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EXPERIMENTAL INFECTION OF COLUMBIAN BLACK-TAILED DEER WITH THE LYME DISEASE SPIROCHETE

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ABSTRACT: The course of *Borrelia burgdorferi*-infection in Columbian black-tailed deer (*Odocoileus hemionus columbianus*), its effect on the health of these animals, and their reservoir competence for fleas were evaluated experimentally. Four yearling females inoculated intramuscularly with 10^8 organisms of the CA4 strain of *B. burgdorferi*, and two yearling males unexposed to spirochetes, were monitored daily for 3 mo. Spirochetes were reisolated from the blood of three does at 14 or 70 days postinjection, and from several tissues of the fourth doe at necropsy. Considerable antigenic heterogeneity was observed among the reisolates as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Only two of the four infected deer developed significant antibodies ($\geq$1:128) to *B. burgdorferi* with titers persisting for $\leq$2 mo. Hematological values were highly variable and the degree of variation observed was much greater than that reported previously for Columbian black-tailed deer or other subspecies of mule deer. Infected deer did not manifest signs of Lyme disease. On histologic examination of eight tissues per deer, we observed a minimal hepatic lesion in all animals exposed to *B. burgdorferi*. No spirochetes were detected in 367 fleas (*Pulex irritans*) that had naturally infested these deer; thus this flea probably is an inefficient host of *B. burgdorferi*.

Key words: Lyme disease, spirochetes, deer, fleas, ticks, *Odocoileus hemionus columbianus*.

INTRODUCTION

The Columbian black-tailed deer (BTD), *Odocoileus hemionus columbianus*, is one of several subspecies of mule deer that occur in California (Hall, 1981); its geographic distribution overlaps that of reported cases of Lyme disease. It also is a significant host of the western black-legged tick, *Ixodes pacificus* (Lane et al., 1981; Furman and Loomis, 1984; Westrom et al., 1985), the primary vector of the Lyme disease spirochete (*Borrelia burgdorferi*) to humans in the far-western United States (Naversen and Gardner, 1978; Burgdorfer et al., 1985; Lane and Lavoie, 1988). Moreover, spirochetes and antibodies to *B. burgdorferi* were detected in 27% and 38%, respectively, of BTD in northern California (USA) (Lane and Burgdorfer, 1986). The high prevalence of spirochetemia in BTD, coupled with abundant feeding on this host by *I. pacificus* and other hematophagous arthropods such as fleas, indicated that the BTD is an important host of spirochetes and may serve as a reservoir of spirochetal infection.

The course of *B. burgdorferi* infection, pathologic effects attributable to such infection, and the reservoir competence of BTD for the Lyme disease spirochete have not been determined before. Our objective was to investigate the route of spirochetal infection in experimentally inoculated BTD as well as the clinical and histologic effects of this spirochete on susceptible deer. Simultaneously, we evaluated the ability of the flea *Pulex irritans* to acquire spirochetes while feeding on these deer.

MATERIALS AND METHODS

Six BTD orphaned fawns (four females, two males) provided by the California (USA) State Department of Fish and Game were raised to yearlings; five of the animals were from Sonoma County and the sixth was from El Dorado County. None of these deer had a history of exposure to ticks known to transmit the Lyme disease spirochete. During the study, the yearlings were held in separate pens having areas of 60.2 m^2 with a height of 2.44 m. The pens were constructed of chain-link fencing covered exteriorly with a knitted shadecloth (70%) made from polyethylene and having a diameter of 1.5 mm to exclude all but the smallest hematophagous arthropods. Deer were maintained on a diet of alfalfa pellets, hay, and a mixture of corn, oats and barley. Water was provided *ad libitum*.

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Their body weights were recorded at the start and end of the experiment. The research was approved by the institutional Animal Care and Use Committee as conforming to all applicable animal welfare laws, regulations, and guidelines.

The four females (54 to 57) were inoculated intramuscularly (IM) with 10^8 organisms of a low passage (P-3) uncloned strain of *B. burgdorferi* in 0.5 ml of BSK II medium (Barbour, 1984). The 10^8 spirochetal dosage was chosen because inocula of about 10^7 or 10^8 spirochetes have been used routinely to infect research animals irrespective of their body weight (Johnson et al., 1984; Schwan et al., 1988; Lane, 1990). This strain, isolate CA4, was derived from an adult *I. pacificus* collected in Sonoma County, California. It was chosen because it had been characterized phenotypically and genetically (Lane and Pascocello, 1989; LeFebvre et al., 1990), and was demonstrated to be infective for deer mice, *Peromyscus maniculatus*, and *I. pacificus* (Lane et al., 1994).

