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EXPERIMENTAL INFECTION OF *BORRELIA BURGDORFERI* IN WHITE-TAILED DEER

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ABSTRACT: Four white-tailed deer (*Odocoileus virginianus*) were experimentally inoculated with *Borrelia burgdorferi* to determine serologic response by enzyme-linked immunosorbent assay (ELISA) and immunoblotting. Deer had antibodies by ELISA by 2 to 3 wk post-inoculation (PI) and remained positive for 10 wk. Deer demonstrated immunoblotting reactivity between 10 and 14 days PI and consistently showed antibody response to nine *B. burgdorferi* antigens. Attempts were made to recover the spirochete from blood and tissues; *B. burgdorferi* was isolated from an ear punch biopsy from one of the inoculated deer.

Key words: Lyme disease, *Borrelia burgdorferi*, white-tailed deer, *Odocoileus virginianus*, *Ixodes scapularis*, enzyme-linked immunosorbent assay, ELISA, immunoblotting.

INTRODUCTION

Lyme disease, caused by the spirochete *Borrelia burgdorferi*, is the most commonly reported tick-borne disease in the United States (Centers for Disease Control, 1992). In the northeastern and north central United States, human cases of Lyme disease are associated with the presence of the tick *Ixodes scapularis* (Steere and Malawista, 1979). *Ixodes dammini* recently has been relegated to a junior subjective synonym for *I. scapularis* (Oliver et al., 1993). White-footed mice (*Peromyscus leucopus*) serve as the principal host for immature *I. scapularis* (Piesman and Spielman, 1979) and principal reservoir of *B. burgdorferi* where the disease is endemic in the Northeast (Levine et al., 1985; Anderson et al., 1987). The adult stage of the tick attaches predominantly to white-tailed deer (*Odocoileus virginianus*) (Main et al., 1981; Wilson et al., 1985).

The distribution of *I. scapularis* is highly correlated with the distribution of white-tailed deer (Wilson et al., 1990). In the northeastern United States, where tick infection frequencies routinely exceed 25% in some areas (Piesman et al., 1986; Falco and Fish, 1988), white-tailed deer exposure to *B. burgdorferi* is intense. However, this spirochete rarely has been isolated or detected in white-tailed deer blood or other tissues (Bosler et al., 1983, 1984). Based on field studies with ticks, deer may be incompetent reservoirs for *B. burgdorferi* (Telford et al., 1988). Antibodies to *B. burgdorferi* have been detected in serum from deer sampled in Connecticut (USA) (Magnarelli et al., 1984, 1986, 1991), New York (USA) (Bosler et al., 1984; O'Connell et al., 1986; Daniels et al., 1993), and other northeastern states (Anderson and Magnarelli, 1983; Magnarelli et al., 1991), and in Minnesota (USA) (Gill et al., 1993). Antibodies to *Borrelia* spp. also have been detected in serum or plasma from white-tailed deer in the southeastern United States in North Carolina (USA) (Magnarelli et al., 1986), Texas (USA) (Rawlings, 1986), and Georgia (USA) (Magnarelli et al., 1991; Mahnke et al., 1993), where tick infection rates are comparatively low (Levine et al., 1989; Luckhart et al., 1991). The exposure of white-tailed deer to *B. burgdorferi* potentially serves as a useful indicator for delineation of foci of spirochete transmission when used in conjunction with other data (Mahnke et al., 1993). Our objective was to compare the serologic response in

white-tailed deer with the enzyme-linked immunosorbent assay (ELISA) and immunoblotting technique. We also attempted to recover the spirochete from deer blood, skin, and other tissues.

