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Ineffectiveness of Tigecycline against Persistent *Borrelia burgdorferi*▽

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The effectiveness of a new first-in-class antibiotic, tigecycline (glycylcycline), was evaluated during the early dissemination (1 week), early immune (3 weeks), or late persistent (4 months) phases of *Borrelia burgdorferi* infection in C3H mice. Mice were treated with high or low doses of tigecycline, saline (negative-effect controls), or a previously published regimen of ceftriaxone (positive-effect controls). Infection status was assessed at 3 months after treatment by culture, quantitative *ospA* real-time PCR, and subcutaneous transplantation of joint and heart tissue into SCID mice. Tissues from all saline-treated mice were culture and *ospA* PCR positive, tissues from all antibiotic-treated mice were culture negative, and some of the tissues from most of the mice treated with antibiotics were *ospA* PCR positive, although the DNA marker load was markedly decreased compared to that in saline-treated mice. Antibiotic treatment during the early stage of infection appeared to be more effective than treatment that began during later stages of infection. The viability of noncultivable spirochetes in antibiotic-treated mice (demonstrable by PCR) was confirmed by transplantation of tissue allografts from treated mice into SCID mice, with dissemination of spirochetal DNA to multiple recipient tissues, and by xenodiagnosis, including acquisition by ticks, transmission by ticks to SCID mice, and survival through molting into nymphs and then into adults. Furthermore, PCR-positive heart base tissue from antibiotic-treated mice revealed RNA transcription of several *B. burgdorferi* genes. These results extended previous studies with ceftriaxone, indicating that antibiotic treatment is unable to clear persisting spirochetes, which remain viable and infectious, but are nondividing or slowly dividing.

Management of Lyme disease patients with antibiotics is based upon evidence-based clinical guidelines (78). Clinical assumptions are complicated by the ephemeral, variably recurrent, and diverse nature of both objective clinical signs and subjective symptoms of Lyme borreliosis. What is not known is whether or not antibiotic treatment completely eradicates the infection, and this has generated debate among the medical and lay communities. Various *Borrelia burgdorferi* isolates have been shown to possess differences in susceptibility to antibiotics, including *B. burgdorferi* N40 resistance to erythromycin (60, 73), but there is no evidence of acquired antibiotic resistance by *B. burgdorferi* in response to treatment. It has recently been demonstrated that *B. burgdorferi* has a putative efflux system with significant homology to the tetracycline efflux protein and to a resistance-nodulation-division (RND) efflux system (AcrAB-TolC) (15). Treatment failures for human Lyme borreliosis have been documented with nearly every type of antimicrobial drug, based upon clinical relapse (22), culture (23, 28, 29, 48, 52, 55, 57, 62, 72), or PCR (48, 58).

Antibiotics are likely to kill most *B. burgdorferi* organisms, but the immune system is needed to fully eliminate the remaining spirochetes. However, therein lies the challenge, since *Borrelia burgdorferi* has evolved to persistently infect fully immunocompetent hosts. Persistent infection has been shown to be the rule, rather than the norm, in a variety of laboratory animal species, including mice (7, 9), rats (45), *Peromyscus leucopus* (64), hamsters (20, 32, 63), gerbils (54), guinea pigs (66), rabbits (17), dogs (70), and nonhuman primates (24, 49, 59). Based upon culture and/or PCR, persistent infections have also been documented in humans from both Europe (3, 36, 43, 46, 65, 67, 71, 74) and the United States (14, 19, 47). Therefore, the "mop up" phase, which is dependent upon the immune system, is likely to be ineffective against an agent such as *B. burgdorferi*, which is highly effective at evading host clearance.

Experimental evaluation of antimicrobial effectiveness *in vivo* has been based upon studies in various animal models in which tissues can be invasively collected and cultured. These studies have shown success with a variety of different classes of antimicrobial drugs (31, 33, 34, 41, 44, 50, 53, 56, 82). However, with the advent of increasingly sensitive PCR methods, studies in mice and dogs have revealed persistence of noncultivatable spirochetes, based upon DNA amplification, in tissues following antibiotic treatment (13, 27, 69, 70). These noncultivatable spirochetes are nevertheless infectious, as they can be acquired by larval ticks that feed upon treated mice (xenodiagnosis) and survive molting into nymphal ticks. Nymphal ticks can then effectively transmit spirochetes to naïve mice, which develop disseminated infection. At all phases of these events, spirochetes cannot be cultured, and their numbers are very low, suggesting a viable but slowly dividing or nondividing population (27). These features fit the paradigm of multidrug tolerance or "recalcitrance to eradication" by antibiotics that occurs among a variety of persistent bacterial and fungal infections (reviewed in references 30, 38, and 39).

These results challenge prevailing dogma about effectiveness

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of antibiotics for eliminating *B. burgdorferi* infection, and therefore further work is critically needed. The purpose of the present study was to test the potential for improved effectiveness of a new first-in-class antibiotic, tigecycline (glycylcycline) (21), which has been shown to be highly active and bactericidal *in vitro* against multiple strains of *B. burgdorferi* (80) compared to previously published treatment regimens of ceftriaxone, a β-lactam antibiotic which is similarly highly active and bactericidal *in vitro* (40) and has become the mainstay in the treatment of disseminated and chronic Lyme disease (78). In the present study, antibiotics were administered during the early dissemination (1 week), early immune (3 weeks), or late (4 months) stages of *B. burgdorferi* infection in immunocompetent C3H mice to mimic the stages found in human disease.