Two sets of controls were employed. As controls for the inocula, both yearling males (58, 9481) were injected IM with 0.5 ml of spirochete-free BSK II medium. As controls for the infectivity of *B. burgdorferi* isolate CA4, four female New Zealand white rabbits were injected IM with 10^8 spirochetes. Rabbits were injected with the same inoculum used to infect deer. Thirty days post-injection, the rabbits were sedated with innovar (0.6 ml, Janssen Pharmaceutica, Piscataway, New Jersey, USA) administered subcutaneously and one 2-mm punch biopsy was taken from the center of each ear and put into 1-ml of BSK II medium. Thirty-four days post-injection, after sedation with innovar, whole blood was collected from each rabbit for serologic assay. One drop of whole blood from each rabbit also was placed into 1 ml of BSK II medium for isolation attempts. All cultures were held at 34.5 C and examined for the presence of spirochetes for 4 to 5 wk by darkfield microscopy.

All deer and rabbits were bled prior to inoculation as well as afterward, and their sera harvested by centrifugation (about 350 × *g* for 20 min.). Pre- and postinoculation serum specimens were tested for antibodies to *B. burgdorferi* by indirect immunofluorescence using the B31 strain as antigen (Lane and Burgdorfer, 1986; Lane and Manweiler, 1988). The cutoff titer for the deer assay was 1:128. It was determined prior to inoculation by bleeding all six deer on two occasions (except for deer 9481, which was bled only once) several months apart and ascertaining the lowest serum titer below which 10 of 11 of these control sera would be considered negative. The cutoff titer for the rabbit assay was determined similarly (Lane and Manweiler, 1988). Deer were chemically immobilized with a combination of ketamine (5 to 12 mg/kg; Aveco Co., Fort Dodge, Iowa, USA) and xylazine (1 to 2 mg/kg; Mobay Corp., Shawnee, Kansas, USA) administered IM to effect once each week for 6 wk and biweekly thereafter for another 6 wk. Prior to handling, the front and hind legs of deer were hobbled and the head was protected with a padded drape. Immobilized animals were bled via the jugular or large leg veins on all nine dates to obtain 5 to 10 ml of blood for isolation of spirochetes, complete blood cell counts, preparation of two thin blood films and two thick drops, and serologic evaluation as described by Lane and Burgdorfer (1986) and Lane and Brown (1991).

The complete blood counts and histopathologic analyses were performed by a commercial laboratory (Consolidated Veterinary Diagnostics, West Sacramento, California, USA). Blood was analyzed with a hematology cell counter (Series 9000, Serano-Baker, Allentown, Pennsylvania, USA). Hematological variables evaluated included total and differential leukocyte count, hematocrit, hemoglobin, and erythrocyte count and morphology.

To determine if deer exposed to spirochetes become arthritic within 3 mo, the diameters of the carpus of each foreleg and the hock and stifle of each hindleg were measured biweekly. Also, the measures for the hock and stifle were summed separately for both hindlegs as two more potential indicators of arthritis. The two groups could not be compared statistically with any degree of confidence because of the small number of experimentally inoculated deer and control deer. Instead, 48 separate regressions were performed (Zar, 1984), eight per day with date ($n = 6$) serving as the independent variable in each regression. The regression coefficient (estimated slope), sign of the coefficient, and significance value of the coefficient (P-value) were evaluated.

Following euthanasia with an intravenous injection of pentobarbitol sodium (Anthony Products, Arcadia, California, USA), gross necropsies were performed on all four treatment females and one of the two control males. The remaining control male was retained for use in a study to determine the ability of BTD to infect xenodiagnostic ticks. Specimens of the following eight tissues were preserved in buffered formalin and submitted for histopathologic evaluation: brain, eye, heart, kidney, liver, spleen, synovium, and urinary bladder. The techniques and stains of Luna (1968) were used in the histopathologic analyses. Except for the eye, pieces of the same tissues and about 1 ml of urine were put separately into BSK II medium in isolation attempts

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following the procedures of Lane (1990). In addition, about 0.7 to 1.0 ml of the aqueous humor of the eye and one drop of whole blood were injected individually into BSK II. Cultures were incubated for 3 wk at 34.5 C and examined for spirochetes weekly thereafter for 1 mo by dark-field microscopy. Rabbits were not necropsied because they were employed solely as positive controls for the spirochetal inoculum.