MATERIALS AND METHODS

Borrelia burgdorferi inocula

Tick-derived isolates of *Borrelia burgdorferi*, JD-1 and SH2-82, were used to inoculate deer. Isolate JD-1 was obtained from Dr. Richard Sinsky, University of Alabama, Birmingham, Alabama (USA), and SH2-82 was provided by the Rocky Mountain Laboratories, Hamilton, Montana (USA). Isolates were grown in modified Barbour-Stoenner-Kelly (BSK II) medium (Barbour, 1984) containing the following: 0.15% soft agarose (SeaPrep, FMC Bioproducts, Rockland, Maine, USA), 0.023% L-cysteine hydrochloride, 0.015% DL-dithiothreitol, 1 μg of L-glutamine per ml, 50 μg of rifampin per ml, 20 μg of phosphomycin per ml, and 2.5 μg of amphotericin B (Fungizone) per ml (all from Sigma Chemical Company, St. Louis, Missouri, USA) (Sinsky and Piesman, 1989). Both isolates, passed six times in BSK II, were inoculated intraperitoneally in hamsters, then reisolated in BSK II from hamster bladders or blood and grown to approximately 2×10^7 cells per ml for deer inoculations. Spirochete numbers were estimated by making direct counts of live organisms in 10 μl of each inoculum using darkfield microscopy.

Inoculation and isolation of *Borrelia burgdorferi* from white-tailed deer

Five male white-tailed deer fawns were procured as orphans from the Georgia Department of Natural Resources. Fawns were hand-raised in a tick-free environment and maintained on Purina deer chow (Purina Deer Blend 5607, Purina Mills, St. Louis, Missouri). During the experimental period, deer were housed separately in insect-free, temperature-controlled (18 to 20 C) stalls measuring 4.1 m × 1.4 m × 3.0 m. Before inoculation, isolation of *B. burgdorferi* was attempted by culture of whole blood. Deer also were screened for *B. burgdorferi* by ELISA and for six *Leptospira interrogans* serovars (*pomona, hardjo, grippotyphosa, icterohemorrhagiae, canicola,* and *bratislava*) by microscopic agglutination test (Cole et al., 1973).

At 6 to 9 mo of age, two deer were inoculated with the SH2-82 isolate in BSK II (deer 1 and deer 2), and two deer were inoculated with the JD-1 isolate in BSK II (deer 3 and deer 4) as follows: 1.5 ml was injected subcutaneously behind the neck and 1.0 ml was injected intradermally in multiple sites in the right ear. The left ears of deer 2 and deer 4 also were injected intradermally with 1.0 ml of spirochetes which had been washed three times and suspended in phosphate-buffered saline solution pH 7.2 (PBS). The fifth deer was used as a control and was injected in the same manner with uninoculated BSK II. For all inoculations and sample collections, deer were sedated with a mixture of 70 to 125 mg ketamine (Fort Dodge Laboratories, Inc., Fort Dodge, Iowa, USA) and 25 mg xylazine hydrochloride (Mobay Corporation, Shawnee, Kansas, USA) administered intramuscularly at 2.6 to 4.6 mg per kg. Yohimbine (Sigma Chemical Company) was used as an antagonist and given intravenously at 0.3 mg/kg. Blood was collected weekly in tubes with ethylenediaminetetraacetic acid (EDTA) (Becton-Dickinson, Rutherford, New Jersey, USA) starting at the day of inoculation. For culture, 0.1 ml of whole blood was inoculated into 7.0 ml of BSK II.

At 10 wk post-inoculation (PI), animals were killed with a lethal dose of sodium pentabarbital (Veterinary Laboratories, Inc., Lenexa, Kansas) given intravenously, and necropsies were performed. Bladder, spleen, liver, kidney, brain, cerebral spinal fluid, synovial fluid, aqueous humor, blood, urine, and skin were collected for culture. Sections of whole tissues approximately 1 cm^3 were ground in a blender with 25 ml of tissue culture medium RPMI-1640 (Sigma Chemical Company). After tissue fragments were allowed to settle, the supernatant was inoculated into 7 ml of BSK II in plastic screw-cap tubes to make final dilutions of 1:10 and 1:100 for each tissue. Spinal fluid, synovial fluid, aqueous humor, blood, and urine were inoculated into 7 ml BSK II to make final dilutions of 1:10 and 1:100. From each animal, five skin samples were taken from both ears using a 5 mm skin biopsy punch (Baker/Cummins Pharmaceuticals, Inc., Miami, Florida, USA). Additional skin samples were taken from the caudal thorax region of deer 2 and deer 4 using the 5 mm biopsy punch. The ear and thorax areas were shaved before the samples were taken. Skin samples were cleaned with hydrogen peroxide and 95% ethanol, then placed in 4.5 ml BSK II in snap-cap tubes.

