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EXPERIMENTAL INFECTION OF THE RACCOON (*PROCYON LOTOR*) WITH *BORRELIA BURGDORFERI*

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ABSTRACT: The reservoir competence of the raccoon (*Procyon lotor*) for the Lyme disease spirochete (*Borrelia burgdorferi*) was evaluated in the laboratory during September 1991 to April 1993. Five raccoons were exposed to spirochete-infected (JD1 and Wisconsin 210 Wise strains) *Ixodes scapularis* nymphs (20/raccoon). A second feeding of spirochete-infected (Wisconsin 210 Wise strain) nymphs (20/raccoon) was performed with four of the original raccoons. Xenodiagnosis with cohorts of *I. scapularis* larvae (300/cohort) or nymphs (150/cohort) that were periodically placed on each animal was used to detect infection. We examined 1943 engorged ticks by a indirect immunofluorescence monoclonal antibody assay, but no spirochetes were detected. After exposure to spirochete-infected ticks, blood samples were collected at approximately weekly intervals and ear-skin biopsy samples were taken from each animal every third week. These tissues were placed in Barbour-Stoenner-Kelly media. Spirochetes were isolated in cultures of skin (wk 3, 5, 9, 81, and 83) and blood (wk 5, 8, 9, 11, and 12) of one raccoon and the skin (wk 28 and 31) of another raccoon. Antibody response of each animal was monitored through enzyme-linked immunosorbent assays and immunoblotting of blood serum against *B. burgdorferi* proteins. Except for one animal, raccoons did not have an antibody response until they were fed upon by a second cohort of infected *I. scapularis* nymphs. Based on Western blot analyses, raccoons exposed to *B. burgdorferi* via tick bite responded to the 31- (OspA) and 34-KDa (OspB) antigens. Response to other antigens varied among raccoons. Based on our results raccoons may be inefficient reservoirs for *B. burgdorferi*. Although some raccoons can become infected with *B. burgdorferi*, they may not transfer the infection to attached ticks.

Key words: Raccoons, *Procyon lotor*, *Borrelia burgdorferi*, Lyme disease, *Ixodes scapularis*.

INTRODUCTION

A wide range of vertebrates serve as hosts for *Ixodes scapularis*, the principal vector of the Lyme disease spirochete (*Borrelia burgdorferi*) along the Atlantic coast of the United States. Adult *I. scapularis* have been found on numerous mammalian species, but in areas of the northeastern U.S., where Lyme disease is enzootic, white-tailed deer (*Odocoileus virginianus*) are the primary host for adult ticks (Main et al., 1981). Due to their role as hosts for adults, the distribution of *I. scapularis* is closely correlated with the distribution of white-tailed deer (Spielman et al., 1985). However, the deer apparently are not competent reservoirs of *Borrelia burgdorferi*, the etiologic agent of Lyme disease (Telford et al., 1988). Immature *I. scapularis* have been removed from more than 70 host species (Lane et al., 1991) but white-footed mice (*Peromyscus leucopus*) are the primary hosts for larvae and nymphs in the northeast (Piesman and Spielman, 1979). *Borrelia burgdorferi* is transferred efficiently to immature ticks that attach to infected white-footed mice and these abundant rodents serve as reservoirs of the spirochete in the northeastern U.S. The relative importance of *Peromyscus* spp. as a host species for *I. scapularis* may vary between seasons (Mannelli et al., 1993) and in different localities. When white-footed mouse populations are low in some habitats, chipmunks (*Tamias striatus*) and other animals may substitute for *P. leucopus* as hosts for *I. scapularis* and serve as reservoirs of *B. burgdorferi* (Mannelli et al., 1993).

Efforts to reduce local transmission of the Lyme disease spirochete have focused on reduction of *I. scapularis* by exploiting the relative importance of white-footed mice or white-tailed deer as tick hosts. Ex-

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posing mice to acaricides (Mather et al., 1987) and reducing or excluding deer populations (Wilson et al., 1988) have been effective in reducing the abundance of immature *I. scapularis*. However, these approaches may not eliminate the local presence of ticks because of the availability of alternative hosts (Jaenson et al., 1991). Raccoons (*Procyon lotor*) are ubiquitous throughout the United States; individual raccoons are heavily parasitized by *I. scapularis* in New York (USA) (Fish and Daniels, 1990). Raccoon tick burdens can be intense, and if raccoons are reservoir competent, one infected raccoon could possibly produce many times more *B. burgdorferi*-infected *I. scapularis* nymphs than a single mouse (Fish and Daniels, 1990).

Borrelia burgdorferi has been isolated from raccoons (Anderson et al., 1983). The spirochete also has been detected in ticks removed from raccoons (Fish and Daniels, 1990), and antibodies to *B. burgdorferi* have been detected in sera from raccoons collected in the southeastern U.S. (Magnarelli et al., 1991) and northeastern U.S. (Magnarelli et al., 1984). However, *B. burgdorferi* infected *P. lotor* may be inefficient in infecting attached ticks.

Alternatively, if it is a competent reservoir, the raccoon potentially could play a role as a non-rodent reservoir of *B. burgdorferi*. Accordingly, we evaluated the competence of the raccoon to serve as a reservoir of *B. burgdorferi*. In particular we evaluated the raccoon's susceptibility to infection, its ability to infect attached *I. scapularis*, and its antibody response to the spirochete.

MATERIALS AND METHODS

Five *B. burgdorferi* antibody and culture-negative raccoons (R4, R5, R6, R7, and R8) were used for these studies during September 1991 through April 1993. Two of these animals (R4 and R5) were live-trapped by a North Carolina Wildlife Resources Commission biologist at the Penny Bend Rabbit Research Area at the confluence of the Eno and Little Rivers in Durham County, North Carolina (USA) (35°6′N, 78°52′W). Both were males, more than one year of age, and infested with low numbers of ticks. The ticks were allowed to engorge and drop off the animals; ticks were identified as *Dermacentor variabilis* using a dichotomous key (Sonenshine, 1979). The remaining three raccoons were born in captivity and obtained from the Wistar Institute in Philadelphia, Pennsylvania (USA). All three of these animals were less than a year old and parasite-free. Raccoon 6 was male, and Raccoons 7 and 8 were female.

The raccoons were individually housed at the College of Veterinary Medicine, North Carolina State University, in stainless steel cages (87cm × 45cm × 78cm). During tick-feeding, cages were continuously held over pans filled with water to capture ticks as they dropped off the raccoons (Fish and Daniels, 1990).

Animals were quarantined upon arrival and feces examined for presence of worms or eggs of *Baylisascaris procyonis*, a neurotropic ascarid (Kazacos and Boyce, 1989) that can be prevalent in raccoon populations (Kidder et al., 1989). All raccoons were treated with the antihelmintic, Panacur (Fenbendazole, Hoechst-Roussel Pharm. Inc., Somerville, New Jersey, USA) (Kazacos and Boyce, 1989), at a dosage of 50 mg/kg per day for three consecutive days and once a week for the next 2 wk.

