

Antibiotic Treatment of Animals Infected with *Borrelia burgdorferi*

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INTRODUCTION

Lyme disease is the most common tick-borne infection in North America. Objective clinical manifestations may involve the skin, nervous system, heart, or joints, all of which usually respond well to conventional antibiotic therapy (52). Despite resolution of the objective manifestations of infection after antibiotic treatment, a minority of patients have fatigue, musculoskeletal pain, and/or difficulties with concentration or short-term memory of uncertain etiology (13). Subjective complaints that persist are referred to as post-Lyme disease symptoms; if they persist for >6 months and cause functional impairment they are often referred to as post-Lyme disease syndrome (13, 26, 52). Post-Lyme disease syndrome is sometimes referred to as “chronic Lyme disease,” but this term is poorly defined and is often used to refer to chronic symptoms that are unrelated to *Borrelia burgdorferi* infection (13, 16). Although post-Lyme disease syndrome is a topic of considerable interest and controversy, it is important to point out that the principal evidence in support of the existence of this syndrome is derived from older retrospective studies in which the diagnosis and treatment of Lyme disease would not meet current standards (10, 45). No prospective treatment study of

patients with Lyme disease has been published to prove or disprove that the frequency of such nonspecific symptoms at 6 or 12 months after antibiotic treatment actually exceeds that of the same types of symptoms in individuals without Lyme disease (16, 45). There is also no convincing evidence that post-Lyme disease syndrome will resolve following additional courses of antibiotic therapy (13, 26, 52).

The purpose of this review is to describe the methodology and findings of recently published treatment studies of experimental animals that were infected with *B. burgdorferi* and to evaluate the significance of the results with regard to human Lyme disease. Special emphasis is placed on whether these studies provide any useful insights into posttreatment Lyme disease symptoms and/or post-Lyme disease syndrome. Possible future approaches for clarifying the outstanding questions are discussed.

ANIMAL STUDIES: GENERAL REMARKS

Studies of the effects of antibiotic therapy in animals infected with *B. burgdorferi* have been conducted most often with mice (8, 18, 25, 30, 31, 35, 38, 53, 54) but also with hamsters (21, 22), gerbils (15, 32, 42), dogs (48, 49), and nonhuman primates (39). In most of the studies the animals were immunocompetent, but in several the animals were immunocompromised (25, 37, 48). The criteria for judging that the outcome of treatment was successful varied among the studies. In the initial studies, outcome was based on whether *B. burgdorferi* could

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TABLE 1. Study methodologies employed

Parameter	Use in study by:	
	Hodzic et al. (18)	Bockenstedt et al. (8)
Animal(s)	3–5-wk-old female C3H/HeN mice, C3H-scid mice	4-wk-old female C3H/HeJ mice
Strain of <i>B. burgdorferi</i>	N40 low passage, clonal	N40 low passage, clonal
Route of infection	Subdermal inoculation of 10 ⁴ spirochetes	<i>Ixodes scapularis</i> tick bite
Treatment	Treatment duration, 30 days; ceftriaxone at 16 mg/kg intraperitoneally twice daily for 5 days and then once daily for 25 days; saline for 30 days	Treatment duration, 30 days; ceftriaxone at 16 mg/kg subcutaneously twice daily for 5 days and then once daily for 25 days; doxycycline at 50 mg/kg by gavage twice daily for 30 days; saline for 30 days
Timing of treatment	3 wk after infection, 4 mo after infection	1 mo after infection
Timing of evaluation	1 and 3 mo after completion of a 30-day treatment	1, 3, 6, and 9 mo after completion of a 30-day treatment
Outcome measures	Culture, PCR, xenodiagnosis, serology, histology, immunohistochemistry, transmission by skin allograft to C3H/HeN mice, transmission by xenodiagnostic ticks to SCID mice	Culture, PCR, xenodiagnosis, serology, histology, transmission by skin allograft to SCID mice, transmission by xenodiagnostic ticks to C3H/HeJ mice
Timing of xenodiagnosis	1 and 3 mo after completion of treatment	1, 3, 6, and 9 mo after completion of treatment; mice were immunosuppressed with corticosteroids before xenodiagnosis at 9 mo

be recovered on culture, a straightforward approach widely used with other infectious agents that are cultivable. In more recent studies, detection of DNA of *B. burgdorferi* (8, 18, 30, 35, 48, 49, 54), visualization of the spirochete in tissues by immunohistochemistry (18), xenodiagnosis (detection of *B. burgdorferi* in ticks that had fed on an infected animal) (8, 18), transplantation of tissues from *B. burgdorferi*-infected animals to uninfected animals (8, 18), or other outcome measures were employed, in addition to culture. Judging of outcomes based on so many diverse parameters and imposition of such stringent criteria for therapeutic success appear to be somewhat unique to experimental infection due to *B. burgdorferi*. The justification for adopting this approach may have been the presumption that either a local inflammatory response or a vigorous generalized immune response to the spirochete or some residual antigen(s) is responsible for posttreatment Lyme disease symptoms and/or post-Lyme disease syndrome (13, 26, 52).