MATERIALS AND METHODS

Mice. Five-week-old female C3H/HeN (C3H) mice were purchased from the Frederick Cancer Research Center, Frederick, MD, and C3H.C-*Prkdc^{scid}/IcrSmnHsd* (SCID) mice were purchased from Harlan Laboratories, Inc., Indianapolis, IN. Mice were specific pathogen free, maintained in an isolation room under static filter-top cages, and provided food and water *ad libitum*. Mice were necropsied following an American Veterinary Medical Association-approved euthanasia procedure of carbon dioxide narcosis and exsanguination by cardiocentesis. The presence of infectious spirochetes in tissues of various treated mice was assessed by subcutaneous transplantation of small pieces of tibiotarsal joint and heart base tissue into recipient SCID mice under ketamine/xylazine anesthesia, as described previously (6, 8). Animal use was approved by the University of California at Davis (UCD) Animal Care and Use Committee. UCD has a Public Health Service Animal Welfare Assurance on file and is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) International.

***Borrelia burgdorferi*.** Mice were infected by subdermal inoculation of the dorsal thoracic midline with 10^4 mid-log-phase clonal *B. burgdorferi* N40 (cN40) cells in 0.1 ml of modified Barbour-Stoenner-Kelly (BSK II) medium (5). All mice were inoculated with the same dose of spirochetes and at the same time. Infection status of mice was determined by culture and PCR. Tissues (urinary bladder and inoculation site) collected from mice at necropsy were cultured in BSK II medium, as described previously (7). Based upon serial dilutions of quantified cultured spirochetes, culture in BSK II medium is sensitive for detection down to the level of single organisms (27). Tissues were also processed for *ospA* PCR (inoculation site, heart base, ventricular muscle, left tibiotarsus, and quadriceps muscle).

Antibiotic. Mice were treated with 16 mg/kg of body weight ceftriaxone (Hoffman LaRoche, Nutley, NJ) in a 500-μl total volume of 0.9% normal saline solution administered intraperitoneally twice daily for 5 days and then once daily for 25 days. Mice were treated with low (12.5 mg/kg) or high (50 mg/kg) doses of tigecycline (Wyeth Pharmaceutical, Pearl River, NY), administered subcutaneously once daily for 10 days. The ceftriaxone treatment regimen served as a control group for comparison with previously published studies that demonstrated persistence of *B. burgdorferi* after treatment (13, 27). Tigecycline doses were based on recently published tigecycline pharmacokinetic data in mice (35), which showed the 12.5-mg/kg dose to be similar to the serum drug concentrations achieved in humans.

MICs of antibiotics were determined against *B. burgdorferi* N40 and *B. burgdorferi* B31 using a 96-well microdilution method with minor modifications, as described previously (80). Duplicate wells containing BSK-H medium (Sigma-Aldrich, St. Louis, MO) with and without the appropriately diluted antimicrobial agents were inoculated to final density of 5×10^6 cells/ml of the test organism. The ranges of antibiotic concentrations tested were 0.006 to 25.0 mg/liter. After incubation at 34°C for 72 h, 10-μl aliquots were extracted from each well and live (motile) *Borrelia* cells were examined under dark-field microscopy. The MIC was the lowest concentration of antibiotic at which the number of cells after incubation did not exceed the initial concentration. The minimum bactericidal concentration (MBC) was the lowest antibiotic concentration from which spirochetes could not be cultured after 72 h of coincubation with antibiotics. Following 72 h of incubation with antibiotic, an aliquot (20 μl) from each test well was transferred to 5 ml of fresh BSK-H medium and subcultures were assessed for the presence of motile spirochetes at 21 days.

Ticks. Ticks were derived from a single population of laboratory-reared *Ixodes scapularis* larvae kindly provided by Durland Fish of Yale University. For xenodiagnosis, 40 larval ticks were placed on mice 1 week prior to necropsy and allowed to feed to repletion. Engorged larvae were allowed to molt into nymphs and harden, and samples of each tick cohort from each mouse were individually tested by *ospA* PCR to determine the prevalence of infection within each cohort. Ten nymphal ticks from positive cohorts were allowed to attach and feed upon naïve SCID mice to determine the presence of infective spirochetes. Some of the nymphs were allowed to molt into adult ticks, which were then dipped in 70% ethanol and bisected sagittally using a sterilized razor blade. One-half of the tick was placed in BSK II medium containing 50 mg/ml rifampin, 20 mg/ml phosphomycin, and 2.5 mg/ml amphotericin B; incubated at 33°C for 11 to 14 days; and then examined for spirochete growth under dark-field microscopy. The other half of the tick was frozen in liquid nitrogen and then ground with a mortar and pestle, and DNA was extracted for PCR analysis.