Animals were anesthetized for tick-application, blood withdrawal and skin biopsy by intramuscular injection of a combination of ketamine hydrochloride (15.0 mg/kg; Ketaset, Fort Dodge Laboratories, Inc., Fort Dodge, Iowa, USA) and xylazine (0.5 mg/kg; Rompum, Mobay Corporation, Shawnee, Kansas, USA). Each animal was determined not to have previous exposure to *B. burgdorferi* by testing serum for antibody using an enzyme-linked immunosorbent assay (ELISA) (Luttrell et al., 1994); by immunoblotting serum against Lyme disease spirochete-specific proteins (Luttrell et al., 1994); and by attempting to culture the spirochete from blood samples, ear skin biopsies, and ear tissue aspiration (Schwan et al., 1989). The serum testing also provided baseline values for subsequent serological analyses. All animals were euthanized by intravenous injection of Beuthanasia-D Special (1.0 ml/22 kg; Schering-Plough Animal Health, Kenilworth, New Jersey) at the termination of the project.

Raccoons were exposed to *B. burgdorferi* by placing 20 infected *I. scapularis* nymphs inside the ears of each animal. The ticks were derived from a colony established from adults collected on Great Island, Massachusetts (USA) in 1985. Nymphs placed on R4 and R5 were infected with the JD-1 strain of *B. burgdorferi*. Nymphs placed on R6, R7, and R8 were infected with *B. burgdorferi* Wisconsin 210 Wise strain. The JD1 and Wisconsin 210 Wise strains were used

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because ticks infected with these strains were available from established colonies of ticks.

Xenodiagnosis was used to detect spirochete infection in the raccoons 4, 6, 7, and 8 (Donahue et al., 1987) using cohorts of 300 naive larval *I. scapularis*. Ticks used for xenodiagnosis were derived from established colonies from three sources; Great Island, Massachusetts, Westchester County, New York and Yorktown, Virginia (USA). Due to the unavailability of suitable quantities of ticks, application times varied between raccoons. Because of poor recovery of xenodiagnostic larvae fed during initial trials with R4, 150 *I. scapularis* nymphs were used in an alternative xenodiagnostic procedure for evaluating R5. Transovarial transmission in *I. scapularis* is inefficient (Piesman et al., 1986); consequently, the cohorts of ticks were not evaluated for infection prior to use.

Ticks detaching from the host were collected from the water pans daily, rinsed in distilled water (DW) to remove debris, placed into steam-sterilized glass vials (containing a plaster of Paris: powdered activated charcoal base and a cloth screen top), and held at 21 C and 97% relative humidity (Piesman et al., 1990). These ticks were then evaluated for the presence of *B. burgdorferi*.

The susceptibility of Syrian hamsters (*Mesocricetus auratus auratus*) to infection by *B. burgdorferi* has been described by Johnson et al. (1984). To confirm the infectivity of the *B. burgdorferi* used, 50 nymphs, composed of 10 nymphs from each of five cohorts of infected nymphs were fed on 10 hamsters (H1 to H10) such that each hamster had five nymphs from one of the cohorts. Xenodiagnosis, using a single cohort of 25 naive *I. scapularis* larvae, and culturing techniques were used to verify that the hamsters were infected after tick feeding.

All of the infected nymphs recovered from the raccoons were tested for spirochetes. Tick midgut contents were removed and a smear was made on a slide. These smears were examined for infection by *B. burgdorferi* by indirect immunofluorescence microscopy with a species-specific monoclonal antibody (IFA-MAB) as described by Levine et al. (1989). Murine monoclonal antibody H5332, obtained from Dr. A. Barbour, directed against a species-specific, 31-kilodalton (kDa) protein of *B. burgdorferi* was used. Positive control slides were prepared by using *B. burgdorferi* (strain JD-1 and Wisconsin 210 Wise strain), cultured from the original cohorts of infected nymphs from the Centers for Disease Control, Fort Collins, Colorado (USA). *Borrelia burgdorferi* was diluted to a spirochete density of approximately 100 spirochetes per 400× microscopic field. This dilution was applied to the wells of a 30-well Teflon® printed slide (2mm, Cel-Line Associates, Inc., Newfield, New Jersey). The slides were air-dried, acetone-fixed, and stored at −20 C until needed. A single positive control slide was stained and read concurrently with each slide containing the smears of tick midguts. Half of the engorged larvae collected were dissected and examined by IFA-MAB 12 days after collection (Piesman et al., 1990) to verify tick infection. The remaining larvae were held until they molted; nymphs surviving ecdysis were dissected and examined for spirochete infection by IFA-MAB procedures.

Blood samples (Steere et al., 1983) from the jugular and skin biopsies from the ears (Sinsky and Piesman, 1989) were taken from all raccoons after each exposure to *B. burgdorferi* to monitor the time course of infection. Approximately 0.5 ml of each blood sample was mixed with sodium citrate and three to five drops were then added to 8.0 ml Barbour-Stoenner-Kelly medium (BSK-II) (Barbour, 1984). The remainder of each blood sample was allowed to clot at 4 C and the serum was harvested after centrifugation at 400 × G for 10 min and held at −70 C for subsequent examination for antibodies to *B. burgdorferi* (Schwan et al., 1989).

Ear skin biopsies and needle aspiration material were taken as described by Piesman et al. (1991). These and other tissue samples taken at euthanasia were collected using standard sterile surgical techniques and were placed directly into BSK-II medium or BSK-H medium (Sigma Chemical Company, Saint Louis, Missouri, USA). All cultures were held at 35 C for 6 to 8 wk (Anderson and Magnarelli, 1984) and examined weekly by dark-field microscopy for *B. burgdorferi* growth. Spirochetes observed using dark-field microscopy were confirmed as *B. burgdorferi* by IFA-MAB.

Unsuccessful attempts at obtaining blood samples from the raccoons diminished the consistency of sampling during the first exposure trial. Blood samples were obtained on a more regular schedule as our animal handling techniques improved. Blood samples were collected from R4 during weeks 1, 2, 3, 8, 9, 11, 12, 14, 16, 17, 19, 41, and 71 through 83 (at euthanasia). Blood samples taken from R5 during weeks 1, 2, 4, 6, 7, 8, and 11 (at euthanasia) and from R6, R7, and R8 during weeks 1, 2, 3, 5 through 17, and 25 through 37 (at euthanasia). Ear skin biopsies were taken from R4 during weeks 3, 5, 9, 71, 74, 77, 81, and 83 (at euthanasia) and a needle aspiration sample was taken during week 3. Ear skin biopsies were taken from R5 during weeks 3, 5, and 11 (at euthanasia) and from R6, R7, and R8 during weeks 3, 8, 12, 17, 25, 28, 31, 35, and 37 (at euthanasia). R4 was euthanized during week

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83, R5 during week 11, and R6, R7, and R8 during week 37. Using asceptic techniques, skin, liver, bladder, and kidney samples were taken from each animal at necropsy for culture and histologic examination. Samples of internal organ consisted of sections through each organ so that the surface and interior of each organ could be examined. All samples were no larger than 4 cm^3. Slides for histologic examination were made from skin, liver, kidney, and bladder samples taken from R4, R6, R7, and R8 at euthanasia. Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) (Brown and Lane, 1994). After the initial examination, R4 kidney tissue was sectioned at 3 μm and stained with H&E, periodic acid methionine silver (PAMS), Grocott's modification of Gomori's methionine silver (GMS), and acid fast stain (AFB); additional R4 liver sections were stained with periodic acid-Schiff (PAS) and AFB (Luna 1968; Sheehan and Hrapchak, 1980).

