EXPERIMENTAL BORRELIA BURGDORFERI INFECTION IN PEROMYSCUS LEUCOPUS

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ABSTRACT

We evaluated the susceptibility of laboratory-reared adult and infant white-footed mice (Peromyscus leucopus) to a known pathogenic isolate of Borrelia burgdorferi (N40). Two month-old and 3-day-old Peromyscus were inoculated intradermally with 10k’ to 10 spirochetes. At 21 days for adults or 30 days for infants post inoculation, mice were killed, and tissues were cultured for spirochetes and examined microscopically. Based on serology and culture, adult mice became infected but did not have any gross or microscopic lesions. Mice inoculated as infants became infected, and also developed carditis and multifocal arthritis. Contact transmission between inoculated infants and their naive mothers was not observed. Age at inoculation appeared to be a critical factor in inducing Lyme borreliosis lesions in Peromyscus leucopus, as in other species.

Key words: Peromyscus leucopus, white-footed mice, Borrelia burgdorferi, spirochete, Lyme disease, Lyme borreliosis, arthritis, carditis.

INTRODUCTION
Lyme borreliosis is a complex of clinicopathologic disorders in humans and animals caused by the tick-borne spirochete, Borrelia burgdorferi (Steere et al., 1983). In the northeastern United States, nymph and larval stages of the tick vector, Ixodes scapularis (formerly dammini), have the white-footed mouse (Peromyscus leucopus) as the preferred host (Boster et al., 1983; Levine et al., 1985). Wild-caught and experimentally-inoculated Peromyscus spp. appear to be persistently infected with B. burgdorferi without apparent adverse effects upon the host (Anderson et al., 1986; Wright and Nielsen, 1990). However, erythematous lesions (Anderson and Magnaretli, 1984) and cystitis (Czub et al., 1992) in spirochete-infected Peromyscus have been described.

We previously reported that rats, mice, hamsters and rabbits were susceptible to infection and disease when inoculated as infants with a known pathogenic strain of B. burgdorferi (Moody et al., 1990a, b); in addition, rats and mice were susceptible to infection and disease when inoculated as weanlings (Barthold et al., 1988). Our objective was to determine the susceptibility of adult and infant Peromyscus leucopus to a documented infective and pathogenic B. burgdorferi isolate.