PCR analysis. Quantitative real-time PCR was utilized to detect *ospA* DNA in mouse tissues and ticks, as previously described (25, 26). The *ospA* target was chosen because of the known "target imbalance" of *ospA* over other gene targets in spirochetal DNA obtained from tissues (51), which has been confirmed in multiple studies in this laboratory. All samples were assayed with positive and negative controls. In addition, since copy numbers of target DNA are typically very low in antibiotic-treated mice, the specificity of the real-time PCR was verified on skin, heart base, ventricular muscle, quadriceps muscle, and tibiotarsal tissues from 4 uninfected mice (20 sites total) and subjected to *flaB*, *ospA*, and *dbpA* real-time PCR amplification. All samples were negative for all 3 gene targets, and 16 positive control tissues from previously archived antibiotic-treated mice were positive. DNA was extracted from tissues or ticks with DNeasy kits (Qiagen, Valencia, CA) according to manufacturer's instructions for tissue or insects, respectively. Prior to DNA extraction, tissue samples were weighed. Data are expressed as the number of DNA copies per mg of tissue or per tick.

A multiplex nested quantitative real-time reverse transcriptase PCR (RT-PCR) for cDNA of the *flaB*, *ospA*, *ospC*, *arp*, *dbpA*, *fbp* (*bbk-32*), *oppA-2*, and host β-actin genes was developed, using previously published *B. burgdorferi* N40-specific primer sequences (25). Although nested RT-PCR is not precisely quantitative, this assay was used to detect RNA transcription in tissues of mice subjected to the various treatments. Total RNA from each sample was purified using RNeasy minikits, according to the manufacturer's instruction for tissues (Qiagen, Valencia, CA). Samples were frozen in liquid nitrogen and homogenized with a mortar and pestle (pretreated with 0.1% diethylpyrocarbonate in water overnight, and then autoclaved). RNA was purified using the RNeasy protocol for animal tissues. After elution, RNA was treated with RNase-free DNase I and then repurified using the RNeasy cleanup protocol. Extracted total RNA was stored at −80°C until use. The extracted total RNA was subjected to two separate reactions: one to synthesize cDNA and the other to test for DNA contamination. The reverse transcription step was performed utilizing TaqMan reverse transcription reagents (PE Biosystems, Foster City, CA) with random hexamers. cDNA synthesis and the control reaction were amplified in a standard heat block instrument after incubation for 10 min at 25°C, following transcription at 48°C for 30 min and inactivation at 95°C for 5 min. For the preamplification step, a mixture of outer primers for each target gene (designed with Primers Express software), the Advantage cDNA polymerase system (Clontech, France), deoxynucleoside triphosphates (dNTPs), and cDNA was amplified in a thermocycler: 1 cycle at 94°C for 1 min; 25 cycles at 94°C for 15 s, 55°C for 15 s, and 70°C for 40 s; and 1 cycle at 70°C for 5 min. Following preamplification, individual RT-PCRs were set up using individual sets of inner primers and labeled probes. Amplification, data acquisition, and data analysis were performed in an ABI Prism 7700 sequence detector (Perkin-Elmer, Applied Biosystems, Foster City, CA).

Serology. Antibody titers were determined against *B. burgdorferi* N40 lysates by enzyme-linked immunosorbent assay (ELISA), using duplicate 3-fold serial dilutions of test serum and alkaline phosphatase-conjugated rat anti-mouse immunoglobulin (heavy and light chain), as described previously (18, 27). Each assay included positive and negative controls. Cutoff points for each serum dilution were established by determining absorbance means of normal mouse sera and adding 3 standard deviations (SD) above the means.

Histology. Tissues were fixed in neutral buffered formalin, and rear legs were demineralized in acid. Legs and hearts were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin by standard methods. Legs and hearts were blindly examined and scored for inflammation, as described previously (2, 7, 8, 10, 27).

Statistical analysis. Statistical analysis of quantitative PCR data between treatment groups and time intervals was performed by 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). Anti-

TABLE 1. Antimicrobial susceptibilities of *B. burgdorferi* strains N40 and B31 to four different antibiotics

Antibiotic	N40		B31	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
Tigecycline	0.006	0.095	0.006	0.195
Doxycycline	0.780	25.000	0.390	25.000
Ceftriaxone	0.048	1.500	0.012	0.780
Penicillin	0.024	6.250	0.024	6.250

body titers were compared among treatment groups with Student's unpaired t test. Calculated P values lower than 0.05 were considered significant.

RESULTS

Determination of MICs and MBCs. The MICs and MBCs for tigecycline against *B. burgdorferi* N40 and B31 were compared to doxycycline, ceftriaxone, and penicillin in order to evaluate the relative effectiveness of tigecycline and the relative sensitivity of *B. burgdorferi* N40. Tigecycline and ceftriaxone had the lowest MICs against both *B. burgdorferi* N40 and B31, compared to doxycycline and penicillin (Table 1). When the MBCs of *B. burgdorferi* were compared to peak serum levels obtained in previous mouse studies, the serum maximum concentrations of tigecycline and ceftriaxone were at least 100 times greater than the *B. burgdorferi* N40 tigecycline MBC and 70 times greater than the *B. burgdorferi* N40 ceftriaxone MBC (35, 61, 75). The MIC and MBC for *B. burgdorferi* N40 were essentially similar to those of *B. burgdorferi* B31 for any of the antibiotics tested. Therefore, *B. burgdorferi* N40 was considered suitable for examining efficacy of tigecycline in the mouse model.