Cultures of the JD-1 and Wisconsin 210 Wise strains from *B. burgdorferi*-infected ticks were used to produce the strain-specific antigen used in all IFA-MAB, ELISA, and immunoblots by the methods of Anderson et al. (1983). Sonicated antigen used in the ELISA and immunoblot assays again was prepared by growing both strains of *B. burgdorferi* in BSK-II at 37 C. Spirochetes were harvested in late logarithmic phase of growth by centrifugation at 10,000 × G for 10 min. at 22 C. The pelleted spirochetes were washed three times in sterile 0.15M phosphate-buffered saline (PBS) (pH 7.38). The first wash was at 22 C and the last two were at 4 C. The pellet was resuspended in 1 to 2 ml sterile PBS (depending on pellet size), transferred to 1.5 ml microfuge tubes and heat-killed at 65 C for 30 min in a Type 17600 Dri-Bath (Thermolyne Corp., Dubuque, Iowa). The heat-killed spirochetes were transferred to an ice-water bath and sonicated while in the ice-water bath for alternating 15-sec periods of sonication using a W-140 ultrasonic sonicator (Ultrasonics, Inc., Plainview, New York) at an output setting of five, and cooling, for a total of 15 replications. The sonicated antigen for immunoblots was immediately frozen in 50 μl aliquots at −70C. For ELISA, sonicated antigen was further treated by centrifuging the sonicated material for 15 min. at 10,000 × G to remove cellular debris. The supernatant was sequentially filtered through 0.45 μm and 0.20 μm syringe filters. The protein concentration of the filtrate was determined by the Bradford protein assay (Bio-Rad Laboratories, Richmond, California, USA). The filtrate was diluted to a final protein concentration of 300 μg/ml and frozen at −70 C in 50 μl aliquots until used.

Raccoon sera were tested for antibody to *B. burgdorferi* by immunoblot (Western blot) analysis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970) with a Bio-Rad Mini-Protean II Slab Cell (Bio-Rad Laboratories). The separating gel and stacking gel were 12% and 4% acrylamide, respectively.

Gels were blotted to nitrocellulose membranes as described by Towbin et al. (1979), with the following modifications. After blocking in skim milk (5% in DW) for 1 hr, the membrane was allowed to air dry overnight at −20C. Each membrane was cut into about fifty 0.5-cm strips which were numbered consecutively. One strip each, from each side and from the middle, were incubated with positive control sera and another strip each, from each side and the middle, were incubated with negative control sera. The remaining strips were incubated with experimental raccoon sera at 22 C, while shaking on a Precision, Dubnoff Metabolic Shaking Incubator (GCA Corporation, Chicago, Illinois, USA) for 3 hr. While shaking, all strips were washed with skim milk (1% in DW), three times for 10 min each washing. The strips were incubated with goat anti-raccoon IgG biotinylated antibody (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Maryland, USA) (1:1000 in DW) for 2 hr at 22 C while shaking and then washed once. Strips then were incubated in avidin-alkaline phosphatase conjugate (Bio-Rad Laboratories) (1:2000 in 1% skim milk) for 1 hr, washed and then incubated in alkaline phosphatase (AP) buffer (1M Tris, 1mM MgCl, pH 9.5), AP Color Reagent A and B (Bio-Rad Laboratories) until bands of the desired darkness were obtained. The color reagents were decanted, the strips rinsed four or five times in DW and allowed to air dry. Differences in immunoblot band intensities were used to evaluate the serum antibody reactivity of individual raccoons toward *B. burgdorferi* proteins.

Electrophoresis of the JD-1 and Wisconsin 210 Wise strains of *B. burgdorferi* was performed as described for immunoblotting (Laemmli, 1970). Strains grown in BSK-II and BSK-H were used. Gels were stained in Coomassie brilliant blue R-250 (Laemmli, 1970) for comparison of protein profiles (Bissett and Hill, 1987).

The ELISA assays were conducted using the procedures of Greene et al. (1991). Briefly, 96-well polystyrene plates (ICN Biomedicals Inc., Horsham, Pennsylvania) sensitized with antigen (50 μl diluted 1:160 in coating buffer) were in-

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cubated for 1 hr at 35 C and held overnight at 4 C. Raccoon sera were thawed at 22 C, agitated in a vortex mixer and diluted 1:4 with washing buffer.

The sensitized plates were allowed to warm to 22 C; then they were washed three times with washing buffer. During the last washing cycle, the plates were agitated on a clinical rotator shaker (Fisher Scientific, Pittsburgh, Pennsylvania) at 210 revolutions/min for 10 min. This process was repeated three times for all other ELISA washing steps. After agitation, the buffer was removed, 50 μl of diluted raccoon serum was added to each well, and the plates were covered and incubated for 1 hr at 35 C. Each serum sample was run in duplicate. After incubation, the plates were washed three times, and 50 μl of a peroxidase-labeled affinity purified goat-anti-raccoon IgG (H + L) antibody (Kirkegaard and Perry Laboratories, Inc.), diluted 1:500 with washing buffer, was added to every well. The plates were covered and incubated at 35 C for 1 hr. Each plate was then washed three times and 100 μl each of peroxidase substrate solution (4-chloro-1-naphthol) and peroxidase solution B (H_2O_2) (Kirkegaard and Perry Laboratories, Inc.), were added to each well. Color development was terminated at the desired intensity after about 15 min by adding 100 μl of 1% SDS to each well. The optical density in each plate well was then measured and recorded using a BT 2000 Micro-Kinetics Reader (Bio-Tek Instruments, Inc., Fisher Scientific) and KinetiCalc 2.03 (Bio-Tek Instruments, Inc., Fisher Scientific) software package.