MATERIALS AND METHODS

Ten 2-mo-old (sexually mature) Peromyscus leucopus were obtained from the Harvard School of Public Health (Boston, Massachusetts, USA). Four pregnant P. leucopus females were purchased from the Peromyscus Genetic Stock Center (University of South Carolina, Columbia, South Carolina, USA). Mice were at least second or third generation laboratory-reared and were free of B. burgdorferi infection, according to the vendors’ quality assurance monitoring reports. Young adults were group housed in plastic cages within a flexible film isolator (Standard Safety Equipment Company, Palatine, Illinois, USA). Pregnant females were housed individually in Micro-Isolator cages (Lab Products, Maywood, New Jersey, USA). Sterilized food (Agway, Syracuse, New York, USA) and water
were provided ad libitum to all animals. We used an isolate of B. burgdorferi (N40) cultured from the mid guts of naturally infected nymphal I. scapularis captured in Westchester County, New York (41°30'N, 73°30'W) in 1987 (Barthold et al., 1988). Spirochetes were passed twice and grown in modified Barbour-Stoenner-Kelly (BSK II) medium (Barbour, 1984) at 34°C to a concentration of about 10^8 viable organisms/ml for inoculation, as determined by counting in a Petroff-Hausser bacterial counting chamber (Baxter Diagnostics Incorporated, Scientific Products Division, McGaw Park, Illinois) under darkfield microscopy. The outer surface proteins (osp A and osp B) gene sequences of 156 JOURNAL OF WILDLIFE DISEASES, VOL. 30, NO. 2, APRIL 1994 this isolate have been defined and are typical of Group I (B31) isolates of B. burgdorferi (Sears et al., 1991; Fikrig et al., 1993). We used this N40 isolate since it is infective and pathogenic for laboratory rats, mice, hamsters and rabbits (Moody et al., 1990a, b); in addition, its tow passage history ensured its virulence which may be lost after prolonged in vitro passage (Moodu et al., 1990b). Eight 2-mo-old Peromyscus leucopus of both sexes were inoculated intradermally (ID) while under methoxyflurane anesthesia (Metofane, Pitman-Moore, Inc., Washington Crossing, New Jersey) with 0.1 ml of BSK II medium containing about 2.6 x 10^8 spirochetes. Two additional 2-mo-old male Peromyscus received an equal volume of sterile medium ID. The four pregnant P. leucopus delivered a total of 20 live pups. At 3 days of age, all pups in each litter were individually inoculated with 10^9 N40 in 0.1 ml BSK II medium ID. At either 21 days (adults) or 30 days (infants and their mothers) post-inoculation, the mice were killed with carbon dioxide gas and exsanguinated. Although we had intended to examine both groups of mice at the same interval, a lack of medium components precluded examination at 21 days. However, we have documented that peak lesions following N40 inoculation in laboratory rats and mice occurred during post inoculation days (PID) 14 to 30 without any significant variability observed within that period (Barthold et al., 1990, Moody et al., 1990a). To avoid wastage of inoculated animals, we proceeded to examine mice inoculated as infants at 30 days. At necropsy, the ventral
surface of each mouse was wiped with 70% ethanol prior to opening the body cavity to aseptically collect internal organs for culture. Kidney, spleen, brain, urinary bladder, articular tissue, blood, urine (adults) and ear punches were cultured for B. burgdorferi. The ear pinnae were wiped thoroughly with an alcohol sponge prior to obtaining punches (Fisher Scientific, Springfield, New Jersey, USA) for culture. All instruments were wiped with 70% ethanol and flamed prior to tissue collection for culture. Tissues were diluted 1:10 (w/v) in BSK II medium and homogenized with sterile Tenbroeck grinders (VWR Scientific, Piscataway, New Jersey). Duplicate 0.5 ml aliquots were placed in 7 ml BSK II medium. If the urinary bladder contained urine, 0.2 ml urine was collected by aspiration into a sterile tuberculin syringe with a 27 gauge needle and inoculated into 7.5 ml BSK II medium. Similarly 0.2 ml blood or rongeur-excised tissue from the left tibiotarsal joint were each placed directly into a single tube containing 7.5 ml medium. After incubation at 35 C for 14 days, cultures were examined for spirochetes by dark field microscopy (Barthold et at., 1988; Moody, 1990a). Forty high power fields were scanned per culture. Positive cultures had between one and one hundred B. burgdorferi, whereas negative cultures had no organisms. Mice were considered infected if at least one tissue was culture-positive. We have previously determined that if cultures from N40-inoculated animals are maintained and examined 6 wk later, no negative cultures subsequently became positive (K. Moody, unpubl.). Brain, heart and joints (shoulder, elbow, carpus, metacarpus, hip, knee, tarsus, metatarsus and phalanges) were immersion-fixed in 10% neutral buffered formalin (pH 7.2). Joints were demineralized in decalcifying solution (S/P Decalcifying Solution, Baxter Diagnostics Incorporated, Scientific Products Division, McGaw Park, Illinois). Tissues were embedded in paraffin, sectioned at 5 tim, and stained with hematoxylin and eosin. Serum immunoglobulin G (IgG) antibody to B. burgdorferi was determined with an enzymelinked immunosorbent assay (ELISA) using N40 spirochetes as antigen (Moody eta!, 1990a). The N40 spirochetes were washed twice with sterile phosphate buffered saline (PBS), pH 7.5, and
adjusted to a protein concentration of 75 ug/ml (Moody et al., 1990a). Ninety-six well plates (Nunc Immuno Plate, MaxiSorp F96, USA Scientific Plastics, Ocala, Florida, USA) were coated overnight at 37°C with 50 ll per well of either PBS or antigen diluted 1:30 in PBS. To block binding sites not covered by antigen, 200 ll of PBS containing 3% gelatin were added to each well and incubated at 37°C for 1 hr. Plates were then washed three times in PBS with 0.05% tween which was used for all subsequent washes (PBS-tween, Bio Rad Laboratories, Richmond, California, USA). Sixty microliters of two-fold dilutions of serum starting with 1:80 in PBS containing 0.5% bovine serum albumin were added to wells. After a 1-hr incubation and three washes, 60 ll of unconjugated rabbit anti-Peromyscus immunoglobulins at 1:2500 were added to all wells. After a 1 hr incubation and three washes, 60 ll of biotinylated goat anti-rabbit IgG at 1:15,000 were added. After a 1 hr incubation and three washes, 60 ll of peroxidase-labelled avidin (Cappel Laboratories, Cochranville, Pennsylvania, USA) at 1:15,000 were added. An incubation period of 1 hr and three washes followed. Sixty microliters of 3',5,5-tetramethylbenzidine (TMB, Kirkegaard and Perry Laboratories, Inc, Gaithersburg, Maryland, USA) were added to all wells. After 10 mm, 60 ll of 1 N HC1 were added, and absorbance at 450 nm was recorded (MR600 Spectrophotometer, Dynatech Laboratories, Alexandria, Virginia, USA). Serum titers were considered significant if they were more than two standard deviations above the mean 

