, noninfectious spirochetes in a small proportion of mice treated for 30 days with ceftriaxone or doxycycline. One possible explanation for our findings is that the dose or duration of antibiotics administered was insufficient to eliminate infection. Bactericidal agents that inhibit cell wall synthesis, such as penicillin and ceftriaxone, are most effective against replicating organisms, and a maximal rate of killing cannot be enhanced, even when the dose of antibiotic is increased >30,000-fold [39]. In our study, we extended the ceftriaxone course far beyond that necessary to establish cure, even though antibiotic levels were comparable with those in which a 5-day treatment regimen was successful against infection with cultured spirochetes [12]. We found a similar proportion of antibiotic-treated mice with persisting spirochetes in an earlier experiment in which ceftriaxone or doxycycline was given for 2 weeks starting at 21 days of infection. Thus, longer treatment periods, as reported herein and timing of treatment relative to infection onset do not appear to alter results of xenodiagnostic studies with vectorborne infection. Eukaryotic cells have been shown to protect *B. burgdorferi* against the action of penicillin and ceftriaxone in vitro [40, 41], but whether this happens in vivo remains unknown. However, our study provides evidence that residual spirochetes detected are not infectious to new mammalian hosts and thus differ from the original infecting inoculum.

One mechanism to explain the reduced infectivity of residual spirochetes may be related to genetic recombination or loss of plasmids. Highly infectious spirochetes propagated in vitro lose both pathogenicity and infectivity with increasing passage number, findings correlated with loss of plasmids and evidence of genetic recombination [42–44]. Although the genes necessary for infectivity have not yet been defined, 2 plasmids have been correlated with infectivity of *B. burgdorferi* strain B31. Clones of B31 that lack lp25 grow readily in vitro but exhibit low infectivity and cannot be cultured from distant sites after inoculation into mice [28]. In our analysis of the spirochetes detected after ceftriaxone therapy, we were unable to amplify BBE21.1 specific for lp25 in both B31 and N40 strains. B31 spirochetes that lack lp28-1 also exhibit reduced infectivity [28]. The population of spirochetes in ticks that fed on antibiotic-treated mice had diminished levels of the lp28-1 gene p37–47, with the target below levels necessary for PCR amplification in at least 1 tick.

The inability to amplify the specific gene target on lp25 and in 1 case specimen also on lp28-1 could be due to mutations within the gene itself or recombination events and cannot be

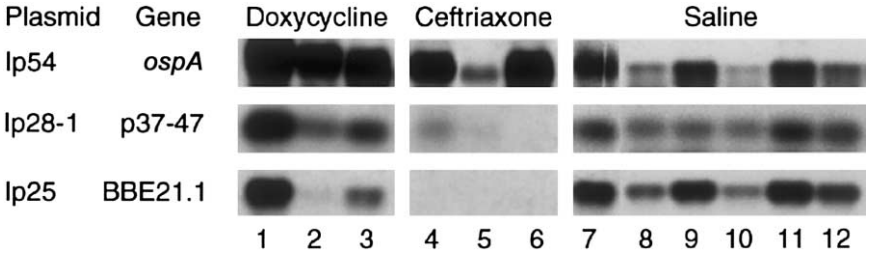

Figure 2. Southern blot of *Borrelia burgdorferi* genes amplified from tick DNA extracts. Gene targets were amplified by polymerase chain reaction and verified by Southern blot analysis. Individual xenodiagnostic ticks were fed on doxycycline-treated mice *(lanes 1–3),* ceftriaxone-treated mice *(lanes 4–6),* or saline-treated mice *(lanes 7–12)* 1 month after antibiotic treatment. Reactions for lanes 1–7 were done on same day; those for lanes 8–12 were done in separate analysis.

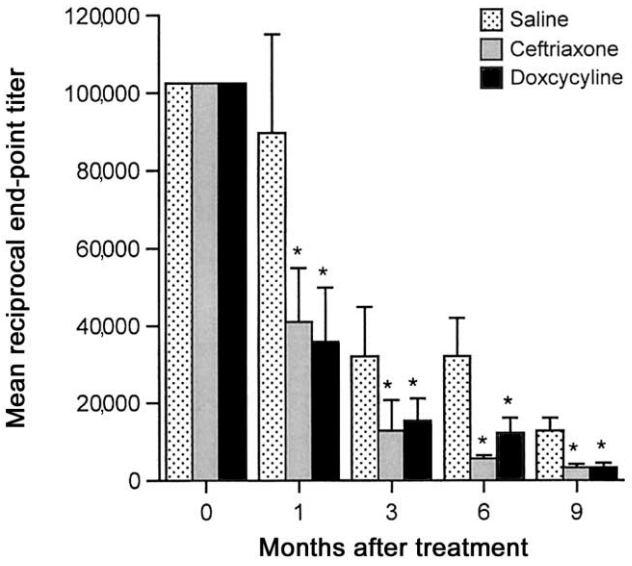

Figure 3. *Borrelia burgdorferi*–specific IgG titers decrease in treated and untreated mice. End-point *B. burgdorferi*–specific IgG titers measured by ELISA are reported as mean ± SD of values from individual mice within each group. *, Significant differences between saline and antibiotic-treated mice (1 and 3 months, saline vs. ceftriaxone or doxycycline, $P = .032$; 6 months, saline vs. ceftriaxone, $P = .029$, saline vs. doxycycline, $P = .032$; 9 months, saline vs. ceftriaxone or doxycycline, $P = .029$; 2-tailed, unpaired Mann-Whitney U test).