All experimental raccoon sera were screened for antibodies to *B. burgdorferi* at a 1:4 dilution against positive and negative control sera. Hyperimmune raccoon sera used as a positive control was produced by sequential inoculation of a raccoon with *B. burgdorferi* (ATCC 35210, American Type Culture Collection, Rockville Maryland). Pre-exposure sera collected before the primary exposure to spirochete-infected *I. scapularis* was used as negative control sera for each raccoon. The ELISA values (EV), described as arbitrary units by Greene et al. (1991) were calculated by the equation:

$$EV = \frac{\text{sample absorbance} - \text{negative control absorbance}}{\text{positive control absorbance} - \text{negative control absorbance}} \times 100.$$

Arbitrary cutoff values were chosen based on our experience with more than 1,000 field collected raccoon samples. An $40 \geq EV \geq 60$ was considered suspect and an $EV \geq 60$ was considered positive for anti-*B. burgdorferi* antibodies. All sera with EVs ≥ 40 were serially diluted from 1:4 to 1:8,192 in washing buffer and assayed by ELISA to determine serum antibody titers.

Serum titers also were determined by IFA microscopy as described by Magnarelli et al. (1984). Five μl of the appropriate serum sample was applied to each well containing about 100 fixed spirochetes in each 400× field. Slides were incubated at 35 to 37 C for 30 min, washed three times (5 min each) in PBS, shaken to remove excess moisture, and allowed to nearly air-dry. Slide wells were covered with 5 μl fluorescein isothiocyanate (FITC)-labeled, goat anti-raccoon IgG (H + L) (Kirkegaard and Perry Laboratories, Inc.) diluted 1:200 in PBS and incubated for 30 min. Slides were again rinsed three times in PBS, allowed to air dry, covered with 80% glycerin:PBS (9:1, pH 8.9) and a cover slip and examined by fluorescence microscopy. The titer was expressed as the reciprocal of the highest dilution (Magnarelli et al., 1984) in which at least 50% of the spirochetes per microscope field distinctly fluoresced. Serum antibody titers could only be determined by ELISA for the sera of R5. Serum antibody titers for the other four raccoons could not be determined accurately by ELISA because at the dilution of 1:256 the absorbance readings of the negative controls were <0.02, and all remaining samples tested had titers higher than 1:256. An ELISA absorbance value <0.02 was not relatively different than background.

RESULTS

Recovery of spirochete-infected *I. scapularis* nymphs varied from 15 to 65% (n = 3 to 13 of 20) (Tables 1 to 5). We applied 2,400 naive larval *I. scapularis* per raccoon to R4, R6, R7, and R8, and 600 naive nymphal *I. scapularis* to R5. The recovery of replete or partially replete ticks used for xenodiagnosis varied between raccoons and cohorts fed on each raccoon (Table 1 to 5). Smaller percentages of the total number of ticks applied were recovered from field-collected raccoons (R4, R5) than from laboratory-reared raccoons (R6 through R8). We recovered 198 (8.3%) replete larvae from R4, 458 (19%) from R6, 820 (34%) from R7, and 787 (33%) from R8, and 88 (15%) nymphs from R5 (Tables 1 to 5). For each cohort of ticks recovered from our two field-col-

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TABLE 1. Recovery of *Ixodes scapularis* from *Procyon lotor* (raccoon 4) and results of immunofluorescence antibody (IFA)(H5332) assays for *Borrelia burgdorferi*.

	Number of ticks applied	Post-infection day	Engorged ticks recovered	Number dissected premolt (12 days)	Number dissected postmolt	Number IFA-positive
Infected nymphs 1[a]	20	0	3 (15)[b]	3 (15)	0 (0)	2 (67)
Larval cohort 1	300	7	25 (8.3)	13 (4.3)	9 (3.0)	0 (0)
Larval cohort 2	300	17	7 (2.3)	4 (1.3)	2 (0.6)	0 (0)
Larval cohort 3	300	29	29 (9.6)	15 (5.0)	4 (1.3)	0 (0)
Larval cohort 4	300	71	31 (10)	16 (5.3)	10 (3.3)	0 (0)
Subtotal for cohorts	1,200		92 (7.7)	48 (4.0)	25 (2.1)	0 (0)
Infected nymphs 4[a]	20	493	13 (65)	13 (65)	0 (0)	11 (85)
Larval cohort 5	300	499	35 (12)	18 (6.0)	9 (3.0)	0 (0)
Larval cohort 6	300	513	32 (11)	17 (5.7)	14 (4.7)	0 (0)
Larval cohort 7	300	527	15 (5.0)	8 (2.7)	7 (2.3)	0 (0)
Larval cohort 8	300	555	24 (8.0)	12 (4.0)	8 (2.7)	0 (0)
Subtotal for cohorts	1,200		106 (8.8)	55 (4.6)	38 (3.2)	0 (0)
Overall total for cohorts	2,400		198 (8.3)	103 (4.3)	63 (2.6)	0 (0)

a Infected with JD-1 strain of *B. burgdorferi*.
b Number (percent).

lected animals, similarly small percentages of engorged larvae (2.3 to 12% from R4) and nymphs (9.3 to 27% from R5) were collected. In contrast, for laboratory-reared raccoons, the number of larvae recovered for each cohort substantially declined following the second feeding of spirochete-infected nymphs. After five feedings, the recovery of engorged xenodiagnostic larvae from the three laboratory-reared raccoons declined to near 10% and paralleled the recovery from the two field-collected raccoons. Molting success varied (29 to 100%) by cohort regardless of the raccoon (Tables 1 to 5).

Sixteen engorged *D. variabilis* (seven males, nine females) were collected from R4 and, two engorged females were collected from R5 upon capture. All *Dermacentor* sp., ticks were examined by IFA-MAB and no spirochetes were detected. At least one *B. burgdorferi* infected *I. scapularis* nymph fed on each raccoon. No spirochetes were detected in any of the xenodiagnostic ticks examined (Tables 1 to 5). Some ticks that were recovered could not be examined by IFA-MAB because they dessicated during holding.

The infectivity of the two strains of *B. burgdorferi* used was verified by feeding *I.*

TABLE 2. Recovery of *Ixodes scapularis* from *Procyon lotor* (raccoon 5) and results of immunofluorescence antibody (IFA)(H5332) assays for *Borrelia burgdorferi*.

	Number of ticks applied	Post-infection day	Engorged ticks recovered	Number dissected premolt (12 days)	Number dissected postmolt	Number IFA-positive
Infected nymphs 1[a]	20	0	4 (20)[b]	4 (20)	0 (0)	3 (75)
Nymphall cohort 1	150	12	17 (11)	9 (3.3)	5 (3.3)	0 (0)
Nymphall cohort 2	150	22	40 (27)	20 (13)	17 (11)	0 (0)
Nymphall cohort 3	150	32	14 (9.3)	7 (4.7)	5 (3.3)	0 (0)
Nymphall cohort 4	150	56	17 (11)	9 (6.0)	8 (5.3)	0 (0)
Overall total for cohorts	600		88 (15)	45 (7.5)	30 (5.0)	0 (0)

a Infected with JD-1 strain of *B. burgdorferi*.
b Number (percent).

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TABLE 3. Recovery of *Ixodes scapularis* from *Procyon lotor* (raccoon 6) and results of immunofluorescence antibody (IFA)(H5332) assays for *Borrelia burgdorferi*.

