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SUSCEPTIBILITY OF THE GRAY WOLF (*CANIS LUPUS*) TO INFECTION WITH THE LYME DISEASE AGENT, *BORRELIA BURGDORFERI*

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ABSTRACT: Four juvenile gray wolves (*Canis lupus*) were inoculated with live *Borrelia burgdorferi*. One received an intravenous inoculum, a second was inoculated subcutaneously, and two more were fed *Peromyscus maniculatus* sucklings which had earlier been inoculated with *B. burgdorferi*. The intravenously inoculated wolf developed a generalized lymphadenopathy and a persistent serum antibody titer to the spirochete which peaked at 1:512. *Borrelia burgdorferi* was visualized in liver sections of this wolf using direct immunofluorescent staining. The subcutaneously inoculated wolf showed a low and transient antibody response which peaked at 1:64, and manifested no clinical or postmortem abnormalities. The wolves which were fed inoculated mice showed no detectable antibody response. They were clinically normal throughout the project, and there were no detectable lesions at necropsy. Two control wolves were inoculated intravenously with formalin killed *B. burgdorferi*. Serum antibody titers of these controls peaked at 1:64 and 1:32, respectively, and fell to 1:16 by day 48 postinoculation. A survey of serum samples from 78 wild-trapped wolves from Wisconsin and Minnesota revealed that one was positive and another was suspect for *B. burgdorferi* infection based on presence of antibody to the spirochete. We conclude that the wolf is susceptible to infection by *B. burgdorferi* and that wolves are being infected in the wild.

Key words: *Canis lupus*, *Borrelia burgdorferi*, Lyme disease, experimental infection, serologic survey.

INTRODUCTION

A recently discovered spirochete, *Borrelia burgdorferi*, has been shown to be the agent responsible for human Lyme disease (Burgdorfer et al., 1982; Steere et al., 1983). Evidence of *B. burgdorferi* infection also has been found in a wide range of wild and domestic animal species, with sequellae ranging from apparently asymptomatic infections in some species, to ocular and arthritic complications in others (Magnarelli et al., 1984; Burgess et al., 1986a, b). Of particular interest to this report is the susceptibility of the domestic dog to clinical disease caused by *B. burgdorferi* infection. This can take the form of fever, lymphadenopathy, arthralgia, and arthritis (Lissman et al., 1984; Kornblatt et al., 1985).

The principal vertebrate reservoirs of *B. burgdorferi* are *Peromyscus* spp. and white-tailed deer (*Odocoileus virginianus*) (Bosler et al., 1984; Levine et al., 1985). The spirochete is primarily spread by the bite of infected ixodid ticks. *Ixodes dammini*, known regionally as the bear tick or the deer tick, appears to be the main vector in the United States although the spirochete also has been found in other arthropods (Burgdorfer et al., 1982; Magnarelli et al., 1986). Oral inoculation of the organism produces infection in *Peromyscus* spp. (Burgess and Patrican, 1987), and contact transmission of *B. burgdorferi* in dogs and in *Peromyscus* spp. has recently been demonstrated under laboratory conditions (Burgess, 1986a; Burgess et al., 1986a). Shedding of the spirochete in the urine of *Peromyscus* sp. also has been shown to occur (Bosler and Schulze, 1986).

The susceptibility of the domestic dog, coupled with the fact that the range of *I. dammini* overlaps with the range of the gray wolf in the upper Midwest (Berg and Kuehn, 1982; Davis et al., 1984), prompted this investigation of whether gray wolves

(*Canis lupus*) may also be at risk of becoming infected with *B. burgdorferi*. If so, it could be a contributing factor to mortality in this endangered species. Furthermore, oral infection, if it could be shown to occur in wolves, would greatly increase their chances of exposure since these predators are known to prey on white-tailed deer and *Peromyscus* spp. (Mech, 1970); both these species are known reservoirs of *B. burgdorferi*.

Our objectives in this investigation were to determine if: (1) the gray wolf is susceptible to *B. burgdorferi* infection, (2) feeding on infected prey could produce infection in wolves, and (3) wolves in the wild were being exposed to the spirochete.

MATERIALS AND METHODS

Four juvenile male wolves (*Canis lupus*) were used for attempts to establish the infection with live *B. burgdorferi*. The original source of the animals was the U.S. Fish and Wildlife Service wolf colony (U.S. Fish and Wildlife Service, Twin Cities, Minnesota 55111, USA) and they were subsequently maintained by the Wisconsin Department of Natural Resources (Madison, Wisconsin 53707, USA). They were approximately 6 mo old, weighed between 21 and 27 kg, and had never received any type of vaccination. After the infection attempts were completed, a male and female juvenile wolf from the same sources as the original four were added to the study to serve as controls. The latter two animals were approximately 5 mo old and had been vaccinated against canine parvovirus. All wolves were kept in arthropod-free rooms, in individual dog runs which had slatted floors to prevent any contact with urine or feces from the adjacent run. Two wolves were housed per room, each wolf in an individual run. They were fed commercial puppy chow (Purina Puppy Chow, Ralston Purina Company, St. Louis, Missouri 63164, USA).