In the treatment of other infections it is probably unrealistic to expect that antimicrobial therapy per se will eliminate every single microorganism from an infected host, and moreover, such an action would rarely if ever be required for a successful outcome. More conventionally, the role of antimicrobial therapy in vivo can be thought of in terms of “tipping the balance” in favor of the host’s own defenses against a particular pathogen. Indeed, for most infections treatment with antibiotics that only inhibit rather than kill a microorganism is just as effective as treatment with bactericidal agents (36).

MOUSE STUDIES THAT INCLUDED PCR AND XENODIAGNOSIS

Study by Hodzic et al.

Cultures and PCR. Hodzic et al. (18) reported on a series of experiments in which they compared therapy with ceftriaxone (administered intraperitoneally) versus saline in C3H/HeN mice that were infected by needle inoculation with an N40 strain of *B. burgdorferi* (Table 1). Although it was demonstrated that as few as a single spirochete could be detected by

the culture methodology employed, in antibiotic-treated animals cultures of multiple tissue sites were consistently negative, regardless of whether the animals had been treated early (3 weeks after infection) or late (4 months after infection). There was a single instance of allograft transmission by an antibiotic-treated mouse to an immunocompetent mouse, but this observation appeared to be aberrant, since at that time point none of allografts from the sham-treated mice transmitted infection to the recipient mice.

Despite the absence of positive cultures, DNA of *B. burgdorferi* could be detected by PCR in some of the ceftriaxone-treated mice. PCR positivity was present principally at the base of the heart and in the tibiotarsus tissue and not in tissue from the ear, inoculation site for *B. burgdorferi*, subinoculation site, ventricle, or quadriceps muscle (18). Uninfected control mice were not studied. To determine if *B. burgdorferi* could be recovered from the tissues with a positive PCR result, a confirmatory experiment was done in which these tissues were cultured, in addition to urinary bladder, ear, and inoculation site, but these cultures were also sterile. The prevalence of mice that were PCR positive was higher following antibiotic therapy at 4 months than following therapy at 3 weeks after the onset of infection (10/13 versus 2/8; $P = 0.03$). Hodzic et al. (18) stated that they were unsuccessful in detection of RNA transcription despite attempts to amplify cDNA, but they did not provide any details.

Spirochetes diminish over time. Although it was not emphasized by the investigators, mice were more likely to be PCR positive and to have a larger number of *flaB* DNA copies/mg tissue shortly after completion of ceftriaxone treatment than at a later time point, suggesting that the presence of spirochetal DNA was decreasing over time for reasons not elucidated. For example, in the mice treated at 4 months after infection, *B. burgdorferi* DNA was detected by PCR in eight (100%) of eight mice that were evaluated at 1 month after completion of antibiotic therapy, compared with two (40%) of five mice that were evaluated at 3 months after completion of antibiotic treatment ($P = 0.03$). PCR-positive samples from mice at 1 month after treatment contained 33.7 ± 60.6 (mean \pm stan-

TABLE 2. Selected pharmacokinetic-pharmacodynamic parameters of ceftriaxone and doxycycline in mice and humans

Data type and drug	Study authors (reference)	MIC ($\mu\text{g/ml}$) for <i>B. burgdorferi</i>	Half-life	Estimated time (h) drug level (including protein-bound fraction) above MIC after 1 dose of drug
Mouse				
Ceftriaxone	Hodzic et al. (18)	0.015	30–40 min (estimated ^a)	<8
Doxycycline	Bockenstedt et al. (8)	Not given	<1 h (estimated ^a)	<5 ^b
Human				
Ceftriaxone	Patel and Kaplan (37)	0.015 ^c	5.8–8.7 h	>24
Doxycycline	Saivin and Houin (44)	0.25 ^b	15–25 h	>24 ^d

^a Based on data reported by the investigators.

^b An MIC of 0.25 $\mu\text{g/ml}$ doxycycline for *B. burgdorferi* was assumed.

^c An MIC of 0.015 $\mu\text{g/ml}$ is assumed for consistency with the study by Hodzic et al. (18).

