Pathological Manifestations in Murine Lyme Disease: Association with Tissue Invasion and Spirochete Persistence

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The clinical manifestations of human Lyme disease present with a spectrum of tissue or organ involvement and severity of symptoms. The murine model of Lyme disease has proved to be an accurate reflection of many of the human symptoms of disease and has been particularly useful for studying development of subacute arthritis and tendonitis. Direct tissue invasion by *Borrelia burgdorferi* and persistence of high levels of spirochetes in tissues are important components of arthritis development. The outer-surface lipoproteins contain a biologically active lipid-modified moiety with potent ability to stimulate inflammatory cytokine production and other inflammatory mediators such as nitric oxide. Localized inflammation stimulated by these lipoproteins may be the trigger for neutrophil infiltration, synovial proliferation, and other events associated with this arthritis. Invasion of maternal uterine tissue, but not direct invasion of fetal tissue, is associated with low levels of pregnancy loss in mice infected during gestation, consistent with the detrimental effect of inflammatory cytokines on pregnancy.

Our laboratory uses the murine model of Lyme disease, described by Barthold and colleagues [1], for the analysis of parameters associated with arthritis and other pathological developments. Disease is induced by intradermal injection of relatively low numbers of spirochetes, and it mimics tick-transmitted disease in the kinetics of arthritis development and in the host's immune response to the organism [2, 3]. Infection with the N40 strain of *Borrelia burgdorferi* results in severe arthritis in mice of the C3H strain and mild arthritis in BALB/c and C57/BL6 mice.

The severe arthritis in C3H mice is characterized by an influx of inflammatory cells and synovial hyperproliferation in several joints and is readily observed as tibiotarsal swelling of the rear ankle joints [1, 2]. Hyperproliferation of the tendon sheath is frequently seen, with foci of chondrocytes and osteoblasts found within this region [3]. Abnormalities in joint tissue begin several days following intradermal injection of *B. burgdorferi* and peak ∼3 weeks following infection [2].

Our laboratory has studied three factors that contribute to the development of arthritis. The first is the invasion of tissues by the spirochete. Organisms can be detected in many tissues by sensitive PCR within 1 week following infection [3]. By 2 weeks postinfection, the rear ankles and heart are the most heavily infected tissues. The second factor important in arthritis development is the induction of localized inflammation in the invaded tissues. The spirochete has outer-surface lipoproteins with powerful stimulatory activities. The lipoproteins are mitogens for B lymphocytes and stimulate the production of cytokines and nitric oxide (NO) by macrophage lineage cells [4–6]. These activities may be directly responsible for the inflammatory events in invaded tissues [7].

The third component of arthritis development is genetically determined by the host, as demonstrated by the severe arthritis seen in infected C3H mice and the relatively mild arthritis seen in BALB/c and C57/BL6 mice [1, 3]. We have compared responses to infection in severely arthritic C3H mice with those in mildly arthritic BALB/c mice to identify host differences that could be crucial to the development of arthritis.

Methodology and Results

Level of Spirochetes Persisting in Tissues Is Correlated with Severity of Arthritis

We initially observed that markers of systemic inflammation were elevated to a greater degree during *B. burgdorferi* infection in severely arthritic C3H mice than in mildly arthritic BALB/c mice [8]. We hypothesized that this could be due either to innate differences between the two strains in their ability to respond to the stimulatory lipoproteins or else to persistence of higher levels of spirochetes and, therefore, higher levels of stimulatory lipoproteins in the severely arthritic C3H mice.

The first possibility was not supported by our in vitro studies, in which the cytokine-inducing properties of the lipoproteins were very similar when measured with cells from C3H and

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BALB/c mice [4–7]. This suggested that there was not an inherent hyperresponsiveness to the lipoproteins responsible for the greater inflammatory responses seen in C3H mice. To address the second possibility—that greater levels of inflammation and pathology were due to the presence of higher numbers of tissue spirochetes—a very sensitive PCR technique for detection of *B. burgdorferi* DNA in virtually any tissue of the mouse was developed.