	Number of ticks applied	Post-infection day	Engorged ticks recovered	Number dissected pre-molt (12 days)	Number dissected post-molt	Number IFA-positive
Infected nymphs 1[a]	20	0	9 (45)[b]	9 (45)	0 (0)	6 (67)
Larval cohort 1	300	5	73 (24)	37 (12)	19 (6.3)	0 (0)
Larva cohort 2	300	20	132 (44)	66 (22)	45 (15)	0 (0)
Larva cohort 3	300	33	62 (21)	31 (10)	20 (6.7)	0 (0)
Larva cohort 4	300	64	72 (24)	36 (12)	31 (10)	0 (0)
Subtotal for cohorts	1,200		339 (28)	170 (14)	115 (9.6)	0 (0)
Infected nymphs 4[a]	20	175	11 (55)	11 (55)	0 (0)	10 (91)
Larval cohort 5	300	181	37 (12)	19 (6.3)	11 (3.7)	0 (0)
Larva cohort 6	300	195	24 (8.0)	12 (4.0)	9 (3.0)	0 (0)
Larva cohort 7	300	209	24 (8.0)	12 (4.0)	8 (2.7)	0 (0)
Larva cohort 8	300	237	34 (11)	17 (11)	10 (3.3)	0 (0)
Subtotal for cohorts	1,200		119 (10)	60 (5.0)	38 (3.2)	0 (0)
Overall total for cohorts	2,400		458 (19)	230 (9.6)	153 (6.4)	0 (0)

[a] Infected with JD-1 strain of *B. burgdorferi*.
[b] Number (percent).

scapularis nymphs (from each of the five groups used in the raccoon infection experiments) on Syrian hamsters (Table 6). All Syrian hamsters infected *I. scapularis* larvae and had positive tissue cultures at euthanasia.

Spirochetes, confirmed as *B. burgdorferi* by IFA-MAB, were only observed in cultures made from tissues of raccoons R4 and R6. Positive cultures from R4 were of blood (wk 5, 8, 9, 11, and 12), needle aspiration material taken from one ear (wk 3), and skin biopsies taken from ears (wk 3, 5, 9, 81, and 83). *Borrelia burgdorferi* was only isolated from R6 skin biopsies (ear) (wk 28 and 31). Positive cultures were derived from R4 after both exposures to *B. burgdorferi*, but spirochetes only were isolated from R6 after the second exposure to spirochete-infected *I. scapularis*.

TABLE 4. Recovery of *Ixodes scapularis* from *Procyon lotor* (raccoon 7) and results of immunofluorescence antibody (IFA)(H5332) assays for *Borrelia burgdorferi*.

	Number of ticks applied	Post infection day	Engorged ticks recovered	Number dissected pre-molt (12 days)	Number dissected postmolt	Number IFA-positive
Infected nymphs 1[a]	20	0	9 (45)[b]	9 (45)	0 (0)	7 (78)
Larval cohort 1	300	5	272 (91)	136 (45)	59 (20)	0 (0)
Larval cohort 2	300	20	188 (63)	94 (31)	73 (24)	0 (0)
Larval cohort 3	300	33	139 (46)	70 (23)	57 (19)	0 (0)
Larval cohort 4	300	64	107 (36)	54 (18)	27 (9.0)	0 (0)
Subtotal for cohorts	1,200		706 (59)	354 (30)	215 (18)	0 (0)
Infected nymphs 4[a]	20	175	12 (60)	12 (60)	0 (0)	11 (92)
Larval cohort 5	300	181	32 (11)	16 (5.3)	11 (3.7)	0 (0)
Larval cohort 6	300	195	18 (6.0)	10 (3.3)	6 (2.0)	0 (0)
Larval cohort 7	300	209	31 (10)	17 (5.7)	10 (3.3)	0 (0)
Larval cohort 8	300	237	31 (10)	16 (5.3)	13 (4.3)	0 (0)
Subtotal for cohorts	1,200		107 (14)	59 (4.9)	40 (3.3)	0 (0)
Overall total for cohorts	2,400		820 (34)	413 (17)	256 (11)	0 (0)

[a] Infected with JD-1 strain of *B. burgdorferi*.
[b] Number (percent).

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TABLE 5. Recovery of *Ixodes scapularis* from *Procyon lotor* (raccoon 8) and results of immunofluorescence antibody (IFA)(H5332) assays for *Borrelia burgdorferi*.

	Number of ticks applied	Post-infection day	Engorged ticks recovered	Number dissected pre-molt (12 days)	Number dissected postmolt	Number IFA-positive
Infected nymphs 1[a]	20	0	4 (20)[b]	4 (20)	0 (0)	2 (50)
Larval cohort 1	300	5	289 (96)	145 (48)	97 (32)	0 (0)
Larval cohort 2	300	20	173 (58)	87 (29)	48 (16)	0 (0)
Larval cohort 3	300	33	106 (35)	53 (18)	29 (9.7)	0 (0)
Larval cohort 4	300	64	94 (31)	47 (16)	33 (11)	0 (0)
Subtotal for cohorts	1,200		662 (55)	332 (28)	207 (17)	0 (0)
Infected nymphs 4[a]	20	175	9 (45)	9 (45)	0 (0)	8 (88)
Larval cohort 5	300	181	40 (13)	20 (6.7)	17 (5.7)	0 (0)
Larval cohort 6	300	195	29 (9.7)	15 (5.0)	12 (4.0)	0 (0)
Larval cohort 7	300	209	24 (8.0)	12 (4.0)	10 (3.3)	0 (0)
Larval cohort 8	300	237	32 (11)	16 (5.3)	9 (3.0)	0 (0)
Subtotal for cohorts	1,200		125 (10)	63 (5.3)	48 (4.0)	0 (0)
Overall total for cohorts	2,400		787 (33)	395 (17)	255 (11)	0 (0)

[a] Infected with JD-1 strain of *B. burgdorferi*.
[b] Number (percent).

All other raccoon tissue cultures were negative.

No gross or microscopic lesions were noted in tissues obtained from R6, R7, and R8. Raccoon 5 was euthanized at an earlier date and microscopic histologic evaluation was not conducted. Liver sections from R4 had lipofuscin and bridging peroportal fi-

TABLE 6. Recovery of *Ixodes scapularis* from hamsters (*Mesocricetus auratus auratus*) 1 through 10 (H1-H10) and results of immunofluorescence antibody (IFA)(H5332) assays for *Borrelia burgdorferi*.