^d In contrast to the case for ceftriaxone, time above MIC is probably not the most relevant pharmacodynamic parameter for doxycycline, but this information illustrates that doxycycline drug exposure is much reduced in mice compared with humans.

dard deviation) *flaB* DNA copies/mg tissue, compared with 4.1 and 2.8 *flaB* DNA copies/mg in the two positive samples from mice at 3 months after treatment. Furthermore, spirochetes were visualized at the base of the heart or in tibiotarsus tissue by immunohistochemistry (using rabbit immune serum to *B. burgdorferi*) in three (37.5%) of eight mice at 1 month post-treatment, compared with one (20%) of five mice at the 3-month posttreatment time point. There was, however, no histologic evidence for inflammation at these or any other tissue sites in these animals (S. Barthold, personal communication, 2009). Serum antibody reactivity to *B. burgdorferi* whole-cell antigen extract declined significantly in antibiotic-compared to saline-treated mice but did not return to preinfection levels (18). This finding was consistent with the reduction in spirochetes that was documented in antibiotic- versus saline-treated animals.

The normal-appearing morphology of the spirochetes visualized histologically posttreatment implies that the borrelial DNA that was detected by PCR was unlikely to be entirely free DNA in tissues. An intraspirochetal location, even if the spirochete was damaged or attenuated, likely accounts for why *B. burgdorferi* DNA could be detected for at least 90 days after treatment in the study by Hodzic et al. (18). The microscopic findings do not provide support, however, for the theory that *B. burgdorferi* persists through the formation of cysts (3).

Xenodiagnosis and transmission of *Borrelia burgdorferi* by ticks following xenodiagnosis. Of the 23 mice in total that were treated with ceftriaxone at 4 months after onset of infection in the study by Hodzic et al. (18), 11 (47.8%) had a positive xenodiagnosis (i.e., *B. burgdorferi* was detected by PCR in *Ixodes* ticks that fed on these mice). In an experiment that attempted to quantitate the number of spirochetes, the number of *flaB* DNA copies per tick was 120.6 ± 75.1 in the nymphal stages of those ticks that had fed on antibiotic-treated mice during the larval stage, compared to $35,747 \pm 40,705$ *flaB* DNA copies in positive ticks from saline-treated mice. *B. burgdorferi* could not be cultured from PCR-positive ticks that had fed on antibiotic-treated mice but could be cultured from ticks that had fed on saline-treated mice.

Ticks that had fed on antibiotic-treated mice were subsequently fed on highly immunodeficient (C3H-*scid*) mice (18). For unstated reasons, ticks were not fed on immunocompetent

mice. Although essentially none of the SCID mice became culture positive (based on cultures of the urinary bladder, ear, and inoculation site), *B. burgdorferi* DNA was detected in one or more tissue sites of these mice, excluding the site of the tick bite (18). None of the SCID mouse tissues, however, showed histologic evidence of inflammation. This was a surprising finding because this strain of mouse (which is deficient in both humoral and cellular acquired immunity) is highly susceptible to infection with *B. burgdorferi* and would have been expected to develop prominent signs of inflammation. Excluding one tissue sample that was an outlier, the copy number of *flaB* DNA in the tissue samples from the SCID mice averaged 656.2/mg. Based on a prior study of SCID mice by some of the same investigators (17), >40,000 *flaB* copies/mg of tissue might have been expected, although this comparison may not be entirely appropriate because *B. burgdorferi* was introduced by needle inoculation in the earlier investigation rather than by tick bite. Whether spirochetes could be visualized by immunohistochemistry in the SCID mice was not stated. Although the investigators mentioned that they attempted allograft transmission to SCID mice, the results were not reported in the paper.

Conclusions of the investigators in the study by Hodzic et al. Hodzic et al. (18) concluded that viable but not cultivable spirochetes persisted after antibiotic treatment in this animal system.

Failure to consider pharmacodynamics of antibiotic therapy is a methodological concern in the study by Hodzic et al. A serious methodological concern pertinent to the study by Hodzic et al. (18) and many of the other animal studies evaluating antibiotic treatment of *B. burgdorferi* infection is the failure to consider adequately the pharmacokinetic-pharmacodynamic properties of the antibiotic regimen used. Both in vitro and in vivo data demonstrate that the time that β -lactam antibiotics are maintained at or above the MIC for the infecting strain of *B. burgdorferi* ("time over MIC" [T/MIC]) is the key pharmacodynamic parameter governing the activity of this class of antibiotics against this microorganism (29, 31, 41, 51, 52). In view of the much shorter half-life of ceftriaxone in C3H mice than in humans, one or two daily doses of ceftriaxone in mice falls far short of the T/MIC that occurs in humans (Table 2) (18, 37, 38, 52).