All cultures were incubated with loosened caps at 33.5 C in 5% CO_2 and examined for spirochetes by dark-field microscopy twice a week for the first 2 wk and then once each week for three more weeks. Spirochetes were identified by an indirect immunofluorescence antibody test (IFA) (Anderson et al., 1985; Magnarelli and Anderson, 1988) with murine monoclonal antibody H5332 (Barbour et al., 1983) and a flu-

orescein-isothiocyanate labeled anti-mouse IgG conjugate (Sigma Chemical Company).

Inoculation and isolation of *Borrelia burgdorferi* from hamsters

Male Syrian hamsters (*Mesocricetus auratus*) (SASCO, Inc., Omaha, Nebraska, USA), aged 6 mo, were used to confirm infectivity of deer inocula. For each deer injected, two hamsters were injected intraperitoneally with 0.5 ml of the same *B. burgdorferi* inoculum.

Hamsters were necropsied at 7-wk PI, and heart blood, urine, bladder, spleen, kidney, liver, brain, and skin were collected for culture. Tissues were prepared as described by Johnson et al. (1984), and tubes of BSK II were inoculated with supernatant fluid from each tissue as described for the deer. Tubes were inoculated with 0.5 ml and 0.05 ml of urine and whole blood. Five punches per ear were taken (Sinsky and Piesman, 1989) and placed in screw-cap tubes (Sarstedt, Inc., Newton, North Carolina) with 1.25 ml BSK II. Cultures were incubated and examined as before.

ELISA

Serum samples collected weekly from inoculated deer and at 0, 2, 5, and 8 wk PI from the control deer were frozen at −10 C. At the end of the study, serum samples from all deer were tested by a modification of ELISA as described by Mahnke et al. (1993). Changes in the protocol were as follows: Antigen was prepared from sonicated *B. burgdorferi*, (A. G. Barbour B31, American Type Culture Collection, Rockville, Maryland, USA) at a protein concentration of 0.31 mg/ml (Greene et al., 1991) and diluted 1:800 in carbonate buffer (pH 9.6). Serum samples were diluted 1:160 in PBS with 0.05% Tween 80 (Fisher Scientific, Pittsburgh, Pennsylvania, USA) (PBS-tween) and tested in triplicate. Two positive control sera and two negative control sera in triplicate at 1:160 dilutions were used on each plate. Positive control sera was obtained from pen-raised deer hyperimmunized with *B. burgdorferi* (Mahnke et al., 1993), and negative control sera were collected from pen-raised deer with no known exposure to *B. burgdorferi*. Each plate also included two enzyme controls consisting of antigen and PBS. Rabbit anti-deer imunoglobulin G (IgG) conjugated to horseradish peroxidase (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Maryland) was diluted to 1:1000. The substrate consisted of o-phenylenediamine (Sigma Chemical Company) diluted 0.1 mg/ml in sodium acetate buffer (pH 4.5) with 0.003% hydrogen peroxide (Sigma Chemical Company).