	Number of ticks applied	Engorged ticks recovered	Number dissected pre-molt (12 days)	Number dissected postmolt	Number IFA positive
Infected nymphs 1[a] on H1	5	4 (80)[b]	4 (80)	0 (0)	4 (100)
Larval cohort on H1	25	7 (28)	4 (16)	3 (12)	6 (88)
Infected nymphs 1[a] on H2	5	5 (100)	5 (100)	0 (0)	4 (80)
Larval cohort on H2	25	11 (44)	6 (24)	5 (20)	9 (81.8)
Infected nymphs 2[a] on H3	5	5 (100)	5 (100)	0 (0)	4 (80)
Larval cohort on H3	25	16 (64)	8 (32)	8 (32)	12 (75)
Infected nymphs 2[a] on H4	5	3 (60)	3 (60)	0 (0)	1 (20)
Larval cohort on H4	25	10 (40)	5 (20)	5 (20)	7 (70)
Infected nymphs 3[c] on H5	5	5 (100)	5 (100)	0 (0)	5 (100)
Larval cohort on H5	25	8 (32)	4 (16)	4 (16)	7 (88)
Infected nymphs 3[c] on H6	5	4 (80)	2 (40)	0 (0)	2 (100)
Larval cohort on H6	25	17 (68)	8 (32)	8 (32)	10 (40)
Infected nymphs 4[a] on H7	5	3 (60)	3 (60)	0 (0)	3 (100)
Larval cohort on H7	25	13 (52)	7 (28)	6 (24)	11 (85)
Infected nymphs 4[a] on H8	5	4 (80)	4 (80)	0 (0)	4 (100)
Larval cohort on H8	25	20 (80)	10 (40)	8 (32)	16 (64)
Infected nymphs 5[c] on H9	5	5 (100)	5 (100)	0 (0)	3 (60)
Larval cohort on H9	25	21 (84)	11 (44)	9 (36)	18 (86)
Infected nymphs 5[c] on H10	5	4 (80)	4 (80)	0 (0)	1 (25)
Larval cohort on H10	25	15 (60)	8 (32)	7 (28)	11 (73)

[a] Infected with JD-1 strain of *B. burgdorferi*.
[b] Number (percent).
[c] Infected with Wisconsin 210 Wise strain of *B. burgdorferi*.

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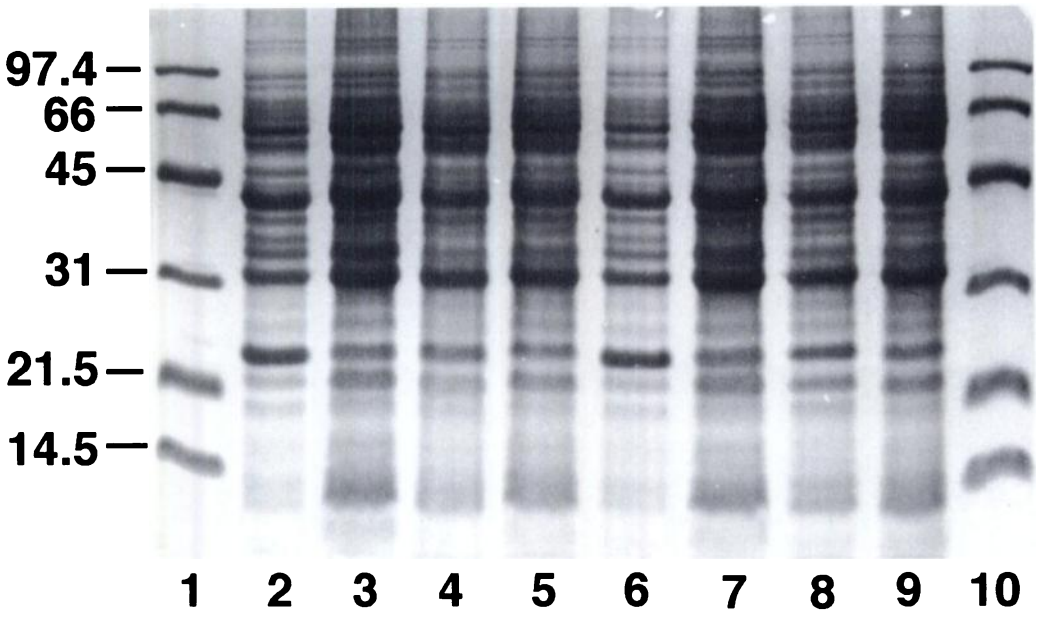

FIGURE 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (12% gel stained with Coomassie blue) protein profiles of the two strains of *Borrelia burgdorferi* grown in Barbour-Stoenner-Kelly medium (BSK-II) and BSK-H. Lanes 1 and 10, low molecular weight standards; lanes 2 and 6, strain JD-1 grown in BSK-II; lanes 3 and 7, Wisconsin 210 Wise strain grown in BSK-II; lanes 4 and 8, strain JD-1 grown in BSK-H; lanes 5 and 9, Wisconsin 210 Wise strain grown in BSK-H. Molecular masses shown on left (in kilodaltons).

brosis by PAMS stain and H&E respectively. Glomulosclerosis was seen by GMS stain and chronic, mild, multifocal, lymphocytic and plasmacytic interstitial nephritis was evident with H&E in kidney sections. Cross-sections of a nematode were evident in urinary bladder tissue sections.

There were no remarkable differences in the protein profiles of the two strains of *B. burgdorferi* used (Fig. 1). No differences within or between strains resulting from the type of culture media used (BSK-II or BSK-H) were detected.

Antibody reactivity to *B. burgdorferi* antigens was depressed relative to positive control sera for all raccoons except R5 following the first feeding of infected nymphs. Serum samples taken from R5 were suspect positive for anti-*B. burgdorferi* antibodies during weeks 4, 6, and 7, but strongly positive during weeks 8 and 11. The remaining raccoons (R4, R6, R7, R8), serologically converted from 4 to 6 wk after the second feeding of infected nymphs. These raccoons had ELISA values (EV) less than 40 during weeks 0 to 73 for R4, 0 to 29 for R6, 0 to 28 for R7, and 0 to 2 and 4 to 27 for R8. These raccoons had suspect EV ($40 \leq EV \leq 60$) during weeks 30 to 35 for R6, 30 for R7, and 28 and 29 for R8. Positive EV (≥ 60) were detected during weeks 74 to 83 for R4, 36 and 37 for R6, 29 and 31 to 37 for R7, and 30 to 37 for R8. Maximum absorbance values for ELISA ranged from 1.25 to >5.5 times greater than values recorded for positive control serum samples.

The antibody titers for R5 sera determined by IFA were equivalent to titers determined for the same samples by ELISA. Antibody titer for weeks 4 and 6 was 1:8. For weeks 7, 8 and 11, the titers were 1:32, 1:128, and 1:64, respectively. Serum antibody titers increased over time for all raccoons. Maximum antibody titers ranged

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TABLE 7. Raccoon (*Procyon lotor*) serum immunofluorescence antibody (IFA)(H5332) titers[a] to *Borrelia burgdorferi*.