Whether the same observations would have been made had the ceftriaxone dosing been administered in a manner that would have faithfully recapitulated the T/MIC seen in humans is unknown. If one of the antimicrobial effects of ceftriaxone is to attenuate *B. burgdorferi* and render it incapable of causing disease, but not completely eliminate it, would the same biologic event occur if ceftriaxone was given for a much shorter period of time, even just 24 h (38)? Is such attenuation reversible? Would doxycycline treatment have had a different effect on *B. burgdorferi* than ceftriaxone treatment, in view of the completely unrelated mechanisms of action of the two drugs?

Study by Bockenstedt et al.

Antibiotic dosing and pharmacokinetic-pharmacodynamic considerations. Some, but not all of these questions, were answered in a study reported several years earlier by Bockenstedt et al. (8). In contrast to the study by Hodzic et al. (18), C3H/HeJ mice in the study by Bockenstedt et al. were infected with an N40 strain of *B. burgdorferi* mice by an *Ixodes* tick bite rather than by needle inoculation (Table 1). At 30 days after infection, mice were treated with either ceftriaxone (by subcutaneous administration) or doxycycline (by gavage) for 30 days. Unlike in the study by Hodzic et al. (18), antibiotic doses were adjusted for age-related weight gain (8). However, Bockenstedt et al. (8) similarly ignored the much shorter half-lives of these antibiotics in mice than in humans and failed to replicate the antibiotic exposure to either drug that occurs in humans (Table 2) (4, 37, 44). The pharmacokinetic-pharmacodynamic parameter that correlates best with the efficacy of doxycycline for the treatment of infections due to other microorganisms is the area under the time-concentration curve of free drug (i.e., drug not bound to protein) divided by the MIC (4), and limited data suggest that this is also true for infection due to *B. burgdorferi* (27).

In a much earlier study, 60% of *B. burgdorferi*-infected mice remained culture positive despite treatment with a 2-week course of doxycycline at a dose of 13 mg/kg twice daily (31). Cognizant of these results and that of a treatment study of *Brucella* infection in mice (12), Bockenstedt et al. increased the dose of doxycycline to 50 mg/kg twice daily (8). The MIC of doxycycline for the N40 strain of *B. burgdorferi* used by Bockenstedt et al. has not been reported, but for other strains of *B. burgdorferi*, MICs of doxycycline are typically 4- to >10-fold higher than the 0.06- μ g/ml MIC of doxycycline for the strain of *Brucella melitensis* in the study relied upon by Bockenstedt et al. (19, 23). Furthermore, 100% of the mice were cured of the *Brucella* infection with a 45-day course of doxycycline (50 mg/kg twice daily), whereas a 21-day course of therapy was less effective and a 30-day course of treatment was not attempted (12). These discrepancies raise concerns about the appropriateness of the dose and duration of doxycycline employed in the study by Bockenstedt et al.

Cultures, PCR, and xenodiagnosis. In contrast to the case for sham-treated mice, antibiotic-treated mice in the study by Bockenstedt et al. did not have either a positive culture of blood, ear, heart, spleen, or urinary bladder or histologic evidence of arthritis or carditis (8). There was evidence by PCR of persistent *B. burgdorferi* DNA in mouse tissues

(heart, bladder, or joint), however, until study completion at 9 months after doxycycline treatment in at least four of five mice, particularly in joint tissue; in contrast, PCR positivity (at the "lower detection limit of the assay") was found only in urinary bladder tissue and only in two of four mice that were treated with ceftriaxone. Uninfected control mice were PCR negative. Although the spirochete could be detected in ticks by xenodiagnosis for up to 3 months after antibiotic therapy, such testing was negative at 6 months in both the antibiotic- and saline-treated mice. Xenopositivity for ticks at 9 months after treatment, however, did occur if the mice were first immunosuppressed with corticosteroids, but only for the ticks that fed on saline-treated mice, not for those that fed on antibiotic-treated animals. In the study by Hodzic et al. (18), xenodiagnosis was attempted only up to 3 months after completion of treatment; thus, the study by Bockenstedt et al. should not be assumed to contradict the study by Hodzic et al. on this point (Table 1).

After larval stage ticks that had fed on infected mice had molted to the nymphal stage in the study by Bockenstedt et al., they were no longer PCR positive and did not transmit infection to uninfected C3H mice (based on the absence of seroconversion in these mice) (8). No attempt was made to culture *B. burgdorferi* from PCR-positive ticks. Importantly, Bockenstedt et al. (8) noted that a similar proportion of antibiotic-treated mice had evidence of persistent infection based on xenodiagnostic studies in an earlier experiment in which ceftriaxone or doxycycline was given for just 2 weeks rather than 4 weeks.