The cut-off point for positive samples was based on sample/negative control (S/N) optical density ratios at a 1:160 serum dilution as determined for 28 white-tailed deer from the mountain region of Georgia (34°30′N, 84°00′W). Deer were selected from this area based on the lack of reported occurrences of *I. scapularis* on these animals (Mahnke et al., 1993). A S/N that exceeded the mean S/N ratio for the 28 deer plus three standard deviations ($\bar{x}$ + 3 SD = 1.37) was considered positive. Experimental deer samples were recorded as negative or positive. Optical densities at 1:160 dilution were compared to more clearly examine changes in antibody concentration (Schwan et al., 1989). To confirm results, serum samples also were sent for ELISA testing to Dr. L. A. Magnarelli, The Connecticut Agricultural Experiment Station, New Haven, Connecticut, for comparison using the methods of Magnarelli et al. (1991).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting

Antigen proteins were separated in 12% separating and 5% stacking polyacrylamide discontinuous gradient gels as described by Laemmli (1970) by using a Mini-Protean II electrophoresis apparatus (BioRad Laboratories, Richmond, California, USA). Antigen was prepared as described by Greene et al. (1988a, b). Briefly, *B. burgdorferi* isolate JD-1 was grown in BSK II, harvested during the log phase of growth, centrifuged for 30 min (10,000 × *G*), and washed in PBS. Whole-cell sonicate was used as antigen. Protein concentrations of the final sonicate preparation were standardized (BioRad Protein Assay Kit, BioRad Laboratories) at 500 μg/ml in PBS. Fifty microliters of the standardized protein was applied on 0.75 mm-thickness gels.

For immunoblotting, separated proteins were electrotransferred onto nitrocellulose membranes (0.2 μm pore size; BioRad Laboratories) in transfer buffer (25mM Tris-192 mM glycin, 20% methanol) (Towbin et al., 1979). After transfer, the membranes were cut into strips and placed in separate tubes. The strips were incubated in 5% skim milk in Tris-HCL buffer (Sigma Chemical Company) containing 0.1% Tween 20 (Difco Laboratories, Detroit, Michigan, USA) (milk TBS) for 1 hr at 25 C with gentle shaking. Sera were diluted to 1:100 in 5% milk TBS and incubated with the strips at 25 C for 3 hr. After washing the strips with 1% milk TBS, secondary antibody against deer IgG (H&L chain specific) conjugated to alkaline phosphatase (Kirkegaard and Perry Laboratories) was diluted to 1:400 in 5% milk TBS and incubated with the strips 1 hr at 25 C with gentle shaking. After another wash, the strips were developed in 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) substrate (BioRad Laboratories).

Monoclonal antibodies (provided by Alan

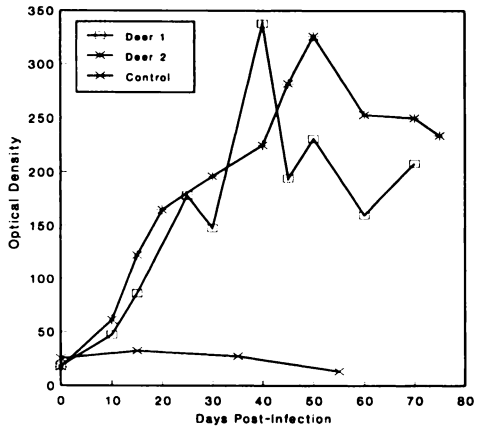

FIGURE 1. Optical densities determined by ELISA for antibodies in white-tailed deer 1 and 2 experimentally infected with isolate SH2-82 of *Borrelia burgdorferi*. Sera were diluted 1:160.

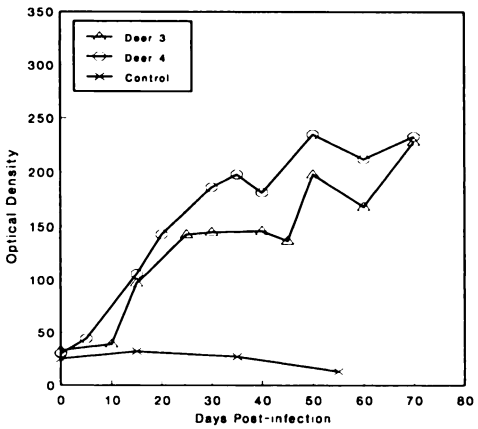

FIGURE 2. Optical densities determined by ELISA for antibodies in white-tailed deer 3 and 4 experimentally infected with isolate JD-1 of *Borrelia burgdorferi*. Sera were diluted 1:160.