equated with plasmid loss. However, these results suggest that the population of spirochetes detected after ceftriaxone therapy is genetically different from the infecting population. A similar trend with loss of the lp25 gene target was seen in spirochetes that persisted after doxycycline treatment. It should be noted that the ticks used to experimentally infect mice for this study had acquired spirochetes after feeding on mice originally infected with a low-passage, virulent clone of *B. burgdorferi* strain N40. Passage between mammal and ticks or spirochete replication within a given host may alter clonality and allow for variants to arise.

The ability to detect noninfectious variants suggests that genes required for spirochetes to infect and disseminate within the mammal may no longer be necessary for spirochetes, once host-adapted, to persist for a time in the chronic phase of untreated *B. burgdorferi* infection. Precedent for this notion can be found in the case of *Chlamydia* species [45]. During normal vegetative growth, *Chlamydia* species organisms express high levels of the glycolytic and pentose phosphate pathway enzymes but apparently use only the gene encoding for an ATP/ADP exchange protein to obtain energy from the host during the persistent phase. Because of limited DNA in samples, we were unable to expand the scope of our analysis to include other genes and plasmids that may be important for spirochete infectivity.

Although we have shown that spirochetes detected after antibiotic therapy exhibit reduced infectivity, this finding does not explain why they were not killed during treatment. Antibiotics may be effective at eliminating infectious, replicating spirochetes but not nonreplicating organisms with limited or deficient metabolic activity. Such impotent spirochetes may be viewed as innocuous by the host immune system, especially if proinflammatory surface lipoprotein expression is diminished, and might be relatively impervious to drugs that depend on protein or cell wall synthesis for their actions. Our study provides indirect evidence in support of this hypothesis. Although we were able to detect spirochetes from a subgroup of antibiotic-treated mice, these spirochetes did not survive the molt of larvae to nymphs and could not be transmitted to new mammalian hosts, a process that involves spirochete replication. It is unclear whether larval ticks must harbor a critical number of infectious spirochetes to remain infected at the nymphal stage. We analyzed larvae 8 days after tick feeding; it is possible that spirochetes never successfully established infection in the tick and died during the 1–2-month molting period. We did not attempt to culture spirochetes from ticks used for xenodiagnosis so that replicating variants might be demonstrated.

Ticks feeding on mice 6 months after sham treatment could no longer acquire spirochetes, and *B. burgdorferi*–specific antibody titers had declined substantially. These findings suggest that tickborne spirochetes and their immunogenic antigens diminish over time in mice, even without specific therapy. The host immune response probably controls persistent, infectious spirochetes, because transient cortisone immunosuppression of saline-treated mice enhanced spirochete transmission to ticks. This was not the case for antibiotic-treated mice, even though spirochete DNA was present in 6 of 10 mice at the end of the experimental period.

There is precedent for persistence of attenuated pathogens after antimicrobial therapy for infection with *Mycobacterium tuberculosis.* Patients receiving antituberculous drugs may continue to have positive results of sputum smear testing but are less infectious [46, 47]. In addition, sputum specimens from patients treated for 9 months with antituberculous therapy may remain positive for acid-fast bacilli but test negative by culture, suggesting that the observed organisms either are nonviable or have reduced infectivity [48]. Our results in mice show that although it is possible to detect some spirochetes for a limited time after antibiotic therapy, they do not have the same transmissibility or pathogenicity as those of the original infectious inoculum. The mouse is the natural reservoir for *B. burgdorferi,* and spirochetes, even if attenuated, may be particularly suited to persisting in specific microenvironments within this host. There is currently no evidence that infectious spirochetes survive antimicrobial therapy, and our findings show that attenuated spirochetes do not cause persistent disease.

In summary, we provide xenodiagnostic evidence that a subpopulation of spirochetes can persist for a limited time after antibiotic treatment of *B. burgdorferi*–infected mice but are of low infectivity and are not transmissible to new mammalian

hosts. Taken together, these findings—limited duration of xenodiagnosis detecting spirochetes in antibiotic-treated mice, loss of gene targets by some residual spirochetes, the inability of such spirochetes to survive in ticks and infect new mammals, and the absence of cultivable spirochetes and disease in antibiotic-treated mice—all suggest that residual spirochetes are avirulent. We propose that these spirochetes constitute an attenuated population that is no longer infectious and will eventually die or be killed by host defenses without causing significant pathology. Further studies are needed to determine whether genes required for infectivity may no longer be necessary for spirochetes to persist in a host-adapted state.

Acknowledgments

We thank Gary Wormser, Janine Evans, Stephen Malawista, and Ruth Montgomery for editorial comments, Michelle Papero for maintenance of the tick colony, and Debbie Beck for tissue processing of mouse specimens.

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