RESULTS

The N40 isolate was infectious for young adult Peromyscus as indicated by serology and culture at PID 21 (Table 1). At least one, and up to five, organs per mouse were culture-positive for B. burgdorferi. Kidney tissue had the highest prevalence of positive cultures (seven of eight), with three of the eight spleens, ear punches, and urinary bladders being positive followed by two of the seven joints tested and one of
eight for both blood and brain samples. Of the two urine 
samples available at necropsy, neither had detectable levels of 
spirochetes in culture. Both control mice were culture-negative 
for all organs. Alt eight mice inoculated as young adults 
developed IgG antibodies to B. burgdorferi with the 
range of titers from 1:5120 to 1:81,920, with a geometric mean 
titer (GMT) and standard error of the mean (SEM) of 13,512 
± 25. Both inoculated and control groups of young adult 
Peromyscus remained clinically normal throughout the 
experiment and had normal tissues on gross and microscopic 
examination. None of the inoculated Peromyscus had any 
lesions at the inoculation site. Infant-inoculated Peromyscus 
were susceptible to both infection and disease caused by B. 
burgdorferi (Table 1). One mother cannibalized her litter, leaving 
15 B. burgdorferi-inoculated pups for examination. 
Spleen, ear punches, kidney, and urinary bladder were the most 
common organs from which B. burgdorferi was isolated, 
although spirochetes also were cultured from the tibiotarsal 
joint, brain, and blood. At PID 30, all infant-inoculated P. 
leucopus had histologic evidence of arthritis in multiple joints. 
Arthritis was particularly common in the tibiotarsal joints. 
Microscopic joint lesions consisted of synovial hypertrophy and 
hyperplasia with exudation of fibrin and neutrophils into the 
joint spaces (Figs. 1, 2). Mice also had inflammation of tendons, 
ligaments, tendon sheaths and bursae. All mice had at least 
one joint severely affected histologically, with 13 of 15 having 
arthritic lesions in more than one peripheral joint. Naive 
mothers in contact with their B. burgdorferi- inoculated pups 
were culture negative at necropsy; alt tissues were normal on 
gross and microscopic examination. FIGURE 1. Elbow joint from 
Peromyscus leucopus inoculated at 3 days of age with Borrelia 
burgdorferi and examined 30 days later. There is periarticular 
inflammation with exudation of fibrin and neutrophils into the 
joint space. H&E stain. Bar = 190 zm. 

DISCUSSION

Prior to 1987, wild-caught Peromyscus were described as having 
one or more organs culture-positive for B. burgdorferi but
without any of the cardiac, neurologic or arthritic sequelae of human Lyme borreliosis (Levine et al., 1985; Anderson et al., 1987a). Excepting one report of spirochete-positive erythematous skin lesions in Peromyscus (Anderson and Magnarelli, 1984), no other pathologic abnormalities had been reported. Several investigators have attempted to induce infection and disease with Borrelia burgdorferi in Peromyscus species. Burgess and Patrican (1987) described oral nasal inoculation of P. maniculatus and B. burgdorferi following which six of 10 mice developed hind limb lameness; mice developed antibodies, but all tissues were grossly and histologically normal without evidence of spirochetes. Burgess et al. (1990) also described neurologic lesions in wild-caught P. leucopus, and attributed the motor dysfunction to B. burgdorferi; however, all potential etiologic agents were not systematically ruled out. In contrast, Wright and Nielsen (1990) experimentally infected laboratory-reared P. leucopus with live B. burgdorferi from several sources. Inoculation was by subcutaneous and oral routes; contact, tick attachment, venereal, and placental transmission studies also were conducted. Although all inoculated mice developed antibodies and B. burgdorferi was identified histologically in the spleen, kidney and liver, none of the mice had any clinical or pathologic changes. The sole lesion reported to date in B. burgdorferi-inoculated P. leucopus has been cystitis (Czub et al., 1992).