Experimental design and rationale. Groups of *B. burgdorferi*-infected C3H mice were treated with high-dose tigecycline, low-dose tigecycline, ceftriaxone, or saline. Saline-treated mice served as negative-effect controls, and ceftriaxone-treated mice served as positive-effect controls, using a previously published regimen that resulted in persistence of noncultivable spirochetes (13, 27). Treatment was commenced at 1 week, 3 weeks, or 4 months of infection. Based upon previous studies in C3H mice, these stages of infection represented (i) an early preimmune phase of infection (1 week) in which spirochetes are in the process of disseminating from the site of inoculation, (ii) an early immune phase of infection (3 weeks) in which the host immune response is under way and beginning to resolve joint and heart inflammation and globally reducing spirochete burdens in tissues, and (iii) a late phase of infection (4 months) in which inflammation is generally absent and infection is persistent (7, 9, 25). Early and late stages of infection were evaluated because treatment regimens for later-stage Lyme disease in humans require longer and sometimes repeated courses of antibiotics (68, 78). Mice were necropsied at 3 months after completion of treatment, which provided an interval that would test for survival and outgrowth of spirochetes well after antibiotic levels had dissipated.

Preimmune phase (1 week) treatment. All of the antibiotic-treated mice were culture negative, but approximately half of the mice in each of the antibiotic treatment groups had one or more tissues with detectable *ospA* DNA (Table 2). Copy numbers per mg of PCR-positive tissue (negative samples not included in calculations) did not differ significantly ($P \geq 0.05$) among antibiotic treatment groups, but were significantly lower than mean copy numbers per mg of PCR-positive tissues from saline-treated mice ($P = 0.25$). Cumulative percent positive PCR results from all tissues tested from mice treated with antibiotics suggested that antibiotic treatment reduced dissemination or the number of tissues involved compared to that in saline-treated control mice ($P < 0.001$).

TABLE 2. *ospA* copies in tissue from mice treated with high-dose tigecycline, low-dose tigecycline, ceftriaxone, or saline at 1 week of infection and then sampled 3 months after completion of treatment

Treatment	Mouse	No. of *ospA* copies/mg tissue[a]				
		Inoculation site	Heart base	Ventricular muscle	Tibiotarsus	Quadriceps muscle
Tigecycline						
High dose	1	3	–	–	–	–
	2	–	–	–	–	–
	3	7	–	–	6	12
	4	–	6	11	–	–
	5	–	–	–	–	–
	6	–	–	–	–	–
	7	2	–	10	–	–
	8	–	–	3	1	12
Low dose	1	–	–	–	–	–
	2	–	38	–	8	–
	3	–	17	–	–	–
	4	9	–	5	–	1
	5	–	–	–	–	–
	6	–	–	–	–	–
	7	–	–	–	440	–
	8	–	–	–	–	–
Ceftriaxone	1	–	–	–	–	–
	2	–	–	–	–	–
	3	–	–	–	–	–
	4	–	3	–	–	–
	5	–	–	–	–	–
	6	–	3	–	–	–
	7	–	–	13	–	–
	8	–	–	–	–	–
Saline	1	2	23	149	–	–
	2	–	–	13	–	–
	3	27	81	90	120	37
	4	11	142	11	42	14
	5	32	9	55	–	61
	6	8	302	10	–	2
	7	NA[b]	–	10	–	60
	8	6	37	15	–	1

[a] –, sample tested negative.
[b] NA, sample not available.

Early immune phase (3 week) treatment. All of the antibiotic-treated mice were culture negative, but all mice, regardless of antibiotic treatment, had one or more *ospA* PCR-positive tissue sites (Table 3). There were no significant differences in *ospA* copy numbers per mg of PCR-positive tissue among antibiotic treatment groups ($P > 0.05$). Cumulative percent positive PCR results from all tissues tested from mice treated with antibiotics suggested that antibiotic treatment reduced dissemination or the number of tissues involved compared to that in saline-treated control mice, but differences were only marginally insignificantly different ($P = 0.069$). There was no significant difference ($P = 0.261$) between *ospA*

TABLE 3. *ospA* copies in tissue from mice treated with high-dose tigecycline, low-dose tigecycline, ceftriaxone, or saline at 3 weeks of infection and then sampled 3 months after completion of treatment

Treatment	Mouse	No. of *ospA* copies/mg tissue[a]				
		Inoculation site	Heart base	Ventricular muscle	Tibiotarsus	Quadriceps muscle
Tigecycline						
High dose	1	–	4	–	62	–
	2	–	–	–	3	–
	3	–	32	–	–	–
	4	–	21	4	10	–
	5	–	4	2	8	–
	6	–	4	–	7	–
Low dose	1	–	386	–	9	–
	2	–	1	–	–	–
	3	–	11	–	–	–
	4	–	–	–	8	–
	5	–	2	–	49	–
	6	–	122	–	–	–
	7	–	3	–	2	–
	8	–	43	–	4	–
Ceftriaxone	1	–	13	–	11	–
	2	–	1	–	6	–
	3	–	1	–	38	–
	4	–	25	–	8	–
	5	–	1	–	13	–
	6	–	6	–	5	–
	7	–	5	–	73	–
	8	–	–	–	1	–
Saline	1	–	23	37	55,900	–
	2	24	95	237	1,230	31
	3	45	19	140	100	619
	4	829	505	2,520	718	63
	5	92	1	326	56	105
	6	289	416	1,500	493	118
	7	118	36	805	215	62
	8	147	7	1,100	2,100	81

[a] –, sample tested negative.

