Distribution of the Lyme Disease Spirochete *Borrelia burgdorferi* in Naturally and Experimentally Infected Western Gray Squirrels (*Sciurus griseus*)

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Abstract

The dynamics of *Borrelia burgdorferi* infections within its natural hosts are poorly understood. We necropsied four wild-caught western gray squirrels (*Sciurus griseus*) that were acquired during a previous study that evaluated the reservoir competence of this rodent for the Lyme disease spirochete. One animal was infected experimentally, whereas the others were infected in the wild before capture. To investigate dissemination of *B. burgdorferi* and concurrent histopathologic lesions in different tissues, blood specimens, synovial and cerebrospinal fluid, ear-punch biopsies, and diverse tissue samples from skin and various organs were taken and examined by culture, polymerase chain reaction, and histology. *Borrelia*-positive cultures were obtained from three of the squirrels, that is, from skin biopsies (7 of 20 samples), ear-punch biopsies (2 of 8), and one (1 of 5) lymph node. Sequencing of amplicons confirmed *B. burgdorferi* sensu stricto (s.s.) infection in 9 of 10 culture-positive samples and in DNA extracted from all 10 positive cultures. The experimentally infected squirrel yielded most of the positive samples. In contrast, bodily fluids, all other organ specimens from these animals, and all samples from one naturally infected squirrel were negative for *Borrelia* for both assays. None of the necropsied squirrels exhibited specific clinical signs associated with *B. burgdorferi*. Similarly, necropsy and histological examination of tissues indicated the presence of underlying infectious processes, none of which could be ascribed conclusively to *B. burgdorferi* infection. Based on these results, obtained from a small number of animals investigated at a single time point, we suggest that *B. burgdorferi* s.s. infection in *S. griseus* may result in rather localized dissemination of spirochetes, and that mild or nonclinical disease might be more common after several months of infection duration. Since spirochetes could be detected in squirrels 7–21 months postinfection, we conclude that *S. griseus* can infect *Ixodes pacificus* ticks with *B. burgdorferi* s.s. *trans*-seasonally.

Key Words: *Borrelia*—Epidemiology—Lyme disease—Rodents—Ticks.

Introduction

RECENT STUDIES PROVIDE STRONG EVIDENCE that the western gray squirrel (*Sciurus griseus*) is a primary reservoir host of the Lyme disease spirochete *Borrelia burgdorferi* in certain types of dense woodlands in northern California. A previous molecular epidemiological survey of 222 western gray squirrels from 15 counties in California revealed an overall prevalence of *B. burgdorferi* infection of 30%, and that this spirochete can be maintained in a squirrel–*Ixodes pacificus* tick–squirrel transmission cycle (Lane et al. 2005, Salkeld et al. 2008).

Little is known about the natural course of *B. burgdorferi* infection in its vertebrate hosts. In northwestern California, studies on naturally and experimentally infected dusky-footed wood rats (*Neotoma fuscipes*), deer mice (*Peromyscus maniculatus*), and Columbian black-tailed deer (*Odocoileus hemionus columbianus*) revealed that animals infected via needle inoculation or tick feeding did not develop clinical manifestations consistent with Lyme disease (Brown and Lane 1994, Lane et al. 1994). Experimental infections of mice with human isolates in the northeastern United States indicated strain differences in fitness, pathogenicity, and ability to disseminate (Wang et al. 2001, 2002, Haninková et al. 2008).

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Here, we made an attempt to study the within-host dynamics of *B. burgdorferi* in infected western gray squirrels. Specifically, we ascertained whether one experimentally and three naturally infected animals exhibited signs of clinical Lyme disease, and investigated the distribution of *B. burgdorferi* in various tissues, using polymerase chain reaction (PCR), culture, and histopathology.