This PCR analysis detects DNA from as few as three spirochetes. PCR was carried out as described [3] on DNA prepared from infected mouse tissues. Organs from infected mice were digested with 2.5 mL of a 0.1% collagenase A (Boehringer Mannheim, Indianapolis) solution in PBS (pH, 7.4) for 4 hours at 37°C. The collagenase-digested samples were mixed with an equal volume of proteinase K (0.2 mg/mL; Boehringer Mannheim) in 200 mM of NaCl, 20 mM of Tris-HCL (pH, 8.0), 50 mM of EDTA, and 1% of SDS. Digestions were carried out at 50°C for 16 hours, with occasional mixing.

DNA was recovered by extraction with an equal volume of phenol:chloroform and precipitation with ethanol. Samples were digested with DNAse-free RNAse (1 mg/mL; Sigma, St. Louis), reextracted, and recovered by ethanol precipitation. The DNA yield from various tissues was determined by absorbance at 260 nm and ranged from 100 μg from the bladder to 3 mg from the spleen. Approximately 400 μg of DNA was recovered from the heart and ankle.

For PCR amplification, DNA was diluted to 50 μg/mL, and 3 μL (150 ng) was added to each amplification reaction. The 150 ng of DNA sampled in each reaction represented from 1:666 to 1:20,000 of the DNA obtained from an entire organ or tissue site. Amplification mixtures consisted of reaction buffer (50 mM of Tris [pH, 8.3]; 3 mM of MgCl$_2$; 20 mM of KCl; and 0.5 mg of bovine serum albumin), 70 pmol of each oligonucleotide, 0.8 mM of dNTPs (deoxynucleotide triphosphates) (Boehringer Mannheim), 2.5 μCi of ^{32}P-dCTP (deoxycytidine triphosphate) (New England Nuclear Research Products, Wilmington, DE), and 0.72 units of Taq DNA polymerase (5,000 units/mL; BRL-GIBCO, Gaithersburg, MD). Controls lacking DNA were always included to monitor purity of PCR reagents, as was DNA purified from tissues of uninfected mice.

Ten-μL reactions were carried out in sealed glass capillary tubes. Primer sequences have been described [3]. Nidogen, a single-copy mouse gene, was amplified with specific primers to ensure equivalent DNA content in each sample. Amplification was carried out in the 1605 Air Thermocycler (Idaho Technologies, Twin Falls, ID). Reaction products were separated on a 6% polyacrylamide sequencing gel and identified by autoradiography. In some cases the amplification products were analyzed in a Molecular Dynamics PhosphoImager equipped with Image Quant Software (BioRad, Hercules, CA).

The PCR analysis revealed the presence of *B. burgdorferi* DNA in many tissues from severely arthritic C3H/HeJ mice as early as 1 week following infection with the N40 strain of *B. burgdorferi* (figure 1). The heart, ear, and ankle were particu-

larly heavily infected, although *B. burgdorferi* DNA was also detected in the spleen, liver, brain, kidney, bladder, uterus, and lymph nodes. In contrast, much lower levels of spirochete DNA were detected in tissues of infected BALB/c mice, which develop less severe arthritis when infected with *B. burgdorferi* than do C3H/HeJ mice. This difference was evident throughout the 4-week analysis (figure 1).

The differences in detectable *B. burgdorferi* DNA in C3H and BALB/c mice were observed with primer sets designed for the chromosome-encoded flagellin gene and for the plasmid-encoded outer-surface protein A (OspA) gene [3]. A competitive target PCR method, using a unique sequence flanked by the OspA primer sequences, allowed determination of the absolute number of OspA gene sequences in infected tissues [3]. Ankles and hearts from C3H/HeJ mice were found to harbor 1×10^7 copies of the *B. burgdorferi* OspA gene, while these tissues from BALB/c mice contained 5- and 10-fold less *B. burgdorferi* DNA, respectively.

These studies indicated that there were indeed quantifiable differences in the levels of spirochetes in many tissues of C3H and BALB/c mice, with the severe arthritis of C3H mice correlating with the presence of 5- to 10-fold higher levels of spirochetes. These findings also point to the tremendous sensitivity of PCR for detecting *B. burgdorferi* in tissues. In our studies, disrupted mouse tissues greatly inhibited *B. burgdorferi* growth in culture. For example, while only 200 cfu can be cultured from an infected C3H mouse spleen 2 weeks postinfection, PCR detection allows estimation of 1×10^7 OspA gene sequences in this tissue.