The protein profiles of four reisolates (passage 3 or 4) from the blood of deer 54 and 57, and from the kidney and synovium of deer 55, were compared with the profile of the original inoculum (passage 3) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Lane and Pascocello, 1989).

Since all six deer were infested naturally with the flea *Pulex irritans*, we determined if this flea could acquire spirochetes while feeding on experimentally inoculated deer. The precise origins of their infestations were unknown but deer presumably acquired fleas from one or more of the does that birthed them and potentially from each other during captivity. Fleas were collected weekly from each animal (including the two controls) five times beginning one week postinjection (PI). As many fleas as possible were aspirated from each deer during about 5 min with a non-commercial, battery-operated portable suction device. Fleas were transported alive to the laboratory where they were examined for the presence of spirochetes. An average of seven fleas from each deer was preserved in 70% ethanol as voucher specimens and sent to Harold J. Egoscue, Grantsville, Utah (USA), for specific determination. Live fleas were tested for spirochetes by direct immunofluorescent examination of whole body smears using a polyvalent conjugate (Lane and Loye, 1989). We used polyclonal antibodies instead of a monoclonal antibody because the former stains all borreliae and direct immunofluorescence involves one less step than indirect immunofluorescence. Also, test-specificity was not a concern in the present study since all deer were not exposed previously to spirochetes.

RESULTS

None of the four deer experimentally inoculated with *B. burgdorferi* had signs associated with Lyme disease, such as inappetence, arthritis, lameness, lethargy or depression. The 48 regression analyses involving six leg joints revealed no arthritis in either the treatment or control animals. Further, the baseline weights ($\bar{x} \pm$ SD) of the four treatment does (29.0 ± 2.8 kg) increased a mean of 3.2 kg (11%), whereas those of the two control bucks (32.2 ± 3.2 kg) increased a mean of 5.0 kg (16%) within 3 mo.

Borrelia burgdorferi was reisolated from the blood of three of the four treatment does either 14 (deer 54 and 57) or 70 days (deer 56) postinoculation (PI), but spirochetes were not observed in paired thick drops prepared biweekly. Blood obtained on the same schedule from both control deer did not yield *B. burgdorferi* in culture or on paired thick drops.

Spirochetes were reisolated from three of the four New Zealand white rabbits that had been inoculated with isolate CA4 as positive controls for the inocula, and antibodies to *B. burgdorferi* were detected in all four rabbits at titers ranging between 1:256 and 1:512. Prior to injection, none of the rabbits had detectable antibodies to *B. burgdorferi* at a dilution of 1:64.

Five deer did not have preexposure serum antibody titers to *B. burgdorferi* at dilutions ≥1:64; the sixth animal (deer 56) had a detectable titer of 1:64 on just one of two preexposure blood-collection dates. Based on repeated testing of serum specimens on separate dates, the within-test variation was ≤two-fold. Two of the four females developed elevated titers of 1:128 or 1:256 within 4 wk PI (Fig. 1). The titer of female 56 increased to 1:256 by day 28 and decreased to 1:64 by day 70 PI. The titer of female 57 rose slightly to 1:64 by day 14, peaked at 1:128 on day 28, and diminished to <1:64 by day 56. Deer 54 and 55 remained seronegative as did both control animals.

When we compared the preexposure value of blood collected immediately before inoculation to the mean (range) of six postexposure values for 12 hematological variables, there were no clear-cut patterns over time; deer 56 had a reduction in the white blood cell count from 11.0 to 5.3 (3.8 to 8.6) × 10^3 cells/ml postexposure. Three of the four treatment deer had elevated segmented neutrophils (61 to 85%) on one or two occasions 2 to 6 wk postex-

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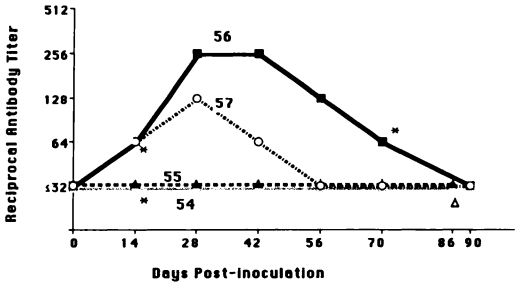


FIGURE 1. Antibody profiles of four yearling female black-tailed deer experimentally inoculated with the CA4 strain of *Borrelia burgdorferi*. An asterisk (*) indicates the day postinoculation (PI) when spirochetes were reisolated from the blood of deer 54, 56, and 57; the open triangle represents the day PI when spirochetes were reisolated from various tissues of deer 55 at necropsy.