TABLE 4. *ospA* copies in tissue from mice treated with high-dose tigecycline, low-dose tigecycline, ceftriaxone, or saline at 4 months of infection and then sampled 3 months after completion of treatment

Treatment	Mouse	No. of *ospA* copies/mg tissue[a]				
		Inoculation site	Heart base	Ventricular muscle	Tibiotarsus	Quadriceps muscle
Tigecycline						
High dose	1	–	–	–	7	–
	2	–	–	–	1,730	–
	3	–	–	2	50	–
	4	–	–	–	3	–
	5	–	–	4	10	–
	6	–	–	–	94	4
	7	–	–	14	1	1
	8	–	–	–	9	–
	9	3	–	–	14	–
Low dose	1	–	–	–	1	8
	2	1	–	–	5	–
	3	–	–	3	5	–
	4	2	14	2	142	–
	5	3	–	–	10	7
	6	3	–	–	49	–
	7	2	–	–	192	–
Ceftriaxone	1	–	–	–	15	–
	2	–	–	3	13	–
	3	–	–	22	9	–
	4	1	–	–	4	–
	5	–	–	–	17	10
	6	–	–	4	34	–
	7	1	–	20	22	–
	8	6	–	–	13	–
	9	7	–	5	33	–
	10	4	42	–	62	–
Saline	1	1,640	–	9,690	214	1,970
	2	20,600	3,510	16,200	864	4,430
	3	13,800	–	197,000	440	808
	4	613	–	33,300	378	7,650
	5	41,700	1,380	4,400	2,820	30,700
	6	15	132	479	602	137
	7	962	–	1,300	950	392
	8	75	247	39	634	135
	9	59	–	118	578	1
	10	11	399	3,350	1,100	116

[a] –, sample tested negative.

copies in saline-treated mice versus antibiotic-treated mice due to the wide range of values in the saline group, reflecting the nonuniform distribution of spirochetes in tissues.

Late persistent-phase (4 months) treatment. As with the other intervals, all of the antibiotic-treated mice were culture negative. Regardless of antibiotic treatment, all mice had one or more *ospA* PCR-positive tissues (Table 4). There were no significant differences in *ospA* copy numbers per mg of PCR-positive tissue among antibiotic treatment groups ($P > 0.05$), and differences were marginally insignificant compared to saline-treated control mice ($P = 0.063$). Cumulative percent positive PCR results from all tissues tested from mice treated with antibiotics suggested that antibiotic treatment reduced dissemination or the number of tissues involved compared to that in saline-treated control mice, but differences did not reach statistical significance ($P = 0.086$).

Differences among treatment intervals. Analysis of mean copy numbers of *ospA* DNA in PCR-positive tissues across all treatment intervals and between treatment groups revealed significant ($P = 0.023$) differences among antibiotic-treated mice and saline-treated control mice. Likewise, there was a significant difference ($P < 0.001$) in the percent PCR-positive tissues across intervals between antibiotic-treated mice and saline-treated control mice.

Antibody response. ELISA of antibody to *B. burgdorferi* lysates (Fig. 1) indicated different titers among saline-treated mice, depending upon phase of infection. These differences reflected the different intervals after infection (approximately 4, 5, and 8 months). With the exception of mice treated with low-dose tigecycline during the late persistent phase of infection, antibiotic treatment resulted in significant reduction ($P \leq 0.05$) in titers at 3 months after antibiotic treatment compared to those in saline-treated mice. Antibody titers were less reduced compared to saline-treated controls when mice were treated during the late persistent phase of infection.

RNA transcription. Because of the high prevalence of positive DNA results among heart base tissues during the early immune phase interval, heart base tissues from DNA-positive samples were analyzed for RNA transcription by nested RT-PCR (Table 5). Samples were tested for the *flaB*, *ospA*, *ospC*, *arp*, *dbpA*, *bbk-32*, *oppA-2*, and mouse β-actin (mouse positive control) genes. These targets were selected because they are considered to be constitutively expressed (*flaB*), downregu-

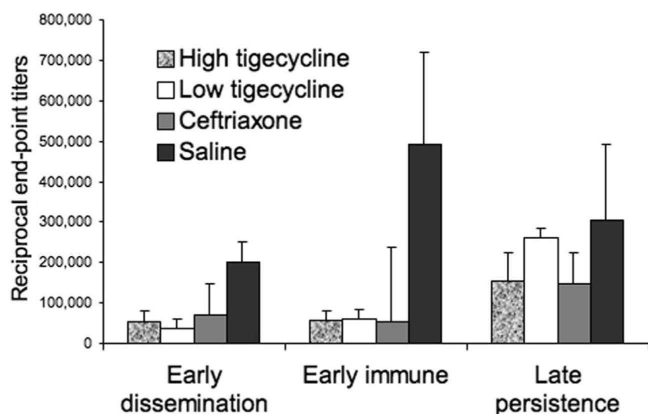

FIG. 1. *Borrelia burgdorferi* immunoglobulin G ELISA titers (mean reciprocal dilution + SD) for mice treated with high-dose tigecycline, low-dose tigecycline, ceftriaxone, or saline during the dissemination (1 week), early immune (3 weeks), and late persistent (4 months) phases of infection. Sera were collected 3 months after completion of antibiotic or saline treatment.