Restraint for parenteral inoculations and blood collections was accomplished with an intramuscular dose of a mixture of ketamine hydrochloride (Ketaset, Bristol Laboratories, Syracuse, New York 13221, USA) at 10 mg/kg and xylazine hydrochloride (Rompun, Haver-Lockhart, Shawnee, Kansas 66203, USA) at 0.7 mg/kg. This produced a light plane of anesthesia lasting 20 to 30 min. All wolves were negative for the presence of serum antibodies to *B. burgdorferi* and negative on blood culture for the spirochete when tested 9 and 17 days prior to beginning the project.

Inoculation and sampling

The *B. burgdorferi* used for all inoculations was originally isolated from the blood of a *Peromyscus leucopus* trapped at Ft. McCoy, Wisconsin (USA; 44°00′N, 90°40′W) and maintained through 15 to 20 culture passages in BSK II media (Barbour, 1984). Spirochete counts for the inocula were determined by the method of limiting dilution, using darkfield microscopy for a visual count of the organisms.

Two of the wolves were inoculated parenterally with live *B. burgdorferi*. Wolf number 1 received approximately 8,000 live spirochetes in phosphate buffered saline (PBS) by intravenous (i.v.) cephalic injection. Wolf number 2 was injected subcutaneously (s.c.) in two sites lateral to the dorsal midline with approximately 4,000 live organisms in PBS per site. Wolves number 3 and number 4 were fed suckling *Peromyscus maniculatus* which had been injected s.c. with approximately 3,000 live organisms in PBS. These mice were inoculated about 1 hr prior to the wolf feedings. Wolf number 3 ate four of the suckling mice and wolf number 4 ate six of them.

In order to determine serum antibody response to *B. burgdorferi* antigen, the two control wolves were inoculated with killed whole *B. burgdorferi* organisms. Cultures were killed by the addition of formalin to a final formalin concentration of 2%. The culture tubes were mixed thoroughly and kept at 22 C for 12 hr. In order to insure that all spirochetes were dead, 0.1 ml aliquots of the formalinized cultures were then inoculated into tubes of fresh BSK II media which were incubated at 34 C and examined weekly for borrelial growth. The formalinized cultures were inspected also for motility by darkfield microscopy. The formalin killed spirochetes were pelleted by centrifugation (9,000 *g* for 20 min) and then washed twice in PBS prior to their final resuspension in PBS. Both wolf number 5 and number 6 received approximately 8,000 dead spirochetes by i.v. cephalic injection.

Blood was drawn at 7 to 14 day intervals for determination of *B. burgdorferi* antibody titers and for culturing. The animals also were given physical examinations at this time; these consisted of assessing mucous membrane color, pulse quality and regularity, lymph node and tonsil size, and abdominal palpation.

Serology and bacteriology

Antibody titers to *B. burgdorferi* were determined by means of an indirect fluorescent antibody test (IFA), using a method previously

described (Steere et al., 1983). The whole-cell antigen used was culture passage seven of the Ft. McCoy isolate of *B. burgdorferi*. Fluorescein isothiocyanate (FITC)-conjugated goat anti-dog IgG (Cappell Worthington Laboratories, Malvern, Pennsylvania 19355, USA) at a dilution of 1:40 was utilized for the test. This conjugate previously had reacted equally with dog serum and wolf serum in a gel immunoprecipitation test (Roitt, 1984). Positive controls consisting of high-titered dog serum, and negative controls which consisted of both known negative dog serum and preinoculation wolf serum were run for each IFA test. The titer was considered to be the serum dilution at which definite fluorescence was observed.

Borrelia burgdorferi isolation attempts were performed by inoculating 0.1 ml of whole blood, citrated blood, and serum into duplicate culture tubes of BSK II media. All culture tubes were incubated at 34 C and checked twice weekly for 6 wk by darkfield microscopy.

Lymph node aspiration

On day 36 postinoculation (PI), the right popliteal lymph node of the i.v. inoculated wolf (wolf number 1) was aspirated. The area was first shaved and aseptically prepared. The node was aspirated using a 20 gauge needle. The aspirate was examined by darkfield microscopy and direct immunofluorescent staining using FITC-conjugated rabbit anti-*B. burgdorferi* serum (supplied by W. Burgdorfer, Rocky Mountain Laboratories, Hamilton, Montana 59840, USA). Portions of the aspirate were inoculated into BSK II media and streaked onto a 5% sheep blood agar plate.