Barbour, University of Texas, Austin, Texas) to outer surface protein A (OspA; H5332) 31 kilodaltons (kD) and the flagellin protein (H9724) 41 kD were used to differentiate between adjacent protein bands. The antibodies were diluted at 1:300 in 5% milk TBS and incubated with the nitrocellulose strips for 3 hr at 25 C. After being washed with 1% milk TBS, the strips were incubated in 5% milk TBS containing biotinylated anti-mouse immunoglobulins (Sigma Chemical Company), and then incubated in 5% milk TBS containing alkaline phosphatase-conjugated avidin (BioRad Laboratories) for 1 hr at 25 C. The strips were developed in BCIP/NBP substrate.

RESULTS

Borrelia burgdorferi was cultured at 10 wk PI from a single ear punch biopsy from deer 2 inoculated with SH2-82. This isolate was made from the left ear which had been inoculated with washed spirochetes suspended in PBS. Identification of the culture as *B. burgdorferi* was verified by IFA using the H5332 monoclonal antibody. All other cultures of blood and tissues from injected deer remained negative for 5 wk.

Spirochetes were isolated from the urinary bladder, skin, or kidney from seven of eight hamsters, thereby verifying infectivity of the inocula used to inject the deer.

All deer inoculated with *B. burgdorferi* had a serological response with ELISA. Antibody titers were positive ($S/N \geq 1.37$) by 2 to 3 wk PI and persistent for 10 wk. The control deer was negative by ELISA for the entire study. The results of L. A. Magnarelli (pers. comm.) were identical on positive and negative results, and he found reciprocal titers ranging from 160 to 2,560. Because titers were not determined by us for each serum sample, we compared optical densities obtained from our ELISA testing to examine temporal changes in antibody response. There were distinct differences between the responses to the two *B. burgdorferi* isolates. Deer injected with SH2-82 had a steady rise in circulating antibodies after 1 wk PI, with sharp peaks between 38 and 48 days PI (Fig. 1). Deer injected with JD-1 exhibited a more gradual increase in antibodies which began at 10 days PI and had slight peaks at approximately 50 days PI (Fig. 2). The responses of deer 1 and 2 appeared to be dropping at the end of 10 wk, whereas those of deer 3 and 4 were still increasing. The control deer remained at or below the initial level of all injected deer.

Inoculated deer consistently had an antibody response to nine *B. burgdorferi* antigens: 66, 43 (flagella:41kD), 42, 37, 35.4 (Osp B:34), 31 (Osp A:31), 19.8, 18.5, and

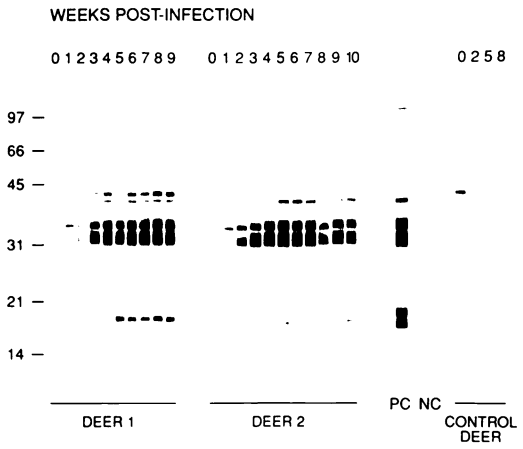

FIGURE 3. Immunoblots of white-tailed deer 1 and 2 experimentally infected with isolate SH-2 of *Borrelia burgdorferi* and of control deer. PC = positive control, NC = negative control. Molecular weight markers (in kilodaltons) are indicated on the left.