Materials and Methods

Origin of squirrels

Four male western gray squirrels were acquired from a previous study that investigated the role of *S. griseus* as a reservoir host of *B. burgdorferi* (Salkeld et al. 2008). Animals were trapped in oak-woodland sites at the University of California Hopland Research and Extension Center in Mendocino County in May and June 2006 (squirrel #3 and #4), and January and June 2007 (#1 and #2), and housed in an animal facility at the University of California. Three of these animals (#2, #3, and #4) were infected with *B. burgdorferi* sensu stricto (s.s.) at the time of capture, as determined by PCR on ear-punch biopsy (EPB) samples, and were used for tick-xenodiagnostic studies. The minimum duration of infection for these three animals was determined considering that the last date of exposure to infected ticks in the wild could have been shortly before capture. A fourth wild-caught, uninfected squirrel (#1) was infected by exposure to an experimentally *B. burgdorferi* s.s.-infected nymph in the laboratory in August 2007. The infection status of each animal was regularly monitored by PCR testing of EPBs and tick xenodiagnosis over the entire time of captivity (7–22 months).

Due to the difficulties inherent in capturing and housing squirrels, and because of the low numbers of uninfected animals in our primary field-study site, we were unable to include a negative control animal. We originally had one control animal, but it died during captivity.

Use of these animals was reviewed and approved by the Animal Care and Use Committee at the University of California at Berkeley.

Preparation and performance of necropsy

Squirrels were kept in nest boxes and monitored daily by trained animal husbandry technicians for clinical signs of disease. Appetite was monitored generally by observing the level of maintenance chow daily, and whether twice-weekly supplements were consumed within 1 day.

Upon completion of xenodiagnosis experiments, squirrels were euthanized in March 2008 by injecting 100 mg/kg pentobarbital (Nembutal 50 mg/mL; Premier Pharmacy, Weekiwee, FL) intraperitoneally, after light anesthesia with isoflurane-soaked gauze (Isothesia; Abbott Laboratories, North Chicago, IL) placed into an enclosed chamber. If necessary, percutaneous follow-up injections of pentobarbital were administered. The weight of each squirrel was recorded, as well as description of the pelage, orifices, proportions of body parts, extremities, joints, lymph nodes, and general body condition.

Collection and processing of samples

Fur and skin of areas to be biopsied were scrubbed and shaved with 75% providone iodine scrub (Butler Company,

Columbus, OH) and 70% ethanol, and aseptic techniques were used during necropsy. Blood samples were taken by cardiac puncture during the last stage of euthanasia, and one to three drops of whole blood were each inoculated directly into a microcentrifuge tube containing 1.25 mL of BSK II culture medium. Additionally, 300–500 μ L of whole blood was each placed into ethylenediaminetetraacetic acid and serum-collection tubes. Coagulated blood was centrifuged, 20 μ L of the resulting serum was used for culture, and the remaining serum and clots were stored at -80°C until tested by PCR. If accessible, synovial fluid was taken by puncture from scapulohumeral and stifle joints and cerebrospinal fluid (CSF) from the atlanto-occipital joint. Approximately 5 μ L of each puncture was inoculated into 1.25 mL of culture medium. Remaining synovia and CSF were stored at -80°C .

Per squirrel, three sets of tissue samples were taken, each consisting of 2-mm EPBs from both pinna, paired skin-punch biopsies from the axillae and back and one from the tail, 4 mm³ biopsies of the right eye, brain, heart muscle, heart valve, pericardium, liver, intestines, kidney, and urinary bladder, and a 2 mm³ biopsy of spleen. If found, lymph nodes (*Lnn. axillaris* and *Lnn. poplitei*) were resected.

One set of skin and organ biopsies per squirrel was placed individually into 1.5 mL microcentrifuge tubes containing 1.25 mL culture medium, another set was kept on wet ice until freezing at -20°C before DNA extraction, and samples from a third set of biopsies were fixed in 10% formalin and submitted to Northwest ZooPath (Monroe, Washington) for histopathologic examination.

Culture of spirochetes from bodily fluids and tissue biopsies

Samples were cultured in BSK-H medium (Sigma, St. Louis, MO) containing 6% filtered rabbit serum and 25 μ L of rifampicin per milliliter. Cultures were incubated at $\sim 34^{\circ}\text{C}$, and examined weekly for 1 month by dark-field microscopy for the presence of spirochetes at 400 $\times$ magnification. All spirochete-positive cultures were frozen as stocks and recultured for molecular characterization.