lated (*ospA*), or upregulated (*ospC*, *arp*, *dbpA*, *bbk-32*, and *oppA-2*) during infection (25) or were involved in environmental adaptation as one of only a few transporters encoded by the *B. burgdorferi* genome (*oppA-2*) (42, 76). Most of the samples from saline-treated mice were positive for all targets (except *ospA*). RNA was detected variably among tissues from antibiotic-treated mice, depending upon the RNA target. Notably, none of 16 samples from antibiotic-treated mice had detectable levels of *flaB*, and the most common target detected in tissues from antibiotic-treated mice was *oppA-2*. These results indicate viability and metabolic activity among the persisting spirochetes.

Xenodiagnosis and transmission of spirochetes. Xenodiagnosis was performed on mice treated with high-dose tigecycline or saline during the late persistent phase of infection. Larval ticks were fed to repletion on each of the mice (one week prior to necropsy) and allowed to molt, and 15 to 23 nymphal ticks from mice in each of the treatment groups were allowed to feed upon individual SCID mice. Two SCID mice were infested with ticks from high-dose tigecycline-treated mice, and 3 SCID mice were infested with ticks from saline-treated mice. None of the tissues from SCID mice fed upon by ticks from antibiotic-treated mice were culture positive, whereas all 3 of the SCID mice fed upon by ticks from saline-treated mice were culture positive. Ventricular muscle and quadriceps muscle from each of the 2 SCID mice fed upon by ticks from tigecycline-treated mice were *ospA* positive (15.8 to 19.5 copies/mg tissue). All 3 SCID mice that were fed upon by ticks from saline-treated mice had multiple tissues that were *ospA* positive (range, 2,870 to 162,000 copies per mg tissue; mean ± SD, 40,673 ± 60,284). Inflammatory lesions were present in the hearts and joints of only the SCID mice fed upon by ticks from the saline-treated mice. Thus, mice treated with tigecycline during the late phase of infection remained infected with spirochetes that could be acquired by ticks and could be transmitted by ticks to SCID mice, but the spirochetes were present in very low numbers and did not induce disease.

Xenodiagnosis was also performed on 8 ceftriaxone-treated mice treated during the early immune phase of infection. This treatment group was selected to examine the phenomenon of tick acquisition and transmission through molting into adult stage, in order to confirm and extend previous studies with ceftriaxone (13, 27). Forty larval ticks were fed to repletion on each mouse (one week prior to necropsy), and allowed to molt into nymphs. Cohorts of nymphs from each mouse were sampled to determine acquisition of infection by the ticks, and 3 of the cohorts were found to contain PCR-positive ticks. Six of 7, 6 of 8, and 3 of 4 nymphs from the 3 cohorts were *ospA* PCR positive. Copy numbers of *ospA* DNA ranged from 31.1 to 1,840 copies per tick. The remaining nymphal ticks were pooled, and 15 nymphs were allowed to feed on each of 7 SCID mice. The SCID mice were necropsied at 30 days after tick feeding, and tissues were cultured (skin near tick feeding sites, quadriceps muscle, heart, joint, and urinary bladder) and sampled for *ospA* PCR reactivity (tick feeding site, ventricular muscle, quadriceps muscle, joint, and ear). Ticks on all 7 SCID mice fed to repletion and were collected, but 4 of the SCID mice died prior to necropsy (water bottle accident). None of the tissues from the remaining 3 SCID mice was culture positive, but all tissues from the SCID mice were positive for *ospA* DNA, ranging from 1.8 to 98 *ospA* copies per mg tissue. These results indicated that ticks feeding upon ceftriaxone-treated mice acquired infection and transmitted infection to SCID mice, as previously shown (27). None of the mice had inflammatory lesions in their joints or hearts.

In an effort to determine if spirochetes from ceftriaxone-treated mice survived molting from the nymphal stage into adults, the remaining nymphs that fed upon the SCID mice were allowed to molt into adults. The adult ticks were bisected, one-half was cultured, and the other half was assayed for *ospA* DNA. All 78 adult ticks were culture negative, but 4 of the 78 ticks (2 of which fed upon the same mouse) were *ospA* PCR positive, with copy numbers ranging from 154 to 234 (mean ± SD, 150.2 ± 101.7) *ospA* copies/tick. Thus, spirochetes survived molting in at least some of the ticks, but continued to be resistant to culture, suggesting a continued state of nonreplication or low replication.