The inoculum of *B. burgdorferi* used in the experiment depicted in figure 1 was 2×10^5 spirochetes. However, we have recently observed increased virulence of the N40 isolate following growth in commercially prepared Barbour-Stoenner-Kelly (BSK-II) medium (Sigma). In fact, the relative resistance of BALB/c mice can be overcome by this dose (2×10^5) of the more virulent inoculum of *B. burgdorferi,* and arthritis in BALB/c mice is mild only when animals are infected with ⩽2,000 spirochetes. In addition, BALB/c mice with severe arthritis have high levels of spirochetes in their tissues, similar to those found in severely arthritic C3H mice (authors' unpublished observations). Thus, the correlation between the two traits—severe arthritis and persistence of high levels of *B. burgdorferi*—is further supported by evidence in BALB/c mice infected with large numbers of spirochetes.

Role of NO in the Murine Defense Against *B. burgdorferi* and in the Pathophysiology of Arthritis Development

NO, a potent inflammatory mediator, is involved in the murine defense against many pathogens and is particularly important in the defense against intracellular pathogen infections of mice [9–12]. NO has also been directly implicated in the development of two rodent models of arthritis: streptococcal cell wall–induced arthritis in rats and autoimmune arthritis in
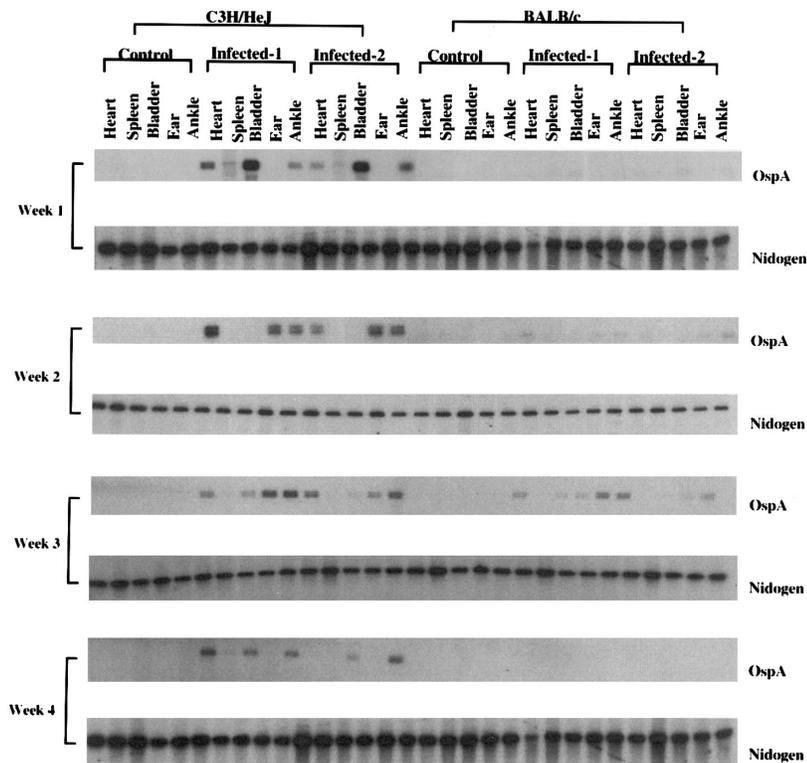
Figure 1. Detection of *B. burgdorferi* DNA in tissues from infected C3H/HeJ and BALB/c mice. DNA was prepared from the indicated tissues of control and infected mice, and PCR analysis was performed as described. Oligonucleotide primers were used to detect the OspA gene of *B. burgdorferi* or the murine gene nidogen, indicated on the right side of the figure. Mice were killed at various times following infection, as indicated on the left side of the figure. PCR amplification was for 28 cycles with the OspA2 and OspA4 primers and for 22 cycles with the nidogen primers. Amplification was performed in the presence of ^{32}P-dCTP (deoxycytidine triphosphate). Products were analyzed on a 6% polyacrylamide gel and exposed for autoradiography. (Figure reprinted from [3].)

lpr/lpr mice [13, 14]. The inducible NO synthase is an enzyme capable of generating high levels of NO in response to inflammatory stimuli, including cytokines and bacterial products [15]. The outer-surface lipoproteins of *B. burgdorferi* are potent stimulants of NO production [5, 16]. Furthermore, the addition of NO gas to cultures of *B. burgdorferi* inhibits growth in vitro [5, 17].