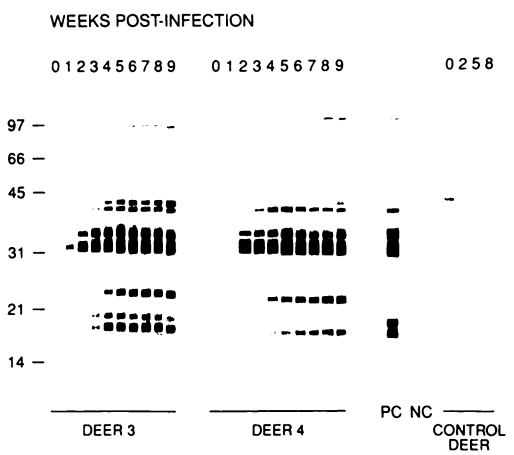

FIGURE 4. Immunoblots of white-tailed deer 3 and 4 experimentally infected with isolate JD-1 of *Borrelia burgdorferi* and of control deer. PC = positive control, NC = negative control. Molecular weight markers (in kilodaltons) are indicated on the left.

17.5 kD proteins (Figs. 3, 4). The Osp A and B antigens were recognized earlier than other antigens, at 1 wk PI for deer 1, 2, and 3 and at 2 wk PI for deer 4. Sera of deer 1, 3, and 4 had antibody against *B. burgdorferi* flagellin proteins, and the intensity of reactivity on this band increased with time PI. Antigens not uniformly recognized by sera from the inoculated deer included 81, 61, 50.6, 40.5 and 23.1 kD. In summary, 12 bands were demonstrated by deer 1; 10 bands by deer 2; 13 bands by deer 3; and 12 bands by deer 4. The flagellin proteins were the only ones recognized by sera from the control deer.

DISCUSSION

The experimental inoculation of white-tailed deer with *B. burgdorferi* resulted in a low rate of recovery of the organism from tissues. Of the several tissues cultured from each deer, only one sample from one deer was positive. The single isolation was made from the skin of an ear that had been injected with washed spirochetes. It is unknown if the spirochetes had multiplied or had remained in place at the site of injection. The low recovery rate may be due to a lack of sensitivity of our culture techniques or to a failure of the spirochete to efficiently multiply in this host. Limitations of our procedures may relate to low numbers of spirochetes present in tissues. In humans, numbers of spirochetes are small, making the culture of organisms difficult (Steere et al., 1983). This may be true for deer as well. A larger amount of inoculum from tissue suspensions as well as longer incubation times may produce a higher rate of recovery. Another possible factor affecting culture success may be the artificial route of infection. Although large doses of viable organisms were administered to the deer via subcutaneous and intradermal injections, the transmission of spirochetes may be enhanced by the natural route of tick feeding as opposed to the artificial route (Ribeiro et al., 1985).

Spirochetes were not isolated from blood samples taken weekly from any deer. *Borrelia burgdorferi* spirochetemias often are low in wild and experimental animals (Barbour and Hayes, 1986; Kazmierczak et al., 1988; Greene et al., 1988b) including deer (Bosler et al., 1984). The first blood samples taken at 7 to 10 days PI may have missed early peaks of circulating organisms.

In a related study, laboratory-reared *I. scapularis* were allowed to feed on deer 2

at 43 and 69 days PI and on deer 4 at 35 and 61 days PI (Oliver et al., 1992). Of the 89 larvae, nymphs, and adult ticks which fed on these animals, only 8 (9.0%) tested positive in polymerase chain reaction assays. Considering the competence of this vector and the sensitivity of this assay, this is a very low level of transmission and does not determine spirochete viability. Further experimental studies should be done to determine tick transmission of *B. burgdorferi* to deer and the ability of ticks fed on infected deer to transmit Lyme borrelia to other animal hosts.

