Fetal Outcome in Murine Lyme Disease

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Lyme disease is an inflammatory syndrome caused by infection with *Borrelia burgdorferi*. Although this syndrome has important implications for human pregnancy, little is known about gestational infection with *B. burgdorferi*. Fetal death occurred in 33 of 280 gestational sacs (12%) in 39 C3H/HeN female mice infected by intradermal injection of *B. burgdorferi* 4 days after mating (acute infection), compared with 6 of 191 sacs in 25 control mice (*P = 0.0001*). Forty-six percent of acutely infected mice suffered at least one fetal death, compared with none of the control animals (*P = 0.0002*). There were no fetal deaths in 18 C3H/HeN mice infected 3 weeks prior to mating (chronic infection). A sensitive PCR technique detected *B. burgdorferi* DNA in the uteri of acutely infected mice but did not detect DNA in the uteri of controls or chronically infected mice. Spirochete DNA was only rarely detected in fetal tissues, and its presence was not required for fetal death. The inclusion of an internal competitive PCR target indicated that the lack of *B. burgdorferi* sequences in fetal DNA was not due to the presence of a PCR inhibitor. Histologic analysis of gestational tissues from infected animals demonstrated nonspecific pathology consistent with fetal death. These findings indicate an association between murine fetal death and acute infection with *B. burgdorferi* early in gestation but not with chronic infection. Our data suggest that fetal death is due to a maternal response to infection rather than fetal infection. These findings could provide an explanation for observations in humans in which 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.

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**MATERIALS AND METHODS**

**Mice.** C3H/HeN, C57BL/6, and BALB/c mice were obtained from the National Cancer Institute or Jackson Laboratories. Animals had free access to food and water and were exposed to a 12-h light/12-h dark cycle. Six to 8-week-old virgin C3H/HeN female mice were mated with 6- to 12-week-old C57BL/6 males, and the day of coital plugging was established as day 0 of pregnancy. This allogeneic mating is well characterized and is not normally abortion prone. C3H/HeN × C3H/HeN and BALB/c × BALB/c matings were also used. Studies were approved by the University of Utah Institutional Animal Care and Use Committee.

**Bacteria.** *B. burgdorferi* was the uncloned N40 isolate at passage 5 from an infected mouse, kindly provided by Stephen Barthold, Yale University (2). Cultures were maintained as 0.5-ml frozen stocks at −70°C. Fresh aliquots were seeded in 15 ml of BSK-II medium (Sigma) (1) and cultured at 30°C for 3 to 5 days prior to injection.

**Infection of mice with *B. burgdorferi*.** C3H/HeN or BALB/c females were infected by intradermal injection with *B. burgdorferi* (2 × 105 to 3 × 107 of low-passage-number N40 isolate) or vehicle (BSK-II medium; Sigma) in the shaven back either 3 weeks before mating (chronic infection) or 4 days after mating (acute infection). Methylene blue (Pitman-Moore, Mundelein, Ill.) was used as an inhalational anesthetic during the injections. Additional mice were infected with *B. burgdorferi* 5 days prior to mating.

**Determination of fetal status.** Pregnant animals were sacrificed on days 14 to 16 of pregnancy, and the fetal status was determined. Live pups (pink, round uniform sacs, formed fetus with a heartbeat) were distinguished from fetal deaths (malformed, hemorrhagic sacs, formed fetus with no heartbeat), which rarely occur spontaneously in these matings, and resorptions (very small, pale, grey sacs, no discernible fetus), which probably occur early in gestation and are occasionally seen in normal murine pregnancy. Gestational sacs were examined under a 10× dissecting microscope. Fetal outcomes between groups were compared by using chi-square analysis and Fisher’s exact test.

**Histology.** Gestational sacs from animals infected on day 4 of gestation were taken for histologic analysis. Samples were fixed in 10% neutral buffered formalin solution and embedded in paraffin blocks, and sections were stained with hematoxylin and eosin. All histologic examinations were performed by a single observer.