Postmortem examination

Upon completion of the project (day 75 PI for wolves number 1 and number 2; day 61 for wolves number 3 and number 4; day 48 for wolves number 5 and number 6) all animals were euthanized by i.v. barbiturate (Beuthanasia-D; Schering Corporation, Kenilworth, New Jersey 07033, USA) overdose and necropsied. Samples from wolves numbers 1, 2, 3, and 4 were collected for histopathology and for *B. burgdorferi* isolation attempts. These included kidney; spleen; popliteal, prescapular and mesenteric lymph nodes; duodenum; heart; synovium; synovial fluid; urine; and vitreous. Tissue samples were triturated with 2 cc of BSK II media in Tenbroeck tissue grinders (Bellco Glass, Vineland, New Jersey 08360, USA) and 0.1 ml of each of these suspensions was inoculated into culture tubes. Formalin fixed, paraffin embedded sections of the tissues were mounted on slides and stained with hematoxylin and eosin. In addition, formalin fixed, paraffin embedded sections were cut, slide-mounted, de-paraffinized, and stored at −70 C. These were later examined for the presence of *B. burgdorferi* by direct immunofluorescent staining using the anti-*B. burgdorferi* conjugate.

Serologic survey

Serum samples from 78 wild-trapped wolves were provided by the Wisconsin and Minnesota Departments of Natural Resources and were tested for the presence of antibodies to *B. burgdorferi* by the IFA test. These wolves had been trapped in Wisconsin and Minnesota (USA; 46°15′ to 47°42′N, 92°10′ to 94°05′W) between 1977 and 1984. Since *B. burgdorferi* may have some minor antigenic relatedness to the leptospires (Magnarelli et al., 1985), any positively reacting sera were absorbed for 12 hr at 22 C with a dense culture of *Leptospira interogans* serovars *canicola* and *icterohemorrhagica* which had been pelleted by centrifugation (9,000 *g* for 20 min) and resuspended in an equal volume of the test serum. After another centrifugation, the IFA test was then repeated using the serum supernatant.

RESULTS

Antibody response and clinical signs

The *B. burgdorferi* antibody titers of the four parenterally inoculated wolves are shown in Figure 1. As a point of reference, in the domestic dog, a titer of 1:64 (6 $\log_2$) or above is considered evidence of an infection with *B. burgdorferi* (Magnarelli et al., 1985).

The titer of wolf number 1 (live spirochetes i.v. inoculated) rose to a peak of 1:512 (9 $\log_2$) by day 43 PI and remained high through day 75 PI when the animal was euthanized. This wolf developed lymphadenopathy beginning on day 15 PI, with enlarged popliteal and prescapular nodes. The lymph nodes decreased in size by day 50 PI, though the popliteal lymph nodes remained bilaterally enlarged through day 75 PI. Direct immunofluorescent staining and darkfield microscopic examination of the popliteal node aspirate performed 36 days PI were negative for *B. burgdorferi*. Growth was not noted on the blood agar plate streaked with the

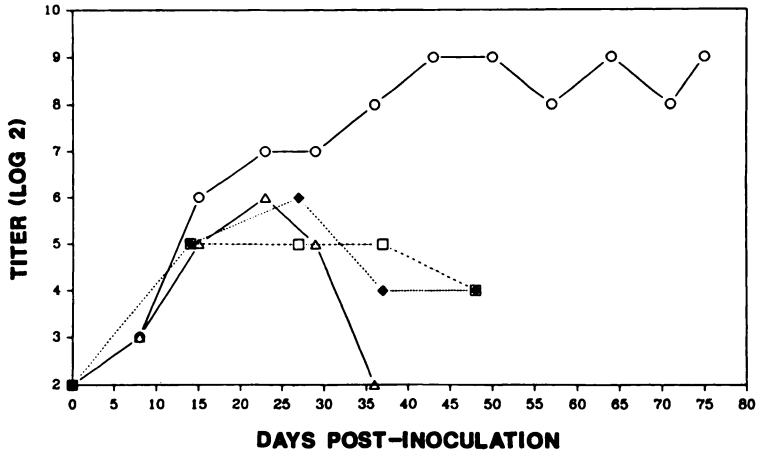

FIGURE 1. Antibody titers to *Borrelia burgdorferi* in the four parenterally inoculated wolves as determined by indirect immunofluorescent antibody tests. O——O intravenous inoculation with living organisms, △——△ subcutaneous inoculation with living organisms, □- - -□ intravenous inoculation with killed organisms (number 5), ◆- - -◆ intravenous inoculation with killed organisms (number 6).

aspirate. Physical examinations of this wolf were unremarkable except for the lymphadenopathy, and lameness or behavioral change were not observed for the duration of the project.