Experimentally inoculated deer mounted specific serologic responses detectable by ELISA and confirmed by immunoblotting. The responses occurred rapidly and increased to significant peaks by 6 to 7 wk PI in all deer. These patterns are similar to those in dogs experimentally inoculated with *B. burgdorferi* cultures (Appel et al., 1993). These animals showed a strong antibody response between 1 and 4 wk PI which eventually declined to minimal levels after 6 mo. In the same study, tick-infected dogs had a slower but more persistent response. We did not keep the *B. burgdorferi* inoculated deer longer than 10 wk PI, so long-term responses could not be assessed. However, antibody response appeared to be declining in deer 3 and 4 by the end of the study.

All deer inoculated with *B. burgdorferi* also demonstrated a well-defined response in immunoblots. The first proteins detected in deer sera were present 7 to 14 days PI, which was earlier than the initial positive result by ELISA. Antibody responses of all the deer generally were consistent; however, differences were noted that possibly reflect variation between isolates or individuals. For example, deer 3 and 4 showed dark bands at 23.1 kD and at 97 kD whereas deer 1 and 2 reacted to these proteins lightly or not at all. The variations evident on the immunoblots and ELISA may be due to different proteins in the isolates or to inoculum viability. Counting spirochetes with darkfield microscopy is not highly accurate so the actual numbers of viable organisms inoculated may have varied. Although the number of passages was low for both isolates, antigenic changes have been noted with in vitro cultivation of *B. burgdorferi* (Schwan et al., 1988).

Antibodies to OspA and OspB were the earliest to appear which is in contrast to deer exposed naturally to *B. burgdorferi*. Field sera from white-tailed deer that were tested by Gill et al. (1993) did not react to the outer surface proteins. Similarly, immunoblots of experimentally infected dogs show patterns different from those of dogs exposed via ticks, either naturally (Greene et al., 1988b) or experimentally (Appel et al., 1993). Although the response did not occur in these dogs as early as in the deer, dogs inoculated with *B. burgdorferi* cultured in vitro had strong reactions to OspA and OspB. Dogs infected by ticks react to these proteins only occasionally. Inbred mice experimentally infected with *B. burgdorferi* also showed similar differences. Mice that were needle-inoculated reacted to the outer surface proteins whereas tick-infected mice did not (Gern et al., 1993). In humans with natural infections of Lyme disease, antibodies to these outer surface proteins occurred later in the infection, and antibodies to the flagella antigens were the first to appear (Craft et al., 1986). Thus the methods of experimental inoculations could be responsible for this contrast in responses. The chemical treatment and washing of organisms, large number of organisms given in a single dose, route and frequency of infection are some factors which may influence this effect (Greene et al., 1988b; Gern et al., 1993).

The chief role of white-tailed deer in Lyme disease epizootiology appears to be as the primary host of the adult tick stage (Anderson, 1988). Attempts to culture *B. burgdorferi* from deer blood and tissues have been unsuccessful (Bosler et al., 1983, 1984; Loken et al., 1985), and field studies indicate that white-tailed deer may be incompetent reservoirs for Lyme disease in

the field (Telford et al., 1988). Spirochetes have been observed in nymphal and adult ticks which have fed upon deer (Magnarelli et al., 1986; Kocan et al., 1992; Amerasinghe et al., 1992), but it is likely that these ticks picked up *B. burgdorferi* from feeding on other hosts, particularly mice. That deer react to *B. burgdorferi* in a manner detectable by routine serologic testing is well established (O'Connell et al., 1986; Magnarelli et al., 1991). Suggestions that this information be used in surveillance programs to detect potential foci of infection for humans are valuable, but serologic analyses should not be used alone. As others recommend (Magnarelli et al., 1991; Daniels et al., 1993), results from testing deer for Lyme disease should be used in conjunction with other data such as vector detection and distribution, occurrence of human and domestic animal cases, and recognition of reservoir hosts.

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