These findings suggested that *B. burgdorferi*–induced NO could be a component of the inflammatory response involved in the development of arthritis or could be a crucial component of the anti-*Borrelia* host defense. The murine model of Lyme disease was used to determine the role of inflammatory induced NO during infection by *B. burgdorferi*. Studies were designed to determine if differences existed in NO production in *B. burgdorferi*–infected C3H and BALB/c mice and whether any differences were related to the infectious or pathological process [18]. The quantitative PCR technique was used to evaluate the level of invading spirochetes as an indicator of the effect of inhibition of NO production in the infected animal.

C3H/HeJ or BALB/c mice were housed in metabolic cages (Nalgene, Walnut Creek, CA) in groups of four. Urine was collected daily for measurement of nitrate and nitrite, products derived from NO whose presence in urine are indicative of systemic NO production. Mice were fed a chemically defined nitrate/nitrite-free diet containing L-arginine (Ziegler Brothers, Gardner, PA) ad libitum, thus assuring that nitrates/nitrites measured in urine came from endogenous production [19, 20].

Mice were allowed to adapt to this diet for ~5 days before collection of urine for baseline measurement of nitrate/nitrite.

Mice were infected by intradermal injection with the N40 strain of *B. burgdorferi* into the shaven back [3]. Control mice were injected with an equal volume of sterile BSK-H culture medium. The effect of NO on *B. burgdorferi* infection was assessed by administration of an analog of arginine, the natural substrate for the NO synthase enzymes. N^G-L-Monomethyl arginine (LMMA) (Chem-Biochem Research, Salt Lake City) acts as a competitive inhibitor of the NO synthase enzymes and has been shown to efficiently inhibit NO production in mice [19, 20].

The inhibitor was given orally by gavage twice daily (100 μL of a 0.6-M solution of LMMA per dose). Controls received 100 μL of distilled water by gavage twice daily. This dosage of LMMA has been established to provide almost complete inhibition of inflammatory induced NO in vivo without observable secondary toxic effects on mice [19, 20].

Both mildly arthritic (BALB/c) and severely arthritic (C3H/HeJ) strains of mice systemically produced high levels of NO 1 week after infection with *B. burgdorferi,* as determined by the urinary nitrate levels (figure 2). NO production remained high throughout the infection in BALB/c mice, while in C3H/HeJ mice NO production returned rapidly to uninfected levels (figure 2). The return to baseline NO levels in C3H mice coincided with the peak in numbers of spirochetes detectable in tissues by PCR [3]. This suggested that the drop in NO
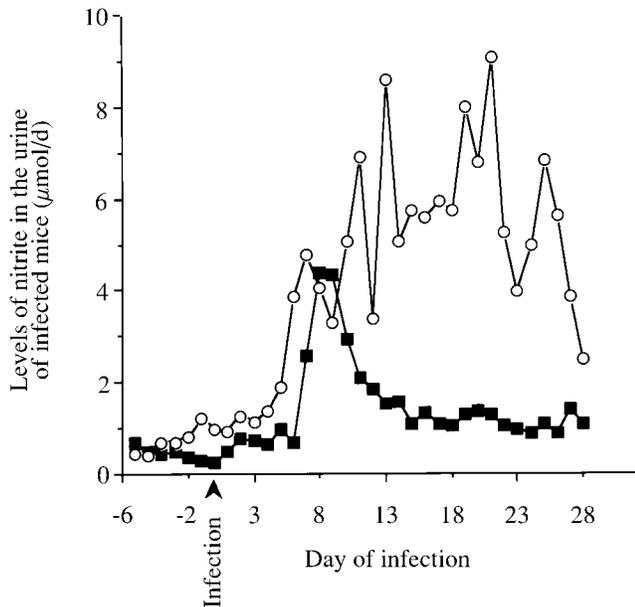
Figure 2. Urinary nitrite levels in infected mice. Urine was collected daily from C3H/HeJ mice (*squares*) and BALB/c mice (*circles*) and analyzed for nitrite levels. Mice were infected with 2×10^5 *B. burgdorferi* cells on day 0 (*arrowhead*). Baseline levels of urinary nitrite were determined for 6 days prior to infection. Nitrite levels were measured in samples of urine from four mice of the same strain, all housed in the same metabolic cage. Values represented are averages of pooled urinary nitrite based on urine volume and number of mice per cage. (Figure reprinted from [18].)

levels in C3H mice might have been responsible for the growth of spirochetes in this strain of mouse, while the continued high levels of systemic NO in BALB/c mice might explain the superior control of spirochete numbers seen in these mice.