The antibody response of wolf number 2 (live spirochetes s.c. inoculated) peaked at 1:64 (6 $\log_2$) on day 23 PI and fell to nondetectable levels (<1:8) by day 36 PI. Clinical abnormalities were not observed during the 75 day course of the experiment.

Wolves number 3 and number 4 who were fed the inoculated *P. maniculatus* never developed a detectable antibody response through day 61 PI. Neither of these wolves showed any detectable clinical signs.

Wolves number 5 and number 6 (killed spirochetes i.v. inoculated) developed antibody responses which peaked at 1:32 (5 $\log_2$) and 1:64 (6 $\log_2$), respectively, and then fell to 1:16 (4 $\log_2$) by day 48 PI. Clinical abnormalities were not observed during this period.

Borrelia burgdorferi isolation attempts

All attempts to culture *B. burgdorferi* from the weekly blood samples, the lymph node aspirate from wolf number 1 and the postmortem tissues obtained from wolf numbers 1 through 4 were unsuccessful.

Necropsy findings

Pronounced enlargement of both popliteal lymph nodes was noted on wolf number 1. On cut section, these nodes were congested with a dark yellow fluid. Histologically, the popliteal nodes showed lymphoid hyperplasia, with a marked increase in plasma cells and macrophages in the medulla. Several brightly fluorescing spirochetes were observed by direct immunofluorescent staining of histologic sections of the liver of this wolf.

Necropsy of the remaining five wolves revealed no abnormalities. The histopathologic examinations of wolf numbers 2, 3 and 4 were unremarkable.

Serologic survey of wild-trapped wolves

Of the 78 sera tested from free-ranging wolves, two were reactive in the IFA test (3%). One of these was an adult female trapped in Wisconsin in 1982 (titer of 1:1,024), and one juvenile male trapped in Minnesota in 1977 (titer of 1:64).

DISCUSSION

The development of a high antibody titer over an extended period (60 days), coupled with the visualization of spirochetes in the liver, demonstrates that the wolf

inoculated i.v. with live *B. burgdorferi* had become infected. In contrast, the control animals who received the same strain and number of killed organisms by the same route showed relatively low and short-lived antibody responses. Therefore, we conclude that the wolf is susceptible to infection with the Lyme disease spirochete.

The wolf inoculated s.c. with live organisms developed a transient antibody response, comparable in magnitude to the control animals, which indicated that it had not been infected. Although *B. burgdorferi* infections in nature occur primarily through infected tick bites, the exact mechanism by which the organism is introduced is unknown. It has been suggested that the tick regurgitates the spirochetes into the skin or the bloodstream of its mammalian host (Steere et al., 1983). Therefore, whether the s.c. inoculation or the i.v. inoculation most closely approximates natural infection is unknown.

Our inability to culture *B. burgdorferi* from either the blood or tissues of the IV inoculated wolf may be a reflection of the difficulty in isolating the organism from infected hosts. The rate of success in recovering *B. burgdorferi* from both human and canine patients with Lyme borreliosis is uniformly low because of the brief, intermittent nature of the spirochetemia and the paucity of organisms (Steere et al., 1984a; Kornblatt et al., 1985).

Although our attempt to orally infect the wolves by feeding inoculated *P. maniculatus* was unsuccessful in this experiment, oral infection of *Peromyscus* spp. with *B. burgdorferi* has been demonstrated (Burgess and Patrician, 1987). It is possible that the small size of the suckling mice did not give the borrelia the protection from gastric acidity which a larger bolus of food might have provided. Another ramification of their small size is that the wolves ingested them with little or no mastication. This resulted in minimal exposure of oral and pharyngeal tissues to the spirochete.

The question of whether or not wolves infected with *B. burgdorferi* actually develop disease was not resolved by this study. Asymptomatic infections and infections whose only manifestation is arthritis seen months after the initial exposure are known to occur in both dogs and man (Steere et al., 1984b; Magnarelli et al., 1985; Burgess, 1986b). Obviously, this complicates experiments which utilize a small number of animals over a limited period of time. However, the lymphadenopathy shown by the i.v. inoculated wolf in this study is a symptom shared by both humans and dogs with Lyme borreliosis (Steere et al., 1984b; Kornblatt et al., 1985).

The detection of *B. burgdorferi* antibodies in the free-ranging wolves indicates that these animals are being naturally exposed to the organism. The high titer (1:1,024) of the wolf trapped in Wisconsin is evidence that some animals are developing an actual infection with *B. burgdorferi*. The low titer (1:64) of the wolf trapped in Minnesota could have resulted from sampling during an early or a very late stage of infection. Alternatively it could have resulted from a transient exposure to the spirochete without the development of an actual infection, similar to what occurred in the s.c. inoculated wolf.

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