DNA extraction of spirochetes from bodily fluids, tissue biopsies, and culture

Clinical specimens were tested individually for the presence of *B. burgdorferi* sensu lato (s.l.). DNA was extracted with the DNeasy Blood and Tissue Kit from recommended amounts of tissues (Qiagen, Valencia, CA). Extracts were prepared from 50 μ L of serum and 50 and 100 μ L of clots and ethylenediaminetetraacetic acid blood from each animal, following the manufacturer's non-nuclear blood sample protocol. DNA was eluted in final volumes of 100 and 200 μ L of elution (AE) buffer, respectively. Likewise, DNA extract of each 10 μ L synovial and CSF was eluted twice with 100 μ L of AE buffer to increase the overall DNA yield. Isolation of DNA from skin and organ biopsies was carried out following the manufacturer's spin-column protocol for animal tissues, with a few modifications. Approximately 10 mg of thawed spleen and 25 mg of all other tissue specimens were triturated with sterile, disposable plastic pestles in 20 μ L tissue lysis (ATL) buffer before adding the remaining 160 μ L of ATL buffer.

Samples were incubated overnight, and DNA was eluted twice in a final volume of 200 μ L.

Frozen stocks of spirochete-positive cultures were thawed and grown in ~5 mL of BSK-H medium, 200 μ L of the cultures were spun down twice at 14,000 g for 5 min, the supernatants were discarded, and the pellets washed with sterile phosphate-buffered saline. After the second supernatant was removed, 200 μ L of phosphate-buffered saline and 20 μ L of Proteinase K were added. Further processing was performed according to the spin-column protocol for animal blood or cells, and DNA extracts were stored at -20°C .

Detection of Borrelia in blood, synovial and CSFs, skin and organ biopsies, and isolates from necropsy

PCR was performed following the methods of Lane et al. (2004). *B. burgdorferi* s.l. infection was detected with a nested PCR that amplifies an approximately 220 bp target within the *rfl* (5S)-*rfl* (23S) rRNA intergenic spacer region. Cultured *B. burgdorferi* s.s. strain CA4 and UV-treated nuclease-free sterile water (Growcells.com, Irvine, CA) were used as positive and negative controls, respectively, during each PCR run. Each sample was tested in duplicates, and possible inhibition of amplification was controlled by testing a subset of PCR-negative samples in 10-fold dilutions and spiking them with 10-fold dilution series of known amounts of cultured spirochete genomic DNA.

Amplicons were purified and sequenced as described previously (Salkeld et al. 2008). All sequenced samples were aligned with *B. burgdorferi* sequences obtained from previous studies on western gray squirrels, representative *rfl*-*rfl* sequences available in GenBank for *B. burgdorferi* s.s., *Borrelia californiensis*, *Borrelia* genomospecies 1 and 2, *Borrelia bissettii*, and unclassified borrelial strains found in North America, using Clustal X multiple sequence alignment program (v1.83.1). The final alignment was edited manually using Mesquite v1.12 and used to generate a neighbor-joining phylogenetic tree using PAUP* 4.0.

Results

Isolation of Borrelia

Spirochetes were observed in cultures from three of four animals, all within 1 week of necropsy (Table 1). *Borreliae* were detected in skin biopsies (7 of 20), EPBs (2 of 8), and a (1 of 5) lymph node (*Lnn. poplitei*). Most positive cultures were obtained from skin samples (five of five) or EPBs (two of two) derived from the experimentally infected squirrel. In contrast, *B. burgdorferi* s.s. was isolated from two of five skin biopsies and one lymph node from two naturally infected squirrels (#4 and #2). Efforts to cultivate spirochetes from blood, synovial fluid, or CSF or from the internal organs of all squirrels were unsuccessful.