Allotransplantation and serology. Bockenstedt et al. (8) also stated (without providing data) that they were unable to transmit infection from antibiotic-treated mice to SCID mice by allotransplantation, an experiment that was omitted in the study by Hodzic et al. (Table 1) (18). Antibiotic therapy led to a rapid decline in anti-*B. burgdorferi* whole-cell lysate antibody titers, but levels were still elevated through 9 months; titers were indistinguishable between mice that tested positive by xenodiagnosis and those that did not test positive. Over the 11-month experimental period, antibody titers also declined in the saline-treated group of infected mice, although they remained at significantly higher levels than in the antibiotic-treated mice.

Conclusions and comparisons with the study by Hodzic et al. In contrast to Hodzic et al. (18), Bockenstedt et al. (8) concluded that "residual" spirochetes are avirulent and will eventually die or be killed by the host without causing disease. There are numerous methodological differences between the studies by Hodzic et al. and Bockenstedt et al. (Table 1). The latter investigation employed C3H/HeJ mice (which have a mutation in Toll-like receptor 4 [40]) as opposed to C3H/HeN mice; mice were treated only at 30 days postinfection (rather than at 3 weeks and 4 months postinfection by Hodzic et al. [18]); xenodiagnosis was performed at 1, 3, 6, and 9 months (it was done only at 1 and 3 months in the study by Hodzic et al.); and refeeding of xenodiagnostic ticks was performed with C3H mice as opposed to SCID mice. Whether these methodological differences account for all of the discrepancies between the two studies is not clear.

DOG STUDIES

Straubinger et al. (47–49) studied the efficacy of a 30-day course of amoxicillin (amoxicilline) (oral), doxycycline (oral), ceftriaxone (intravenous), or azithromycin (oral) for the treatment of dogs that were infected with *B. burgdorferi* by tick feeding using field-collected ticks. Their results are in general agreement with those of Hodzic et al. (18) and Bockenstedt et al. (8) in that they showed that the dogs were more likely to be PCR positive than culture positive after antibiotic treatment. PCR positivity persisted for up to 455 days after the conclusion of antibiotic therapy (47). Like the mice in the studies discussed above, antibiotic-treated dogs usually remained well without development of objective clinical disease. This remained true even when the animals were immunosuppressed with corticosteroids.

In the first treatment study reported by Straubinger et al. (49), 3 (27.3%) of 11 dogs that were treated with either doxycycline or amoxicillin starting at approximately 60 days after tick exposure had a positive culture for *B. burgdorferi* posttreatment. In a second study by these investigators (48), none of four dogs that were treated with the same dose of doxycycline at 120 days after tick exposure were culture positive, nor were any of the dogs that were treated with azithromycin ($n = 4$) or ceftriaxone ($n = 4$). The larger number of culture-positive dogs in the earlier study might have been a chance event or due to a greater burden of spirochetes at the 60- versus 120-day time point. However, for unclear reasons the doxycycline blood levels reported in the second study were considerably higher than those that were found in the first study. Thus, it is tempting to speculate that the higher culture positivity rate in the first study was due to insufficient antibiotic treatment. Similarly, it is of interest that in the first study by Straubinger et al. (49), the single culture-positive dog that received amoxicillin (one of five infected dogs treated with amoxicillin was culture positive) was one of the two dogs that were treated with twice-daily dosing of amoxicillin; the other three dogs were treated with three doses per day.

The studies by Straubinger et al. (47–49) potentially add important clarifications to the mouse experiments discussed above (8, 18). First, they show that PCR positivity in the absence of culture positivity may occur in dogs as well as C3H mice. Second, the results indicate that this phenomenon can also occur after antibiotic treatment with either amoxicillin or azithromycin. Third, the results demonstrate that this phenomenon is not restricted to either the N40 strain of *B. burgdorferi* or even to a laboratory strain of the spirochete. Last, the exposure of the dogs to doxycycline or azithromycin in the second treatment trial conducted by these investigators (48) seemed more comparable to that seen in humans, suggesting that the reason for persistence of *B. burgdorferi* DNA is not necessarily improper dosing of the antimicrobial. The studies by Straubinger et al., however, documented a high degree of variability in antibiotic levels in blood specimens of different animals receiving the same dosage regimen. For example, in the first study in which the efficacy of doxycycline was compared with amoxicillin (49), antibiotic levels were assessed at multiple time points for four dogs that were treated with doxycycline and for four other dogs that received amoxicillin. Considerable variability (up to twofold or greater) in drug levels

was observed between the dog with the highest drug level and the one with the lowest level for each of the antibiotics. Variability in drug levels between animals was not investigated in the mouse studies performed by either Hodzic et al. (18) or Bockenstedt et al. (8).