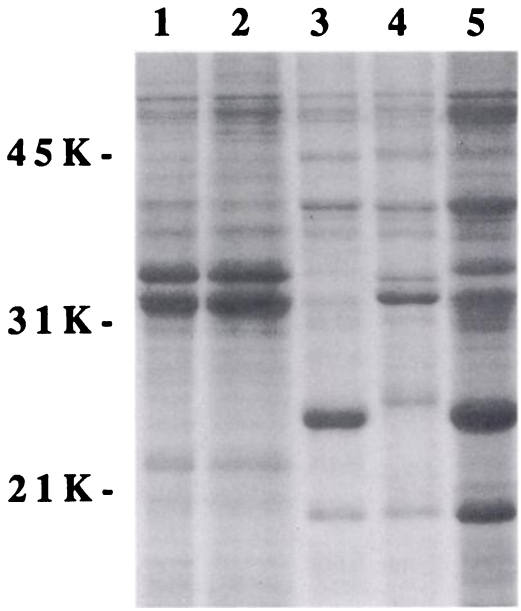


FIGURE 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis Coomassie blue-stained proteins of whole-cell lysates of *Borrelia burgdorferi* isolate CA4 from *Ixodes pacificus* (lane 5) and reisolates of CA4 following inoculation into Columbian black-tailed deer (lanes 1 to 4). Lanes: 1, CA4 from synovium of deer 55; 2, CA4 from kidney of deer 55; 3, CA4 from blood of deer 57; 4, CA4 from blood of deer 54. Prior to inoculation and following reisolation, CA4 was grown in BSK II medium. Passage levels of spirochetes were P-3 or P-4. The approximate positions of the low-molecular-weight protein standards (10^3) are shown to the left of the first lane.

posure. Control deer 9481 had a consistent eosinophilia ($\bar{x} \pm$ SD, $22 \pm 7.7 \times 10^3$ cells/ml) of unknown cause.

Based on gross necropsies and histological examination of eight tissues from each deer, no abnormalities or lesions could be ascribed conclusively to Lyme disease. Minimal hepatic lesions were observed in the four treatment deer, but not in the one control deer (58) that was necropsied. Mild synovial changes and splenic lesions occurred in all necropsied deer. Multifocal minimal myofiber degeneration was recognized in the heart of deer 54.

Viable spirochetes were reisolated from the brain, liver, kidney and synovium of deer 55 on day 86 PI. No spirochetes were recovered from, or detected in, tissues cultured from the other deer at necropsy.

Low passage (P-3) CA4 spirochetes that had been inoculated into all four treatment deer and into the rabbit controls had abundant polypeptides with apparent sizes of 18, 25, 33, 35 and 41 kilodaltons (kDa) (Fig. 2, lane 5). In contrast, spirochetes reisolated from the blood of two deer 2 wk PI (lanes 3, 4) or from the synovium and right kidney of another deer 12 wk PI (lanes 1, 2) either lacked or possessed lighter bands for three of these five polypeptides. These included the 18, 25 and 41 kDa bands for two tissue-reisolates (lanes 1, 2), and the 18, 33 and 35 kDa bands for one (lane 3), and the 18, 25 and 35 kDa bands for the other, blood-reisolate (lane 4). Spirochetes reisolated from synovium and kidney had more abundant 33 and 35 kDa bands than the spirochetal inoculum (lanes 1, 2, 5). A polypeptide with a size of 22 kDa was present in two reisolates (lanes 1, 2) but not in the inoculum (lane 5). Also, one of the reisolates from blood (lane 4) had light bands with sizes of 26 and 34 kDa.

Spirochetes could not be detected in whole body smears prepared from 478 *Pulex irritans* fleas collected from all six deer. This total included 367 fleas from the four treatment does and 111 fleas from the two control bucks. Twenty-three fleas were ob-

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tained from deer 54 and 57 on the same date (day 14 PI) they were spirochetemic.