Polymerase chain reaction

PCR-positive samples were detected in the three squirrels that also yielded positive cultures. *B. burgdorferi* s.l. could be amplified in DNA extracts from 6 of 20 skin biopsies, 2 of 8 EPBs, and the culture-positive lymph node (Table 1). Most of the PCR-positive samples were detected in the experimentally infected squirrel, including four of five skin biopsies and both EPBs. Within naturally infected squirrels, positive biopsies were obtained from skin of the back and the tail of animal #4, and from a lymph node derived from squirrel #2.

We could not amplify borrelial-DNA in any other samples, and the naturally infected squirrel, #3, was negative in both assays. Additionally, we reconfirmed the presence of *B. burgdorferi* s.s. in DNA extracts from all 10 re-cultured spirochete-positive stocks by PCR. Testing of 10-fold serial dilutions of PCR-negative samples confirmed the negative results, which indicated that there was no inhibition in the PCR.

Sequencing and use of the neighbor-joining method with uncorrected (*p*) distances characterized all amplicons as *B. burgdorferi* s.s. (unpublished data). There were no coinfections with any other genospecies of *B. burgdorferi* s.l.

TABLE 1. CULTURE AND PCR RESULTS FOR DETECTION OF *BORRELIA BURGDORFERI* IN NECROPSY SAMPLES FROM ONE EXPERIMENTALLY AND THREE NATURALLY INFECTED WILD-CAUGHT WESTERN GRAY SQUIRRELS (*SCIURUS GRISEUS*)

Squirrel no.	Route of infection	Minimal duration of infection ^a (months)	Number of positive/number of tested necropsy samples													
			Ear		Skin		Lymph nodes		Blood		Synovia		CSF		Organs ^b	
			Culture	PCR	Culture	PCR	Culture	PCR	Culture	PCR	Culture	PCR	Culture	PCR	Culture	PCR
1	Experimental ^c	7	2/2	2/2	5/5	4/5	0/2	0/2	0/4	0/10	0/2	0/2	0/1	0/1	0/10	0/10
2	Natural	9	0/2	0/2	0/5	0/5	1/1	1/1	0/4	0/10	0/1	0/1	0/1	0/1	0/10	0/10
3	Natural	22	0/2	0/2	0/5	0/5	NA	NA	0/4	0/10	NA	NA	0/1	0/1	0/10	0/10
4	Natural	21	0/2	0/2	2/5	2/5	0/2	0/2	0/4	0/10	0/1	0/1	0/1	0/1	0/10	0/10
Total no.			2/8	2/8	7/20	6/20	1/5	1/5	0/16	0/40	0/4	0/4	0/4	0/4	0/40	0/40

Spirochete-positive cultures were obtained within 1 week, and infection with *B. burgdorferi* sensu stricto was confirmed by PCR for all samples.

^aInfection status and duration was determined by PCR-positive results for xenodiagnostic *Ixodes pacificus* nymphs, and/or squirrel ear punch biopsies. Duration of infection of the experimentally infected animal was known, whereas durations of infections for the naturally infected squirrels are minimal estimates, assuming that the last possible infection date was the day of captivity, being the last estimated exposure to ticks in the wild.

^bBiopsies of eye, brain, pericardium, heart valves, heart muscle, spleen, intestines, liver, kidney, and urinary bladder.

^cVia experimentally infected *I. pacificus* nymphs.

NA, sample was not available; CSF, cerebrospinal fluid; PCR, polymerase chain reaction.