Raccoon 4		Raccoon 5		Raccoon 6		Raccoon 7		Raccoon 8	
Week	Titer[b]	Week	Titer[b]	Week	Titer[b]	Week	Titer[b]	Week	Titer[b]
76	512	4	8	30	256	29	1,024	3	256
77	1,024	6	8	31	512	30	1,024	6	256
78	512	7	32	32	1,024	31	1,024	28	512
79	512	8	128	33	512	32	2,048	29	512
80	1,024	11	64	34	512	33	2,048	30	1,024
81	2,048	—[c]	—	35	1,024	34	1,024	31	1,024
82	2,048	—	—	36	2,048	35	2,048	32	1,024
83	2,048	—	—	37	2,048	36	4,096	33	1,024
—	—	—	—	—	—	37	4,096	34	2,048
—	—	—	—	—	—	—	—	35	2,048
—	—	—	—	—	—	—	—	36	4,096
—	—	—	—	—	—	—	—	37	4,096

[a] Expressed as the reciprocal of the highest dilution of a serum sample for which 50% of the spirochetes per microscope field could be seen by IFA.
[b] Mean of duplicate samples.
[c] —, not done.

from 1:128 for R5 to 1:4,096 for R7 and R8 (Table 7). Generally, antibody activity as measured by ELISA was not congruent with serum antibody titers determined by IFA. For R5, samples with an $40 \leq EV \leq 60$ were equivalent to IFA-MAB antibody titers of 1:8 or 1:32. For the other raccoons, samples with an $40 \leq EV \leq 60$ were equivalent to IFA antibody titers ranging from 1:256 to 1:2,048.

Positive control sera had strong antibody reactivity to antigens estimated to be 18, 21, 31, 34, 41, 43, 45, 48, 50, 55, 61, 66, and 96 kDa antigens (Fig. 2). Negative control sera had no apparent antibody reactivity. Raccoon 4 developed strong antibody reactivity to 18-, 21-, 31-, 34-, 41-, 50-, and 55-kDa antigens during weeks 73 to 81. Sera from R5 primarily reacted to 31 and 34 kDa antigens during weeks 3 through 11. Serum samples from R6 had strong reactivity to 18-, 21-, 31-, 34-, and 96-kDa antigens during weeks 29 through 37. Strong serologic reactivity to 18-, 21-, 31-, 34-, 41-, and 96-kDa antigens was observed in the sera of R7 during weeks 28 to 37. Raccoon 8 had strong serum reactivity to 18-, 21-, 31-, 34-, 41-, 45-, 50-, 55-, 61-, 66-, and 96-kDa antigens during weeks 29 through 37, but only exhibited weak reactivity to the 41-, 66-, and 96-kDa antigens during weeks 1 through 16. Most of the antibody reactivity observed in the experimental raccoon sera was very similar to the immunoreactivity of the positive control sera; thus the experimental raccoon sera probably contained antibodies produced to the same antigens that elicited an immune response from the control animal.

DISCUSSION

Transfer of *B. burgdorferi* from raccoons to attached *I. scapularis* apparently is inefficient. Although we reisolated the spirochete from two raccoons, larval *I. scapularis* did not acquire the spirochete from these hosts during feeding. In contrast, Fish and Daniels (1990) found that six of 11 raccoons from New York infected attached larval *I. scapularis*. Heavy burdens of infected nymphal and adult *I. scapularis* in Lyme Disease-enzootic areas result in almost continuous exposure to infected ticks during focused periods in the spring and summer (Lord, 1992). The brief exposure to infected ticks in our studies may not have been of sufficient duration or intensity to replicate exposure conditions in natural populations.

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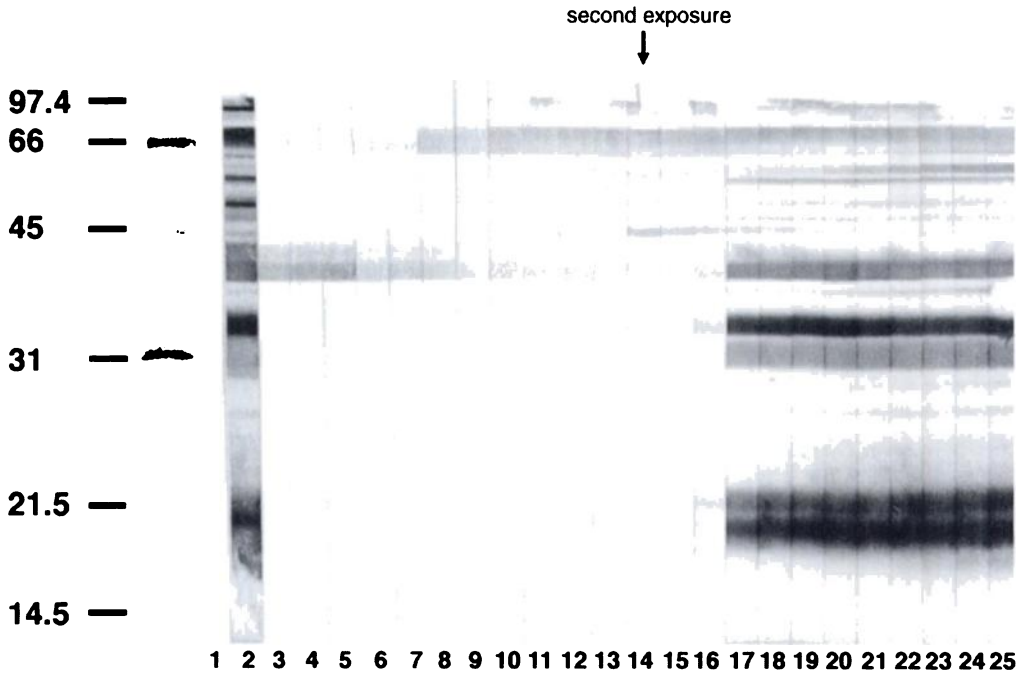

FIGURE 2. Representative immunoblotting profile of raccoon sera (R8) against *Borrelia burgdorferi* antigen over time. Lane 1 was incubated with negative control raccoon serum; lane 2 was incubated with positive control raccoon serum; lanes 3 (pre-exposure), 4 to 13 (primary exposure, weeks 1 and 2, 5 and 6, 8, 11 to 14 and 16), and 14 to 25 (secondary exposure, weeks 26 to 37) were incubated with experimental raccoon sera. Molecular masses of protein standards are shown on the right (in kilodaltons).

Feeding success on the tick-naive raccoons, decreased dramatically with successive applications of *I. scapularis* larvae. The number of engorged larvae recovered from these raccoons declined by as much as 85% over the 37-wk period of the infection trials. Recovery of replete larval ticks from field-collected (R4) and laboratory-reared raccoons (R6, R7, R8), after four feedings of larvae on each animal, were approximately 10% of the number applied. Although some tick loss may be attributable to more intensive raccoon grooming behavior in captivity, we believe that the laboratory-reared raccoons acquired resistance to tick feeding during the infection trials. The field-collected raccoons apparently were somewhat resistant when captured.