Tissue transplant transmission of spirochetes. At the time of necropsy of mice treated during the late persistent phase of infection, samples of tibiotarsal tissue and heart base tissue from each mouse that was treated with high-dose tigecycline,

TABLE 5. Nested real-time RT-PCR amplification of selected *B. burgdorferi* cDNA gene targets (RNA transcripts) in heart base tissues from mice treated with saline, ceftriaxone, high-dose tigecycline, or low-dose tigecycline at 3 weeks of infection

Target gene	No. of samples positive/no. tested			
	Saline	Ceftriaxone	Tigecycline	
			High dose	Low dose
flaB	6/6	0/7	0/4	0/5
arp	6/6	1/7	0/4	0/5
ospA	0/6	0/7	0/4	1/5
dbpA	6/6	2/7	0/4	1/5
ospC	2/6	0/7	1/4	1/5
oppA-2	6/6	4/7	4/4	4/5
bbk32	6/6	2/7	3/4	1/5
β-Actin	6/6	7/7	4/4	5/5

TABLE 6. Summary of culture and *ospA* real-time PCR results from tissues from SCID mice at 4 weeks after transplant of tibiotarsal and heart base tissue from donor mice treated with high-dose tigecycline, ceftriaxone, or saline at 4 months of infection and then collected 3 months after completion of treatment

Donor treatment	No. of mice positive/no. tested by:						
	Culture[a]	*ospA* PCR					
		Inoculation site	Heart base	Ventricular muscle	Quadriceps muscle	Tibiotarsal joint	Cumulative total[b]
High-dose tigecycline	0/9	1/9	0/9	1/9	2/9	1/9	4/9
Ceftriaxone	0/10	0/10	0/10	3/10	1/10	0/10	4/10
Saline	10/10	10/10	10/10	10/10	10/10	10/10	10/10

[a] Combined culture results from blood, inoculation site, and/or urinary bladder.
[b] Cumulative total of mice with 1 or more PCR-positive tissues.

ceftriaxone, or saline were transplanted subcutaneously into a single SCID mouse for each treated donor mouse (Table 6). Previously published studies with ceftriaxone revealed a very low level of transmission by tissue transplant, but those studies used ear tissue, which was less apt to be persistently infected after antibiotic treatment (27). Therefore, tibiotarsal and heart base tissue from treated mice were utilized in the current study, since these sites were more consistently positive after treatment. The recipient SCID mice were necropsied at 4 weeks after transplantation, and blood, urinary bladder, and transplantation sites were cultured. None of 9 SCID mice transplanted with tissue from high-dose tigecycline-treated mice and none of 10 SCID mice transplanted with tissue from ceftriaxone-treated mice were culture positive. Four of nine SCID mice that received transplants from high-dose tigecycline-treated mice had one or more tissue sites that were *ospA* PCR positive. Four of 10 SCID mice that received transplants from ceftriaxone-treated mice had single tissue sites that were *ospA* PCR positive. Copy numbers of *ospA* DNA ranged from 1.2 to 18.6 copies per mg tissue. In contrast, all 10 SCID mice transplanted with tissues from the saline-treated mice were culture positive, including 9 of 10 with bacteremia. Furthermore, all tissue sites contained *ospA* DNA, with copy numbers ranging from 144 to 112,000 copies per mg tissue. Histology of joint and heart tissue of the recipient SCID mice revealed inflammation in all knees and tibiotarsal joints (arthritis) and inflammation in all hearts (carditis) of mice receiving transplants from saline-treated donors, whereas there were no detectable lesions in joints or hearts of SCID mice receiving transplants from mice treated with ceftriaxone or high doses of tigecycline. These results demonstrated that tissues from antibiotic-treated mice contained spirochetes that were capable of infecting SCID mice following transplant of tissues and were capable of dissemination to multiple sites, but they did not induce arthritis or carditis.

DISCUSSION

The current study examined the efficacy of tigecycline, a new first-in-class glycylcycline, which is the most potent antibiotic against *B. burgdorferi* on a weight basis *in vitro*. Tigecycline is a structural analog of minocycline that avoids tetracycline resistance mediated by inhibition of bacterial efflux pumps and ribosomal protection (21). However, using both low and high doses of tigecycline, essentially similar findings of persistence of noncultivable, infectious spirochetes were found, as previously shown with ceftriaxone and doxycycline (13, 26, 27). Thus, the phenomenon of *B. burgdorferi* persistence appears to be universal among these various classes of antibiotics.

Earlier studies on antibiotic treatment of laboratory mice that were based upon culture of tissues as a readout showed effective elimination of infection (44), and this was essentially confirmed with standard PCR assays (41). With the advent of more sensitive real-time PCR, PCR-positive, culture-negative evidence for spirochete persistence after antibiotic treatment has been increasingly recognized, using the mouse model (13, 27, 81). This phenomenon was originally noted in dogs treated with doxycycline or amoxicillin for 30 days, in which joint disease was prevented or cured, but tissues from the majority of dogs remained 23S rRNA or *ospA* PCR positive, yet most tissues were culture negative (70). In a study in which mice were treated with ceftriaxone or doxycycline for 30 days, persistence of noncultivable spirochetes was confirmed by xenodiagnosis, with spirochetes detected in ticks by immunofluorescence and *ospA* PCR, but tick transmission to SCID mice did not take place. It was concluded that antibiotic treatment resulted in spirochete attenuation, with loss of infectivity-associated plasmids (lp25 and lp28-1) (13). That study prompted further investigation in mice treated with ceftriaxone or saline at 3 weeks or 4 months of infection (27). Persisting spirochetes could be detected by PCR but not culture, and infection was verified by xenodiagnosis. However, in contrast to the findings of Bockenstedt et al., ticks transmitted infection to SCID mice, and BBE21.1 (lp25), *arp*, and *vlsE* (lp28-1) gene targets could be amplified from *flaB* DNA-positive tissues, indicating the presence of both plasmids (26, 27). In the present study, tick transmission to SCID mice was confirmed, and spirochetes in the ticks survived molting into the adult tick stage. Furthermore, transmission of infectious spirochetes was shown to occur following transplant of tissues from antibiotic-treated mice into SCID mice. The enhanced success of transplant-related infection in the present study was based upon observations that heart base and joint tissue were the most likely tissues to contain residual spirochetes (26, 27).