It is noteworthy that in untreated dogs more tissue samples were positive by culture than by PCR, in contrast to what was observed in antibiotic-treated animals. In one of the studies by Straubinger et al. (47, 48), PCR positivity after antibiotic treatment was so infrequent and at such a low level quantitatively as to raise the concern that the results were due to amplicon contamination or to a stochastic (random) process, or both. For example, among the four ceftriaxone-treated dogs, only 3 (5.0%) of 60 skin biopsy samples obtained during or after antibiotic treatment were PCR positive and only 1 (1.0%) of 100 tissues obtained postmortem was PCR positive. In the single dog with two positive PCR results, PCR was negative for 3 months after completion of antibiotic therapy, a single sample was PCR positive at 4 months, PCR was negative from month 5 to 12, and PCR was positive again at 13 months. The use of a single uninfected control dog in this experiment was insufficient to exclude the possibility of either low-level contamination or a stochastic event.

OTHER MOUSE STUDIES

PCR has been employed as a measure of outcome in several other treatment studies of *B. burgdorferi*-infected animals. In studies by Yrjanainen et al. (54) and by Malawista et al. (30), mice were treated with a 5-day course of ceftriaxone. In neither of these studies did the investigators observe PCR positivity in the absence of culture positivity. However, in the study by Yrjanainen et al. (54), PCR was applied only to urinary bladder tissue and in the study by Malawista et al. (30), it was applied only to urinary bladder and ear tissue. Conceivably, there would have been a disproportionately higher rate of PCR versus culture positivity if tissue specimens from the base of the heart or tibiotarsal area had been evaluated. Yrjanainen et al. (54) raised the question of whether administration of an antibody to tumor necrosis factor alpha (TNF- α) might reactivate *B. burgdorferi* infection and restore culture positivity. The rationale for why this should occur in *B. burgdorferi* infection, which is a distinctly different kind of infection from mycobacterial or fungal infections, which are known to be reactivated by TNF- α inhibitors (50), was not made clear in the report. It is also unclear why TNF- α inhibition should reactivate *B. burgdorferi* when treatment with corticosteroids (38, 48) or transmission to SCID mice (18) in other studies did not. Furthermore, the frequency of culture-positive animals was not significantly different in those mice that were treated with anti-TNF- α and those not receiving this agent, implying that the higher rate of culture positivity in the mice receiving anti-TNF- α might have been a chance event (51).

In a study by Pahl et al. (35), *B. burgdorferi*-infected mice were treated with a subcurative dose of penicillin G given subcutaneously twice daily for 14 days starting on day 8 after infection. This dose did not eliminate the spirochete, and the differences in numbers of spirochetes in tissue based on PCR in favor of treatment versus control animals vanished by 4 weeks after completion of antibiotic therapy. Although details

TABLE 3. Summary of the principal findings of selected treatment studies of animals infected with *Borrelia burgdorferi*

Finding	References
Posttreatment culture positivity may be associated with certain treatment regimens; usually these regimens do not recapitulate the antibiotic exposure found in humans.....	20-22, 30-32, 35, 42, 54
Posttreatment PCR positivity may occur in the absence of culture positivity; there is no evidence that such animals will become culture positive if immunosuppressed with steroids or if followed for prolonged periods of time.....	8, 18, 48, 49
The frequency of posttreatment PCR positivity without culture positivity tends to decrease over time in mice; in dogs it may persist for at least 455 days.....	8, 18, 47
Posttreatment PCR positivity without culture positivity has been observed with various antibiotic therapies, including ceftriaxone, amoxicillin, doxycycline, and azithromycin; limited data for dogs suggest that the phenomenon is not dependent on the specific strain of <i>Borrelia burgdorferi</i>	8, 18, 47-49
Posttreatment PCR positivity without culture positivity is not associated with evidence of inflammation or clinical illness in animals, even if the animals are immunosuppressed.....	8, 18, 47, 48
Posttreatment PCR positivity without culture positivity is associated with positive xenodiagnosis; transmission of <i>B. burgdorferi</i> by xenodiagnostic ticks to SCID mice has been demonstrated, but the SCID mice are also culture negative and do not manifest inflammation or disease; transmission of <i>B. burgdorferi</i> by xenodiagnostic ticks to immunocompetent animals has not been demonstrated; transmission by allograft to SCID mice has not been successful.....	8, 18
Whether posttreatment PCR positivity without culture positivity can be attributed to suboptimal dosage regimens (including insufficient antibiotic exposure due to too low a dosage, too infrequent dosing, too short a treatment course, or inconsistency of antibiotic levels among animals) is unknown.....	8, 18, 47-49
Posttreatment PCR positivity without culture positivity is associated with reductions in titers of antibody to <i>B. burgdorferi</i> but not typically with seronegativity.....	8, 18, 48, 49

were not provided, the investigators indicated that *B. burgdorferi* could be cultured from PCR-positive tissues.