Spirochete levels in LMMA-treated mice were compared with those in H_2O-treated controls by PCR detection of OspA DNA sequences. Quantitative PCR was performed on DNA prepared from the hearts and ankles of mice killed at several time points following infection; data are shown in figure 3 for hearts from infected C3H mice. There was no significant increase in spirochete levels in either C3H or BALB/c mice treated with LMMA. Similar results were obtained with ankles from C3H mice and hearts and ankles from BALB/c mice [18]. Thus, inhibition of NO production did not significantly alter the host's ability to control growth of *B. burgdorferi* in vivo.

In addition, NO appears not to be required for the development of Lyme arthritis, as inhibiting its production had no effect on the severe arthritis seen in C3H mice or the mild arthritis seen in BALB/c mice [20]. These results indicate that *B. burgdorferi* is able to persist in the host even in the presence of high levels of NO. Furthermore, NO is not involved in the control of spirochete infection of tissues, nor is it involved in the development of arthritis.

The potent activity of NO against intracellular pathogens and the in vivo resistance of *B. burgdorferi* to NO suggests

that this organism is not located in an intracellular compartment during an essential portion of its infection of the mammalian host. The shutdown in production of NO in C3H mice, in the presence of persisting spirochetes and intense inflammation in the joints, suggests that there was induction of synthesis of regulatory molecules capable of inhibiting production of inducible NO synthase enzymes. Candidate molecules include IL-10, IL-4, and transforming growth factor-β, which are reported to regulate production of inducible NO synthase enzymes and could be responsible for this effect in the C3H mouse [21].

**Effect of *B. burgdorferi* Infection on Murine Pregnancy**

Clinically, *B. burgdorferi* infection resembles many aspects of syphilis. Infection with *Treponema pallidum* during pregnancy is associated with devastating perinatal outcomes [22]. Several adverse perinatal outcomes, including preterm delivery, fetal death and malformation, and congenital infection have been observed in pregnant women infected with *B. burgdorferi* [23–26]. In contrast, adverse fetal outcomes are not increased in women with antibodies indicating previous Lyme disease [27].

The mouse model of Lyme disease was used to study the effect of maternal infection with *B. burgdorferi* on fetal development [28]. Infection was timed around gestation in order to assess the effect of both acute infection and chronic infection on fetal outcome. Documentation of an effect on fetal viability required killing the mother on day 16 (of 21) of gestation. This allowed discrimination of live pups (pink, round uniform sacs; formed fetuses with heartbeats) from fetal deaths (malformed, hemorrhagic sacs; formed fetuses with no heartbeats), which rarely occur spontaneously in these matings, and resorptions (very small, pale, gray sacs; no discernible fetuses), which probably occur early in gestation and are occasionally seen in normal murine pregnancy.

The effect of acute infection on pregnancy was analyzed by infecting C3H mice by intradermal injection of N40 *B. burgdorferi* on day 4 of gestation. Control pregnant mice were anesthetized and injected with BSK-II medium. Mice were killed on day 16, near the 2-week peak of tissue spirochetes determined in other studies (figure 1 and [3, 18]). Fetal deaths occurred in 33 (12%) of 280 gestational sacs in 39 infected C3H/HeN mice, compared with 0 of 191 sacs in 25 control mice ($P = .0001$) (table 1). Forty-six percent of acutely infected mice had at least one fetal death, compared with none of the control animals ($P = .0002$).