Histopathology

All animals appeared to be in good health. Necropsy and histological examination of formalin-fixed tissues indicated the presence of inflammatory processes due to low-grade parasitism but revealed no abnormalities or lesions that could be attributed to an infection with *B. burgdorferi* s.s. The experimentally infected squirrel (#1) showed slightly increased numbers of lymphocytes and plasma cells in the lamina propria of the intestines, mild hyperplasia of histiocytes around the ellipsoids in the spleen, mild focal lymphocytic inflammation in the epicardium, and mild hyperplasia in one lymph node. Histological changes in tissues from naturally infected squirrel #2 included mild hyperplasia of ellipsoids in the spleen, mild portal lymphocytic hepatitis, low-grade mineralization of the renal tubules, mild hyperplasia and hemosiderosis in one lymph node, and mild perivascular lymphocytic dermatitis. An encysted cestode larva was found in two sections of liver from squirrel #3, combined with minimal lymphocytic hepatitis, mild chronic enteritis, and mild lymphocytic tracheitis and conjunctivitis. Naturally infected squirrel #4 provided the best evidence for a primary systemic infection. Perinodular fibrin disposition and necrosis with reactive ellipsoids were found in the spleen, and periportal lymphocytic hepatitis, mild chronic enteritis, mild lymphocytic interstitial nephritis with tubular necrosis and mesangioproliferative glomerulopathy, mild focal lymphocytic pericarditis, moderate bronchointerstitial pneumonia, mild lymphocytic synovitis, mild perivascular lymphocytic dermatitis, and mild plasmacytic lymphadenitis were observed in this animal. Nevertheless, Warthin–Starry stains were negative for bacterial infection, and the immunohistochemistry results also were negative for *B. burgdorferi* s.s. infection.

Discussion

Our findings suggest that *B. burgdorferi* s.s. infection in *S. griseus* is rather localized since dissemination of spirochetes was mainly confined to cutaneous tissues. No clinical signs were observed during health checks that might have been associated with borreliar infection such as lameness, arthritis, lethargy, or depression. Similar results were documented earlier for naturally and experimentally *B. burgdorferi* s.s.-infected dusky-footed wood rats and deer mice, two other hosts of *B. burgdorferi* s.l. in California (Brown and Lane 1994). *Borreliae* were detected more often in cultures of EPBs of woodrats (10 of 11) and deer mice (4 of 4) than in skin biopsies (3 of 36) or blood (1 of 36) from asymptomatic woodrats, and were absent in internal organs.

Our inability to detect *B. burgdorferi* in internal organs after a minimum of 7 months postinfection may lead to the assumption that this spirochete accumulates in skin-associated tissues in the western gray squirrel. Such dermatotropism could facilitate transmission to an attached *I. pacificus* immature. Variations in the level of expression of immunodominant antigens from different *B. burgdorferi* s.l. genotypes in host tissues might be responsible for tissue tropisms, since some gene products, such as decorin binding proteins, are associated with tissue adhesion and dissemination of *Borrelia* in the host (Guo et al. 1998, Hodzic et al. 2003, Coburn et al. 2005). On the other hand, we only examined tissues from a small number of animals at only one single time point, and therefore

have to consider that infection of internal body organs could have occurred earlier and been cleared upon the day of necropsy.

Since we can exclude inhibition of PCR, another possible explanation for the lack of spirochetal DNA in bodily fluids or tissues besides skin might be the presence of DNA in quantities below the sensitivity limit of our assay. In the current study PCR and culture seemed to have a similar diagnostic sensitivity for detecting spirochetes, with 9 PCR-positive versus 10 culture-positive samples, but sensitivities of both assays can vary depending upon the type of clinical sample (Lebech et al. 1995, Aguero-Rosenfeld et al. 2005).

Serological monitoring also might be considered to further investigate the course of *B. burgdorferi* infection in western gray squirrels, as it was done for Columbian black-tailed deer (Lane et al. 1994). A survey of *B. burgdorferi* infection in wild *Peromyscus leucopus* mice in Connecticut found that 75% of 801 samples collected from 514 field mice had seroconverted, and that re-infections of mice occurred during the transmission seasons (Bunikis et al. 2004). Likewise, our examined naturally infected squirrels may have been exposed more than once to infection with different *B. burgdorferi* strains before capture. If so, production of specific antibodies might have been responsible for the apparent absence of clinical Lyme disease.