In a study of *B. burgdorferi*-infected mice, Pavia et al. (38) observed that administration of five daily doses of ceftriaxone was associated with negative cultures of urinary bladder and ear tissue, irrespective of whether the mice were concomitantly treated with corticosteroids. Similarly, Kazragis et al. (25) found consistently negative cultures for *B. burgdorferi* in SCID mice with *B. burgdorferi* infection that were treated with a 9-day course of ceftriaxone. Neither of these studies, however, evaluated PCR positivity or xenodiagnosis. These studies do, however, raise the question of whether the outcome of ceftriaxone therapy of infected animals would be the same with courses of antibiotic therapy much shorter than 30 days in duration.

Table 3 provides a brief summary of the principal findings for the treatment studies of animals infected with *B. burgdorferi* discussed above.

OUTSTANDING QUESTIONS REGARDING PCR POSITIVITY IN THE ABSENCE OF CULTURE POSITIVITY

The following are outstanding questions regarding the phenomenon of PCR positivity without culture positivity after antibiotic treatment of experimental animals with *B. burgdorferi* infection.

(i) Does the duration of treatment affect the development of PCR positivity in the absence of culture positivity? What is the shortest duration of treatment possible?

(ii) Do pharmacodynamic considerations such as total daily antibiotic exposure affect the development of PCR positivity in the absence of culture positivity?

(iii) What are causes of the attenuation of the spirochetes that persist posttreatment? Are they in the process of dying? Are they producing mRNA, and if so, which mRNA? Are they motile? Can they replicate? Are they genetically altered? Can they regain pathogenicity?

(iv) Does development of PCR positivity in the absence of culture positivity occur in SCID mice that are treated with antibiotic therapy; i.e., does the process occur independently of an adaptive immune response?

(v) Is PCR positivity reduced over time in SCID mice; i.e., does PCR positivity resolve spontaneously due to the death of damaged spirochetes?

For mice there is solid evidence for a reduction in the rates of posttreatment PCR positivity and xenopositivity over the course of time. Is this due to clearance of the spirochete by the host's immune response, or is it due to the death of irreversibly damaged spirochetes, or both? This question could be addressed by sequential evaluation of SCID mice that were infected with attenuated spirochetes as described in the study by Hodzic et al. (18).

VIABILITY OF SPIROCHETES

Does the phenomenon of PCR positivity without culture positivity imply viability of *B. burgdorferi*? Determination of whether bacterial cells are alive or dead is often challenging. It is an important question not only as it relates to the effects of antibiotics in an infected human or animal but also in terms of food safety and sterility of pharmaceutical products and implantable prosthetic devices (5, 6, 9, 11). The most straightforward confirmation of viability is demonstration of cell division. The "viable but not cultivable hypothesis" (for microorganisms that are initially cultivable) posits that bacterial cells have differentiated into a long-term survival state, rather than having degenerated into an injured state to be followed by an inexorable further deterioration to death (9, 34, 43). Demonstration of the presumed viability of bacteria other than borreliae that have become noncultivable has generally relied on the use of nucleic acid stains, redox indicators, membrane potential probes, flow cytometry, and reporter gene systems and on demonstration of the ability of the bacterial cell to take up amino acids or sugars, synthesize protein, synthesize

mRNA, or manifest respiration (6, 9). The validity of such methods is controversial, in part because such reactions might transiently be observed in injured cells, making the timing of the testing a critical variable (6, 9). Unequivocal proof of the viable but noncultivable hypothesis for bacteria has generally rested upon restoration of cultivability, even in circumstances in which the organism is first passed through an animal system (6, 9). Antibiotic treatment of animals with *B. burgdorferi* infection clearly differs from the classic description of bacterial "persisters" that can be demonstrated following exposure to certain antibiotics *in vitro*, since in the latter situation reversion back to regular cells with regrowth occurs when the antibiotic levels drop (7, 28).

Evidence that PCR positivity in mice treated for *B. burgdorferi* infection is due to viable but noncultivable spirochetes is based principally on a single experiment in which xenodiagnostic ticks transmitted the spirochete to SCID mice and then the spirochete disseminated to presumably extravascular tissue sites distant from the inoculation site (18). Whether the acquisition of spirochetes by xenodiagnostic ticks *per se* implies viability is unknown. If active motility on the part of the spirochete is required, then acquisition of the spirochete by a tick would imply viability. If spirochetes were viable after antibiotic treatment in the experiments performed by Hodzic et al. (18), Bockenstedt et al. (8), and Straubinger et al. (47–49), they clearly were attenuated. Was the attenuation due to irreversible spirochetal injury? If so, what is the nature of the damage? The loss of certain key genes was suggested by Bockenstedt et al. (8) but could not be confirmed in the study by Hodzic et al. (18).