PCR analysis revealed the presence of *B. burgdorferi* DNA in the uteri of acutely infected mice but not in the uteri of controls (figure 4). Spirochete DNA was only rarely detected in fetal tissues, and its presence was not required for fetal death (figure 4). The effect of chronic infection on fetal outcome was assessed by infection of C3H mice 3 weeks prior to mating. There were no fetal deaths in 18 chronically infected

A. Water treated
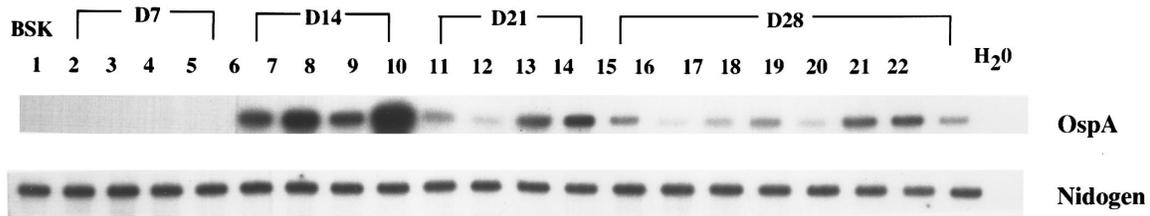
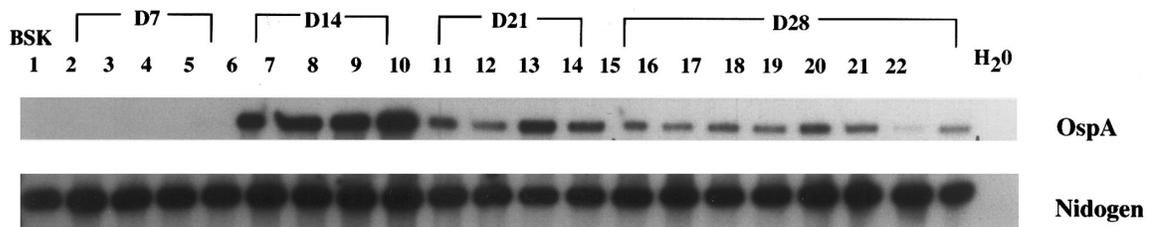

B. Inhibitor treated

Figure 3. Appearance of spirochete DNA in infected hearts was not altered by LMMA treatment. Hearts were taken from infected C3H animals, and the DNA was isolated for PCR analysis. Each time point was run on the same gel to visualize the time course of infection and relative spirochete numbers. Sequences from the mouse gene nidogen were amplified to ensure approximately equal loading of DNA into reaction mixtures. Spirochete numbers in water-treated (*A*) and inducible nitric oxide synthase-inhibitor−treated (*B*) mice are shown. OspA sequences were undetectable until day 14 postinfection (D14; *lanes 6−9*), at which time the number of spirochetes in the heart was maximal. Spirochete number gradually began to decrease with time after infection (*lanes 10−21*). No OspA sequences were detectable in the BSK II medium−injected mouse (*lane 1*) or in the reaction mixture control (*lane 22*). OspA was amplified at 30 cycles. (Figure reprinted from [18].)

C3H/HeN mice [28]. Spirochete DNA was also not detected in the uteri of chronically infected mice [28].

The inclusion of an internal competitive PCR target indicated that the failure to detect *B. burgdorferi* sequences in fetal DNA was not due to the presence of a PCR inhibitor [28]. Spirochetes were also not recovered in culture of fetal tissues, a technique we find to be much less sensitive than PCR for detection of *B. burgdorferi* infection of tissues. Histologic analysis of gestational tissues from infected animals demonstrated nonspecific pathology consistent with fetal death [28]. Polymorphonuclear

Table 1. Fetal outcome after acute infection of C3H mice with *B. burgdorferi* on day 4 of gestation.

Treatment	No. of mice	No. of gestational sacs	Number (%) of			
			Live fetuses	Fetal deaths	Resorptions	Affected mice
None	25	191	183 (96)	0	8 (4)	0
B. burgdorferi injection						
2×10^5	12	83	69 (83)	8 (10)*	6 (7)	4 (33)
2×10^6	9	76	61 (72)	14 (18)*	1 (1)	6 (66)
10^7	10	70	62 (88)	6 (9)*	2 (2)	3 (30)
2×10^7	8	51	45 (88)	5 (10)*	1 (1)	5 (63)
All doses	39	280	237 (85)	33 (12)*	10 (4)	18 (46)

NOTE.   Table reprinted from [28].
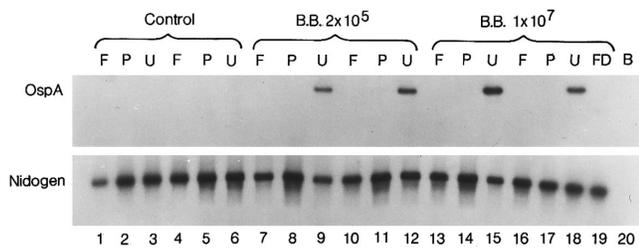* An increase in comparison with data for controls ($P < .001$; χ^2 test).