DISCUSSION

Based on the generally benign results of the hemogram, histologic examination, necropsies, and the absence of clinical signs, isolate CA4 had no detectable pathologic effect on BTD ≤3 mo postexposure. The absence of arthritis may reflect the short observational period. Dogs exposed to *B. burgdorferi*-infected ticks that subsequently experienced lameness did so between 2 and 5 mo postexposure (Appel et al., 1993). In humans, chronic arthritis, which usually involves the knees, typically develops in certain Lyme disease patients ≥1 yr after initial infection (Steere, 1993). The cause of minimal hepatic lesions in the four treatment deer was undetermined, but it may be related to *B. burgdorferi*-infection since a hepatic lesion was not recognized in the only control deer (58) necropsied. Mild synovial lesions present in all deer may be related to trauma induced by frequent handling. Splenic lesions seen in all animals probably were associated with anesthesia or euthanasia, and the multifocal minimal myofiber degeneration seen in deer 54 probably was an incidental finding (R. E. Schmidt, pers. comm.). Lesions were not seen in the brain, eye, kidney, or urinary bladder. In the only other study in which deer were evaluated histologically for *B. burgdorferi* infection, neural lesions were not observed in 42 white-tailed deer infested naturally with the vector tick, *Ixodes dammini* (now *Ixodes scapularis*) in an area hyperendemic for Lyme disease (Levine et al., 1987). The infection status of these deer was undetermined, however.

Hematological values recorded here for BTD varied more than values reported previously for either BTD or Rocky Mountain mule deer (*Odocoileus hemionus hemionus*) (Browman and Sears, 1955; Cowan and Bandy, 1969; Anderson et al., 1970). For example, the pretreatment values for the white blood cell counts (5.6 to 11.0 × 10^3) and the basophils (2 to 5%) were higher in four of the deer than in the BTD or mule deer surveyed earlier. The source of this variability is unknown, though apart from technical variation, factors that play the greatest role in creating such variation are disease, nutrition, excitement, and type of chemical restraint (Kitchen, 1986). The captive deer we evaluated were not tame except for deer 9481; all others were excitable, especially while being chemically immobilized, which may account in large part for the significant variation in the hemogram.

Our experiment needs to be repeated with other isolates of *B. burgdorferi* and particularly with spirochetes injected into deer by tick feeding. White mice infected with *B. burgdorferi* by tick feeding were about 0.2 to 4.5 times more infectious to xenodiagnostic *Ixodes scapularis* ticks than mice injected intradermally with cultured spirochetes (Piesman, 1993). Further, dogs inoculated with cultured *B. burgdorferi* only developed a transient humoral response and never manifested clinical Lyme disease (Appel et al., 1993). We chose to infect BTD by intramuscular injection of low passage, cultured spirochetes rather than by tick-bite because initial attempts to experimentally infect *I. pacificus* larvae with *B. burgdorferi* produced unexpectedly low infection rates (Lane et al., 1994).

Because three of the four treatment does developed short-lived (<1 week) spirochetemias, we believe that BTD occasionally may serve as a source of spirochetal infection for hematophagous arthropods, especially slow-feeding ixodid ticks. In earlier surveys in northern California, 27% of BTD and 50 to 56% of exotic axis deer (*Cervus axis*) and fallow deer (*Cervus dama*) were spirochetemic in late fall to winter (Lane and Burgdorfer, 1986). Although these findings establish unequivocally that deer are important hosts of *B. burgdorferi* or related spirochetes, controlled experiments still are needed to evaluate the relative contribution of deer to

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spirochetal infection in *I. pacificus*. Upon completion of the 3-mo trial, we conducted a feasibility trial with deer 9481 to determine its ability to serve as a source of spirochetal infection for xenodiagnostic ticks. Following IM injection with 10^8 spirochetes of *B. burgdorferi* isolate CA4, approximately 600 *I. pacificus* larvae from an uninfected laboratory colony were put on this animal over a 3-wk period. All 38 replete *I. pacificus* larvae that were recovered tested negative for *B. burgdorferi* DNA with the polymerase chain reaction (PCR) following the methods of Persing et al. (1990).