The finding that most of the positive results were derived from the one experimentally infected animal might be explained by the fact that it harbored a more recent infection of 7 months' duration than the other three animals that were infected for a minimum of 9–21 months. One of the naturally infected animals (#3) had apparently become free from infection after 9 months in captivity. Initially, this squirrel was repeatedly PCR positive in the past and capable of transmitting *B. burgdorferi* s.s. infection to ticks, including those that were used for the transmission experiment (Salkeld et al. 2008). Nonetheless, *B. burgdorferi* s.s. infection in western gray squirrels can be long lasting. Host parasite interactions might vary among host populations. Previous studies on other potential Californian reservoir hosts as dusky-footed wood rats and California kangaroo rats documented that rodents remained infectious for ticks for up to 15 months and 5 years in captivity, respectively (Brown and Lane 1992, Lane et al. 1999). Duration of *B. burgdorferi* s.s. infections in other primary reservoir hosts, such as white-footed mice, does not appear to persist for more than a year, although this may be because investigations have not been carried out for longer periods (Donahue et al. 1987, Wang et al. 2001, Haninková et al. 2008).

In the present study, histopathological changes in the tissues of squirrels were generally mild and indicated a low level of chronic, systemic antigenic stimulation. Given the non-specific nature of these changes and the isolated cestode infestation in squirrel #3, such findings may have been due to parasitism of undetermined etiology. Nevertheless, we cannot exclude a *B. burgdorferi* s.s. infection as a cause of these observations. In another study, Brown and Lane (1994) observed moderate nonsuppurative infiltrates in different organs examined from *B. burgdorferi*-infected woodrats. Minimal lesions in these animals included synovitis, myocarditis, myositis, and interstitial nephritis, whereas none of the woodrats showed any clinical signs that might have been associated with Lyme disease.

The squirrels also might have been exposed to *B. burgdorferi* s.s. strains or genotypes of low invasiveness. Considerable heterogeneity exists among *B. burgdorferi* s.s. in the United States (Wang et al. 2002, Brown et al. 2006, Postic et al. 2007), and genotypic differences in the infecting spirochetes appear to play an important role in the transmission efficiencies to vector ticks (Derdáková et al. 2004) and to the pathogenesis and development of clinical disease in the vertebrate host. Wang et al. (2001, 2002) reported that spirochete dissemination and the resultant burdens in skin, heart, and joints in experimentally infected C3H/HeJ mice depended upon the infecting *B. burgdorferi* s.s. strains, and were correlated with the severity of disease in these rodents. Other studies have demonstrated that immune competence regulates spirochete numbers in the host (Hodzic et al. 2003) and that intrinsic fitness of infecting *B. burgdorferi* isolates may vary by host species (Haninková et al. 2008). The innate immune system of the vertebrate host may impact the selection of certain *Borrelia* strains or genotypes. In that regard, *B. burgdorferi* strains differ in their ability to bind complement factors, which can result in resistance to host complement factors (Kurtenbach et al. 1998, 2002, Stevenson et al. 2002).

Sequencing of positive amplicons acquired during the present study and a subset of those derived from western gray squirrels from our previous molecular epidemiological survey of *Borrelia* infection in *S. griseus* (Salkeld et al. 2008) indicate that this squirrel is infected mainly with *B. burgdorferi* s.s. Further, preliminary genotyping results of these *B. burgdorferi* s.s.-positive samples indicate that the most prevalent *ospC* sequence types found are genetically similar to those detected in the majority of *I. pacificus* nymphs from Mendocino County (unpublished data), an area in California where Lyme disease is highly endemic (Lane et al. 2005, Salkeld et al. 2008).

Conclusion

The fact that viable spirochetes and borrelial DNA were isolated from one experimentally and two naturally infected necropsied squirrels 7–21 months postinfection demonstrates that *S. griseus* can maintain *B. burgdorferi* s.s. interannually and therefore infect *I. pacificus* immature *trans*-seasonally. These findings, in conjunction with the occurrence of highly prevalent *B. burgdorferi* s.s. infections in western gray squirrels in Lyme disease–endemic areas (Lane et al. 2005, Salkeld et al. 2008), reconfirm that *S. griseus* is indeed a primary reservoir host of *B. burgdorferi* s.s. in certain types of woodlands, and that this rodent might be a valuable sentinel animal for detecting the presence of *B. burgdorferi* s.s. in the far-western United States.

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Disclaimer

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Disclosure Statement

No competing financial interests exist.

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