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# 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 $10^6$ to $10^7$ 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, nymphal and larval stages of the tick vector, *Ixodes scapularis* (formerly *dammini*), have the white-footed mouse (*Peromyscus leucopus*) as the preferred host (Bosler 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 Magnarelli, 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 midguts 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 $10^7$ 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

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 low passage history ensured its virulence which may be lost after prolonged in vitro passage (Moody 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 $2.6 \times 10^7$ 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 $10^6$ 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 postinoculation 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 al., 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 μm, and stained with hematoxylin and eosin.

Serum immunoglobulin G (IgG) antibody to *B. burgdorferi* was determined with an enzyme-linked immunosorbent assay (ELISA) using N40 spirochetes as antigen (Moody et al., 1990a). The N40 spirochetes were washed twice with sterile phosphate buffered saline (PBS), pH 7.5, and adjusted to a protein concentration of 75 μg/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 μl per well of either PBS or antigen diluted 1:30 in PBS. To block binding sites not covered by antigen, 200 μl 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 μl of unconjugated rabbit anti-*Peromyscus* immunoglobulins at 1:2500 were added to all wells. After a 1 hr incubation and three washes, 60 μl of biotinylated goat anti-rabbit IgG at 1:15,000 were added. After a 1 hr incubation and three washes, 60 μl of peroxidase-labelled avidin (Cappell 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 min, 60 μl of 1 N HCl were added, and absorbance at 450 nm was recorded (MR600 Spectrophotometer, Dynatech Laboratories, Alexandria, Virginia, USA). Serum titers were considered sig-

TABLE 1. Infectivity and pathogenicity of *B. burgdorferi* (N40) for infant and young adult *Peromyscus leucopus* 30 days following intradermal inoculation.

| Age | Inoculum | Arthritis | Carditis | *B. burgdorferi* culture | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Spleen | Blood | Brain | Joint | Ear | Kidney | Bladder |
| Infant | N40 | 15/15[a] | 0/15 | 15/15 | 2/15 | 2/15 | 8/15 | 13/13 | 14/15 | 10/15 |
| Young adult | N40 | 0/8 | 0/8 | 3/8 | 1/8 | 1/8 | 2/7 | 3/8 | 7/8 | 3/8 |
| Infants' mothers | Control | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/2 | 0/3 | 0/3 |

[a] Number positive/number examined.

nificant if they exceeded by three standard deviations the mean values of sera from 10 uninfected mice at a dilution of 1:80 or greater. No significant variation occurred in reproducibility tests.

## 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. All 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; all tissues were normal on gross and microscopic examination.

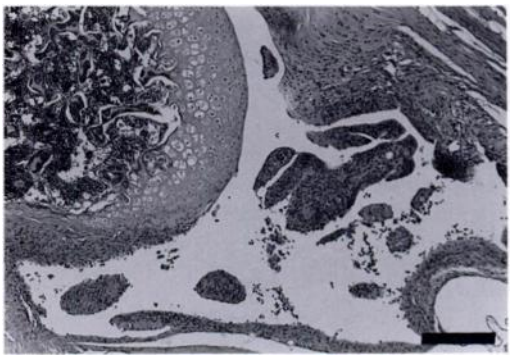

FIGURE 1. Elbow joint from *Peromyscus leucopus* inoculated at 3 days of age with *Borrelia burgdorferi* and examined 30 days later. There is periarticular inflammation with exudation of fibrin and neutrophils into the joint space. H&E stain. Bar = 190 μm.

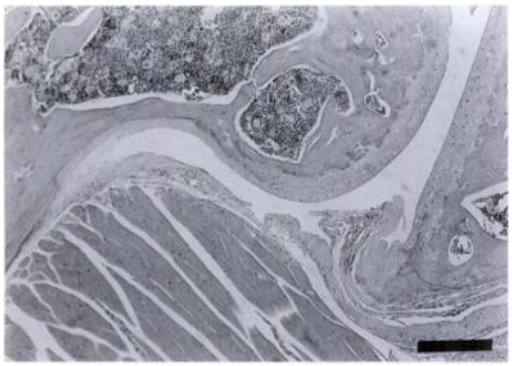

FIGURE 2. Elbow joint from *Peromyscus* inoculated as a young adult with *Borrelia burgdorferi* and examined 21 days later. In contrast to *Peromyscus* inoculated at 3 days of age (Fig. 1), there is no evidence of inflammation. H&E stain. Bar = 190 μm.

## 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 sequellae 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 (Moody et al., 1990b). 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 neonatally-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 car-

diac 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 Schulze (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 (Schwan et 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. burgdorferia* (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 litters 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*

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.

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