In contrast to these previous reports, we found that infant, but not young adult, P. leucopus were susceptible to both infection and arthritis induced by B. burgdorferi. The age of the host at challenge appeared to be critical in the development of Lyme borreliosis lesions in this and other species. Many of the previously cited reports either omitted the age of their Peromyscus spp., or described sexually mature adults. In other laboratory animal species, several investigators reported antibody formation and spirochete recovery following B. burgdorferi inoculation of non-infant animals but with minimal lesion development (Benach et al., 1984; Duray and Johnson, 1986). We previously demonstrated that infant rats, mice, hamsters and three-week-old rabbits developed multisystem infection as well as arthritis and carditis in <30 days when inoculated with low passage B. burgdorferi spirochetes.
In addition, compared with weanling Lewis rats, Lewis rats inoculated as neonates had greater spread and persistence of spirochetes, as well as a higher frequency of gross and microscopic arthritis (Barthold et al., 1988). We also reported that several inbred mouse strains uniformly developed acute polyarthritis when inoculated with B. burgdorferi at 3 days of age; however, when inoculated as weanlings, the severity of polyarthritis and carditis become genotype-dependent (Barthold et al., 1990). The arthritis seen in neonataly-inoculated P. leucopus was similar to the lesions described in hamsters, rabbits, rats and mice (Moody et al., 1990b). The white-footed mice, however, developed no

Cardiac or neurologic abnormalities; thus, variation in species susceptibility may underlie the lack of cardiac pathology. Urinary bladders were not examined. In this study, recovery of B. burgdorferi from multiple organs was successful. The percentages of positive spirochete cultures from blood, brain and kidney were comparable for both neonates and adults, with much higher recoveries from the spleen, ears, bladder and joint in the younger mice. Spirochete recovery may be species related to some degree inasmuch as we have found better spirochete recovery from rat joint biopsies than from mice (Barthold et al.1990). Peromyscus had a low incidence of positive blood cultures; however, spirochetemia has been an inconsistent finding in this and other species (Anderson et al., 1987b, Moody et al., 1990b), and blood was cultured at a single time point. Urine and bladder cultures have been variably successful indicators of B. burgdorferi infection in Peromyscus leucopus. Bosler and Schutze (1986) reported a 50% incidence of spirocheturia in P. leucopus captured in Shelter Island, New York; however, concomitant infection of B. burgdorferi with Babesia microti may have affected the results. In our study, neither of the two available urine samples from adult-inoculated mice were positive for B. burgdorferi. Other investigators have reported negative urine cultures in P. leucopus, even when the same animals had positive urinary bladder cultures (Schwanet al., 1988; Callister et al., 1989). Indeed, urinary bladders appear to be a valid indicator
of spirochetal infection in Peromyscus. We found that three of eight adults and 10 of 15 infants had positive bladder cultures. Other investigators have reported 57 to 100% positive bladder cultures from P. leucopus naturally or experimentally infected with B. burgdorferi (Callister et al., 1989; Czub et al., 1992). Based on the variability between animals and laboratories in spirochete recoveries, we believe that spleen, ear, kidneys, urinary bladder, and perhaps the tibiotarsal joints, should be cultured or that other specific diagnostic procedures, such as polymerase chain reaction, be included (Barthold et al., 1991, Hofmeister et al., 1992). An additional interesting finding was that although the P. leucopus mothers were housed with their infected titters for 30 days, none of the three mothers examined had positive cultures or lesions to indicate that contact transmission had occurred, although this phenomenon previously has been described in this species (Burgess et al., 1986; Wright and Nielsen, 1990). Despite the unequal observation period following B. burgdorferi inoculation of different aged mice, we believe that the findings are significant. As evidenced by arthritis and carditis development in infants, P. leucopus were not inherently resistant to disease associated with B. burgdorferi. When inoculated as infants, and presumably prior to immune system maturation, many laboratory animal species are susceptible to infection, arthritis, and carditis characteristic of Lyme borreliosis. Susceptibility of neonatal or immunosuppressed animals to pathogens is not unique to B. burgdorferi; however, this is the first time that arthritis and carditis have been elicited in this species, substantiating the importance of age at initial infection. Variation in the virulence and passage level of spirochete isolates used for animal inoculation could be additional factors in previous failures to induce similar Lyme borreliosis lesions in these mice. The total spirochete dose we used for Peromyscus inoculations was comparable to that used by Schwan et al. (1988) and Czub et al. (1992) where no arthritic or cardiac abnormalities were described. This spirochete inoculum may be larger than that acquired naturally in tick-infested Peromyscus spp.; however, we documented that for inbred mice, once a critical number of B. burgdorferi has been administered the total dose is irrelevant for subsequent infection and disease production.
(Barthold, 1991). If wild P. leucopus are naturally infected with B. burgdorferi 160 JOURNAL OF WILDLIFE DISEASES, VOL. 30, NO. 2, APRIL 1994 138. , D. H. PERSING, A. L. ARMSTRONG, AND R. as infants, the resultant pathologic changes herein described could diminish their viability and make them susceptible to increased tick loads, thereby facilitating transmission of this spirochete to other wild, vertebrate hosts, humans, and domestic animals.