RELEVANCE OF ANIMAL STUDIES TO HUMANS

Does the phenomenon of PCR positivity without culture positivity observed in animals after antibiotic treatment also occur in humans treated for Lyme disease, and if so, has it been detected and does it have any relevance to the presence of clinical disease or symptoms? Given the propensity of *B. burgdorferi* to localize over time in collagen-rich tissues of mice, including joints and tendons (18), investigation of patients with Lyme arthritis might provide insights into the existence of this phenomenon in humans. Indeed, rates of PCR positivity in synovial fluid specimens of untreated patients with Lyme arthritis may be as high as 96% (33). In contrast to the animal studies discussed above, however, the yield of culture for *B. burgdorferi* of synovial fluid specimens of untreated patients with Lyme arthritis is close to zero (2, 33). Therefore, Lyme arthritis is not a satisfactory model to evaluate whether antibiotic treatment *per se* induces culture negativity despite continued PCR positivity.

Most patients with Lyme arthritis respond well to a 4-week course of oral antibiotic therapy (52), and PCR testing cannot be carried out after therapy because affected joints are no longer swollen. However, a small subgroup of patients have persistent synovitis for months or even several years after treatment for ≥ 2 months with oral antibiotics or for ≥ 1 month with intravenous antibiotics, or usually after both types of therapy, a condition which is referred to as antibiotic-refractory Lyme arthritis. Antibody responses to *B. burgdorferi* antigens decline similarly in patients with antibiotic-responsive or antibiotic-

refractory arthritis, suggesting that spirochetal killing occurs in both groups (24). Because joint inflammation persists for months after antibiotic treatment, it provides an opportunity to assess the duration of PCR positivity after antibiotic therapy. Of 34 patients with antibiotic-refractory arthritis for whom joint fluid was available after antibiotic therapy, only 2 (6%) had a positive PCR result after 4 to 5 months, and just 1 still had a weakly positive result at 6 months (46). No one had a positive result after the 6-month time point. Thus, *B. burgdorferi* DNA may rarely persist in the joints of patients with Lyme arthritis for several months after recommended treatment with oral and/or intravenous antibiotics (52), but it eventually disappears.

The duration of PCR positivity in urine samples of patients with early Lyme disease associated with erythema migrans has also been evaluated. In one such European study, PCR positivity persisted in the urine for 12 months after antibiotic treatment in 8% of cases (1); cultures were not reported. The amplicons were not sequenced in that study, a potentially important omission since PCR testing for *B. burgdorferi* DNA in urine specimens has been associated with false-positive results (1, 2).

Could the phenomenon of PCR positivity for *B. burgdorferi* DNA in the absence of culture positivity after antibiotic therapy provide an explanation for post-Lyme disease syndrome, as suggested by Hodzic et al. (18)? This seems highly unlikely. Clearly the *B. burgdorferi* cells remaining in animals after antibiotic treatment are biologically different from those in untreated animals. Most importantly, their presence does not elicit a local inflammatory response in mice or dogs, even when the animals are immunocompromised (8, 18, 48). In addition, the decline in antibody response to *B. burgdorferi* in animals after treatment suggests a reduction in the overall immunologic response to the spirochete (39). Since there is no convincing evidence that *B. burgdorferi* is capable of elaborating a systemic toxin (14), it is difficult to imagine how residual spirochetes in the absence of a detectable local or generalized immunologic or inflammatory response by the host could lead to chronic subjective symptoms.

CONCLUSION

In conclusion, a number of recent studies in which *B. burgdorferi*-infected animals were treated with antibiotic therapy have demonstrated the presence of PCR positivity in the absence of culture positivity (Table 3) (8, 18, 48, 49). A serious methodological concern with most of these studies is the failure to consider adequately the pharmacokinetic-pharmacodynamic properties of the antibiotic in choosing the dosage regimen used. In mice that have been treated with antibiotic therapy, residual spirochetes can be taken up by ticks during a blood meal (8, 18), at least for the first several months after treatment, and can be transmitted to SCID mice (18). The biological nature of these spirochetes is unclear. Evidence indicates that they are nonpathogenic. Whether the lack of pathogenicity is simply related to low numbers of residual spirochetes or is due to a more fundamental genotypic or phenotypic alteration is unknown. It is also unknown whether the lack of pathogenicity is irreversible. Since in the mouse studies the number of spirochetes is declining over time, a

reasonable conjecture is that they are in the process of dying. There is no scientific evidence to support the hypothesis that such spirochetes, should they exist in humans, are the cause of post-Lyme disease syndrome.

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