Even if BTD should prove to be a competent experimental reservoir of *B. burgdorferi*, the significance of this cervid in the enzootiology of Lyme borreliosis may be minimal, at least in areas where western fence lizards (*Sceloporus occidentalis*) are abundant. In such areas, BTD are infested infrequently with subadult *I. pacificus* (Westrom et al., 1985) in contradistinction to lizards which harbor numerous larval and nymphal *I. pacificus* (Manweiler et al., 1992). Indeed, only about 5% of *I. pacificus* ticks collected from 71 BTD in all seasons were subadults (Westrom et al., 1985).

The role of white-tailed deer (*Odocoileus virginianus*) in the enzootiology of *B. burgdorferi* in eastern North America also warrants further study (Lane et al., 1991; Oliver et al., 1992). Unidentified spirochetes were isolated from, or detected in, the blood of *O. virginianus* (Bosler et al., 1983, 1984), and ≤23% of xenodiagnostic *I. dammini* subadults fed on white-tailed deer experimentally inoculated with *B. burgdorferi* were positive by PCR (Oliver et al., 1992). Nevertheless, white-tailed deer and several European species of wild ungulates such as roe deer (*Capreolus capreolus*) and red deer (*Cervus elaphus*) appear to be incompetent reservoirs of *B. burgdorferi* (Telford et al., 1988; Jaenson and Tälleklint, 1992; Matuschka et al., 1993). In fact, spirochete-infected *Ixodes ricinus* ticks that feed on wild ungulates even appear to lose their spirochetal infections (Matuschka et al., 1993).

The occurrence of a natural infestation of *Pulex irritans* on all BTD afforded us a unique opportunity to evaluate the ability of this flea to acquire *B. burgdorferi*. *Pulex irritans* is frequently found on BTD in the far-western United States where it has been confused with the morphologically similar *Pulex simulans* (Lewis et al., 1988). Based on our inability to detect spirochetes in 367 *P. irritans* removed from infected BTD, we believe that this flea may be an inefficient host of *B. burgdorferi*. We did not determine what percentage of the fleas tested were replete, but since body fleas normally feed intermittently and frequently, many of them presumably had a blood meal within a few hours before collection. Elsewhere in North America, spirochetes have been infrequently isolated from or detected in the cat flea, *Ctenocephalides felis*, from Texas (Rawlings, 1986; Teltow et al., 1991) and in the rodent flea, *Orchopeas leucopus*, in Connecticut (USA) and in Ontario, Canada (Anderson and Magnarelli, 1984; Lindsay et al., 1991). Based on these findings, we propose that fleas generally are not efficient vectors of *B. burgdorferi*.

On comparing the protein profiles (SDS-PAGE) of the original low passage (P-3) inoculum of isolate CA4 with reisolates (P-3 or P-4) obtained from the blood of two deer and from the right kidney and synovium of a third, we observed changes in polypeptides of apparent sizes ranging from 18 to 41 kDa. The variable 33 and 35 kDa polypeptides represent outer surface proteins A (OspA) and B (OspB) as confirmed with monoclonal antibodies by immunoblotting (Lane and Pascocello, 1989). Several explanations may account for these differences. First, CA4 is an uncloned isolate, and therefore may consist of two or more spirochetal strains. Second, *B. burgdorferi* may undergo antigenic changes *in vivo* following introduction into deer as was demonstrated for the white-footed mouse, *Peromyscus leucopus* (Schwan and

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Simpson, 1991). Third, the reisolates may have experienced antigenic changes as a result of recultivation and further short-term passage in BSK II medium.

Finally, it is notable that among the four spirochete-infected deer, only two animals produced significant serum antibody titers (≥1:128), and these titers vanished in about 1 to 2 mo. These findings may have important implications for the interpretation of serologic surveys involving deer, as they are evidence that antibody prevalence studies may actually underestimate exposure of deer to Lyme disease spirochetes. In Europe, many ungulates exposed to feeding by numerous infected *Ixodes ricinus* ticks also do not develop a detectable humoral response to this spirochete (Matuschka et al., 1993). On the other hand, immunologic studies with needle-inoculated wildlife must be interpreted cautiously since the antibody response of animals exposed to cultured spirochetes may differ from that of animals exposed to infected ticks (Appel et al., 1993). We conclude that, whenever possible, vector ecologists conducting reservoir competence studies should infect vertebrates with spirochetes administered naturally via feeding ticks.

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