Studies that utilized ceftriaxone for treatment of mice with *B. burgdorferi* infection have been challenged because of different pharmacokinetic/pharmacodynamic properties of ceftriaxone in mice compared to humans (79). The treatment regimen used in mice transiently achieves more than adequate serum concentrations, but because the serum half-life is only

1.1 h (75), the regimen does not sustain these serum drug levels. Tigecycline, which has potent MIC and MBC activity, a serum half-life of 11.6 h, and a serum maximum 70 times greater than the *B. burgdorferi* N40 MBC (35), overcomes this argument. Furthermore, the ceftriaxone regimen used in mice still serves as a valid model for the study of persisting *B. burgdorferi*. The current study used the previously published ceftriaxone treatment regimen (27) as a positive-effect control group for comparison with tigecycline.

An interesting observation in the present study was that half of the mice treated with any of the 3 antibiotic regimens at 1 week of infection were cleared of detectable spirochetes in all tissues tested, whereas all of the mice treated with antibiotics at 3 weeks or 4 months remained infected, based upon PCR of one or more tissues. This parallels human clinical experience, in which treatment is generally more effective when administered during early Lyme disease (68, 78). In addition, there were clearly different patterns of tissue infection among mice treated during the preimmune (1 week), early immune (3 weeks), or late (4 months) phases of infection. Tissues were not analyzed for spirochete distribution until 3 months after antibiotic treatment was completed. If spirochetes were actively disseminating after treatment, the different distribution patterns would not have been so apparent. These findings suggest that spirochetes persisted in sites where they encountered the antibiotic.

Using nested RT-PCR, spirochetes in tissues of antibiotic-treated mice were found to transcribe RNA of several selected genes, but patterns of RNA transcription differed from normal spirochetes in saline-treated infected mice. Most notably, none of the samples tested from antibiotic-treated mice indicated transcription of *flaB* (flagellin), which is generally considered to be constitutively expressed by *B. burgdorferi*. The downregulation of genes involved in nonessential functions, such as the flagellin gene, has been noted in persistent forms of *E. coli* following antibiotic exposure (4). In contrast to downregulation of *flaB*, *oppA-2* was the most consistently upregulated gene of the targets tested. OppA-2 belongs to a 5-member family of oligopeptide permease proteins of a single-peptide transport system that is chromosomally encoded in *B. burgdorferi* (42, 76, 77). These genes are differentially expressed as a means of adaptation to the varied environments of culture, resting ticks, feeding ticks, and mammalian host. OppA-2 has been previously shown to be upregulated during mouse infection (77).

Collectively, results from previous studies and the present study indicate persistence of low numbers of noncultivable spirochetes, detected by real-time PCR, following antibiotic treatment. For at least 3 months after cessation of antibiotic, these noncultivable forms can be acquired by ticks (xenodiagnosis), transmitted by ticks, survive the molts between larvae to nymphs to adults, infect recipient mice by tissue transplant, transcribe RNA, and express antigen in ticks and tissues in the form of morphologically identifiable spirochetes (13, 27). The fact that these forms are unable to be cultured does not negate their viability. Studies that have demonstrated equivalent sensitivity of culture to PCR have been based upon limited dilutions of cultured spirochetes (1, 16, 27), thereby favoring cultivable spirochetes. It remains uncertain if noncultivable spirochetes are present during the normal course of persistent infection or if they are induced in response to antibiotic. Despite their persistence, previous studies (13, 27) and the present study indicate that antibiotic treatment results in a significant decline in antibody titers to *B. burgdorferi*, suggesting that the persisting spirochetes remain sequestered or occult to immune surveillance. The significance of antibiotic-persisting *B. burgdorferi* has also been challenged (79), because no disease was evident in SCID mice infected with these organisms. That conclusion is not valid, since inflammation does not necessarily correlate with presence of spirochetes. Inflammation requires invasion of specific tissue types, such as synovium, and no inflammation is found in many other tissues (in fact the majority) infected with *B. burgdorferi* (9). We did not examine specific sites for the presence of *B. burgdorferi* by microscopy or hold the SCID mice for longer intervals to determine if disease would evolve.

The phenomenon of bacterial tolerance (persistence) to antibiotic treatment was originally described by Bigger with penicillin and staphylococcus in 1944 (11). Bacterial tolerance to antibiotics differs from the genetically based (chromosomal or plasmid associated) antibiotic resistance. Tolerance to lethal concentrations of various antibiotics, even after prolonged treatment, has been observed in a wide variety of bacteria and clinical conditions (reviewed in references 12, 30, and 37–39). It is not clear whether these persisting forms of *B. burgdorferi* occur in human patients after treatment or give rise to chronic disease later in life. Further studies are under way in the mouse model to determine if the postantibiotic-persistent organisms return to a cultivable and pathogenic state or if they eventually die out.

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