Borrelia burgdorferi was isolated from R4 and from the skin of R6, but was not recovered from any of the other raccoons. Spirochetes have been isolated from white-footed mice and other rodents (Anderson et al., 1985) which can infect attached larval *I. scapularis* (Mather et al., 1989). However, our experience with raccoons more closely resembles that of Appel et al. (1993) in dogs, which were exposed to *B. burgdorferi* by tick bite and inoculation. As in our study, Appel et al. (1993) isolated *B. burgdorferi* from dogs but did not detect spirochetes in xenodiagnostic *I. scapularis* larvae. However, in a more recent study, Mather et al. (1994) was able to recover infected xenodiagnostic larval ticks from dogs infected with *B. burgdorferi* by tick bite.

The tick life stage responsible for pathogen transmission may be important in determining the intensity and development time of infection. Appel et al. (1993) and Mather et al. (1994) found that dogs were infected after they were fed upon by a sin-

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gle cohort of infected adult ticks. In addition, Appel et al. (1993) discovered that feedings of multiple cohorts of infected nymphs were required to induce a similar infection. Greene et al. (1988a) failed to infect four laboratory-reared beagles after each dog was fed upon by *B. burgdorferi*-infected *I. scapularis* nymphs. Based on these observations, we propose that adult ticks may deliver a larger inoculum of spirochetes than nymphs, and that the number of spirochetes inoculated may be correlated with the intensity of infection in the host. Although two of our raccoons (R4 and R6) apparently received sufficient spirochetes to become infected, they did not infect attached ticks. Piesman et al. (1990) found $>10^4$ tick-derived spirochetes inoculated intraperitoneally achieved 100% infection of hamsters, while 10^3 to 10^4 spirochetes infected only 35%, and $<10^3$ spirochetes did not infect hamsters. Although the inoculum size associated with tick-transmitted *B. burgdorferi* has not been defined, infection thresholds are likely to vary by host species.

Antigenic proteins were identified by mobility against molecular weight standards. All five raccoons used in the xenodiagnostic studies were exposed to *B. burgdorferi*. Immunoblot profiles of raccoon sera did not closely ressemble sera of other wildlife or domestic animals species. Sera from each raccoon, including the positive control sera, reacted strongly to the OspA (31-kDa) and OspB (34-kDa) proteins. In contrast, non-human hosts infected via tick bite generally do not develop strong antibody responses to OspA or OspB (Greene et al., 1988b). Reactivity to these proteins is much more common when animals are infected via needle-inoculation (Roehrig et al., 1992). Strong immunoreactivity to these two proteins would be expected for the positive control (hyperimmune) raccoon because it was needle-inoculated with about 10^6 spirochetes. The negative control sera and preexposure sera had no immunoreactivity.

Gern et al. (1993) proposed that naturally infected mice do not produce antibodies to the 31- and 34-kDa antigens because of suboptimal doses of *B. burgdorferi* delivered during tick feeding. An inoculum of $>10^4$ spirochetes was needed to elicit antibody production to the 31- and 34-kDa antigens. Therefore, the innoculum of spirochetes delivered by the laboratory-infected nymphs used to infect the raccoons in our studies apparently was large enough to elicit an antibody response to 31- and 34-kDa antigens. Alternatively, the local immunologic response of raccoon skin may be different than that of white-footed mice and other reservoir competent hosts. Host species have markedly different inflamatory responses to tick attachment (Ribeiro et al., 1985). In addition, tick saliva includes antihemostatic, antiinflammatory, and immunosuppressive factors that facilitate feeding by circumventing the host response to attachment (Ribeiro et al., 1985).

Dogs vaccinated with a commercial *B. burgdorferi* bacterin were protected against challenge with seven consecutive daily doses of 2.5×10^6 *B. burgdorferi* (Chu et al., 1992). The bacteria elicited a strong antibody response to 31- and 34-kDa antigen in these dogs (Chu et al., 1992). High levels of borreliacidal antibodies found in human Lyme disease sera (Callister et al., 1991) are also specific for OspA (Callister et al., 1993). However, antibodies to another common antigen, 41-kDa, did not provide protection against *B. burgdorferi* infection (Schaible et al., 1990). If antibodies to 31- and 34-kDa antigen confer the same type of protection in raccoons, then their occurrence and the lack of recovery of spirochetes through culture or xenodiagnosis would be expected. If, however, these antibodies do not provide complete protection for all raccoons or if infection cycles follow a pattern of relapsing spirochetemia, then spirochetes might sporadically be recovered, as was found for R4 and R6. *Borrelia burgdorferi* spirochetemia has been reported to cycle though alternating spirochete-posi-

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tive and spirochete-negative phases in the hispid cotton rat (*Sigmodon hispidus*) (Burgdorfer and Gage, 1986).

The immune response of animals that were exposed twice to *B. burgdorferi* (R4, R6, R7, and R8) differed markedly from an animal (R5) that was exposed only once. Based on the intensity of antibody reactivity in immunoblots, R4, R6, R7, and R8 were more intensely exposed to *B. burgdorferi* than R5 even though ELISA values were much higher for R5 during the first exposure than for any of the other raccoons. The ELISA and IFA serum titers for raccoons were within the range of values measured for raccoons collected in Connecticut, Maryland, North Carolina, and Florida (USA) (Magnarelli et al., 1991) and naturally-infected dogs (1:64–1: 16,384) (Magnarelli et al., 1985) sampled in Connecticut. Raccoons exposed twice to the *B. burgdorferi* infected nymphs developed threshold serum values (EV > 40) at 4 wk after the second exposure to infected ticks. Although R5 was only exposed once in the laboratory to infected nymphs, it developed a threshold titer after 4 wk. Based on the initial screening and attempted culture of *B. burgdorferi* R5 had no prior exposure to the spirochete; however, it had an anamnestic type of response. *Borrelia burgdorferi* has not been isolated from suspected Lyme disease patients in North Carolina. However, transmission of *B. burgdorferi* has been detected in other areas of North Carolina (Levine et al., 1989). The raccoon may have been previously exposed to the spirochete and the titer of baseline sera, obtained prior to the experiments, may have been below detectable limits. Dogs infected via tick bite did not seroconvert until 4 to 6 wk after being fed upon by infected ticks (Appel et al., 1993). Antibody levels in these dogs increased for 6 to 8 wk and then remained high for up to one year (Appel et al., 1993). The raccoon reservoir competence experiments were not continued long enough to detect a plateau or drop in antibody response.

Based on our studies we believe that raccoons are susceptible to infection with *B. burgdorferi*, but their ability to infect attached ticks may be limited. Additional studies in which raccoons are repeatedly exposed to the spirochete by tick bite are needed to assess if the intensity of exposure is related to reservoir competence. Since different hosts may vary in their ability to infect different species of ticks, additional studies are also needed to assess the ability of raccoons to infect other tick species with the spirochete.

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