Figure 4. Detection of *B. burgdorferi* (B. B.) DNA in tissues from pregnant mice. C3H/HeN mice (mated with C57BL/6 males) were inoculated on day 4 of gestation with BSK-II media (control) or indicated doses of *B. burgdorferi*. PCR amplification was performed for 22 cycles with nidogen gene primers and 35 cycles with OspA primers. PCR of DNA in tissues from 4 of 8 infected and 2 of 4 control mice is shown (F = fetus; P = placenta; U = uterus; FD = fetal death; B = water blank). *Lanes 1–6,* control uninfected mice; *lanes 7–12,* mice infected with 2×10^5 *B. burgdorferi*; *lanes 13–19,* mice infected with 1×10^7 *B. burgdorferi*; and *lane 20,* PCR reactions run on a sample lacking DNA (water blank). (Figure reprinted from [28].)

leukocyte infiltration was not a prominent feature of gestational tissues of *B. burgdorferi*–infected mothers, as it is in arthritic joints [2].

These findings indicate an association between murine fetal death and acute infection with *B. burgdorferi* early in gestation but not with chronic infection. This difference may be explained by the reduced numbers of spirochetes remaining in the chronically infected mice, which were infected with *B. burgdorferi* 3 weeks prior to mating and 5 weeks prior to killing. Our data suggest that fetal death is due to a maternal response to infection rather than actual invasion of fetal tissue.

There is considerable precedent for such a mechanism of fetal death. Systemic maternal infections in women can cause perinatal loss without directly infecting the fetus [29, 30], and the administration of lipopolysaccharide to pregnant mice causes fetal death via a maternal response as opposed to a direct fetal effect [31]. The mechanism of fetal loss in *B. burgdorferi*–infected mice is not known.

The outer-surface lipoproteins are potent stimulants of inflammatory cytokine production, and the direct invasion of the uterus by the spirochetes suggests that these molecules could stimulate cytokine production in this tissue. Maternal production of inflammatory cytokines has been implicated as a cause of murine fetal death [31, 32]. These findings could provide an explanation for observations in humans; sporadic cases of fetal death in women infected with *B. burgdorferi* during pregnancy have been reported, while previous infection has not been associated with fetal death [23–27].

Discussion

Several studies have been described that support the hypothesis that severe pathology in murine Lyme disease is directly correlated with the level of spirochetes found in tissues. Comparison of spirochete presence in C3H and BALB/c mice supports this hypothesis. The severity of arthritis in the rear ankle can be correlated with the number of spirochetes in the ankle joints or in the heart taken from the same mouse. The genetic resistance of BALB/c mice can be overcome by infection with large numbers of *B. burgdorferi*. In this case, both increased severity of arthritis and increased numbers of spirochetes in tissues were observed, further supporting linkage of the two traits. Studies designed to assess the involvement of NO in host defense also supported this hypothesis.

Inhibition of NO did not allow rampant growth of spirochetes in mouse tissues, and it did not result in increased severity of arthritis. Finally, studies on the effect of *B. burgdorferi* on outcome of pregnancy in C3H mice indicated that acute but not chronic infection resulted in a small percentage of fetal deaths. Acute but not chronic infection was also associated with the presence of spirochete DNA in the uterus on day 16 of gestation. This supports the contention that pathological involvement in Lyme disease requires the invasion and persistence of spirochetes in tissues. These conclusions are consistent with involvement of the *Borrelia* outer-surface lipoproteins in the induction of inflammation.

These findings are in agreement with the clinical features of human Lyme disease. Indeed, direct tissue invasion by the spirochete has been associated with many symptoms of Lyme borreliosis. *B. burgdorferi* has been isolated from erythema migrans skin lesions [33], from CSF of patients with neurological complications [34], and from cardiac biopsy specimens of individuals with heart involvement [35]. Although *B. burgdorferi* has been very difficult to culture from synovial fluid, PCR amplification of DNA from synovial fluid has recently been used to detect *B. burgdorferi* sequences [36]. Thus, in human Lyme disease, pathology is also correlated with tissue invasion by the spirochete.

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