**Preparation of DNA from infected tissues.** Uteri, placentas, and fetuses of control and infected mice were removed from mice that were sacrificed at the indicated times. After a saline rinse, tissues were minced with single-use razor blades and digested with 0.1% collagenase A (Boehringer Mannheim) in phosphate-buffered saline for 4 h at 37°C. Collagenase digestion yielded single-cell
TABLE 1. Fetal outcome after acute infection with *B. burgdorferi* on day 4 of gestation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mice</th>
<th>No. of gestational sacs</th>
<th>% of live fetuses</th>
<th>% of fetal deaths</th>
<th>% of fetal resorptions</th>
<th>No. of affected mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (controls)</td>
<td>25</td>
<td>191</td>
<td>183 (96)</td>
<td>0</td>
<td>8 (4)</td>
<td>0</td>
</tr>
<tr>
<td><em>B. burgdorferi</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 × 10^5</td>
<td>12</td>
<td>83</td>
<td>69 (83)</td>
<td>8 (10)^a</td>
<td>6 (7)</td>
<td>4 (33)</td>
</tr>
<tr>
<td>2 × 10^6</td>
<td>9</td>
<td>76</td>
<td>61 (72)</td>
<td>14 (18)^a</td>
<td>1 (1)</td>
<td>6 (66)</td>
</tr>
<tr>
<td>2 × 10^7</td>
<td>4</td>
<td>70</td>
<td>62 (85)</td>
<td>6 (9)^a</td>
<td>2 (2)</td>
<td>3 (30)</td>
</tr>
<tr>
<td>All doses</td>
<td>39</td>
<td>280</td>
<td>237 (85)</td>
<td>5 (10)^a</td>
<td>1 (1)</td>
<td>5 (63)</td>
</tr>
</tbody>
</table>

^a Increased compared with controls; P < 0.001; χ^2.

RESULTS

Pregnancy outcome. C3H mice infected with *B. burgdorferi* began to develop arthritis 2 weeks after inoculation, as evidenced by enlarged and swollen ankle joints. Fetal outcome for acutely infected (day 4 of gestation) pregnant mice (mated with C57BL/6 males) is shown in Table 1. *B. burgdorferi* caused a significant increase in the proportion of fetal deaths at all doses tested (P < 0.001; χ^2). There was no change in the proportion of fetal deaths over increasing doses of inoculum.

Forty-six percent of acutely infected pregnant mice suffered at least one fetal death, compared to none of the controls (P = 0.0002; χ^2).

To determine whether inoculation during pregnancy was required for fetal death to occur, additional C3H/HeN females were infected with 2 × 10^5 *B. burgdorferi* cells 5 days prior to mating with C57BL/6 males. These mice were subjected to an acute infection initiated just prior to gestation. Fetal death occurred in 10 of 72 gestational sacs (14%) in eight mice, compared to 0 of 64 gestational sacs in eight control mice (P < 0.001). Fetal outcome was similar in mice that were acutely infected both just prior to and just after mating, indicating that introduction of the spirochete during pregnancy was not a requirement for fetal death.

In contrast, there were no fetal deaths in 18 chronically infected mice that were inoculated 3 weeks prior to mating (Table 2). Reproductive outcome was normal in these mice despite the development of severe arthritis 2 to 3 weeks after infection.

Additional experiments were performed to ascertain whether the fetal effects of maternal *B. burgdorferi* infection are strain specific. In the model of Barthold et al., inbred strains of mice respond differently to infection (2). For example, C3H mice develop severe arthritis, while BALB/c and C57BL/6 mice develop arthritis of mild and intermediate severity, respectively (2, 3, 35, 36).

In one set of experiments, C3H/HeN females were infected with 2 × 10^5 *B. burgdorferi* cells 4 days after mating with C3H/HeN males (acute infection). Fetal death occurred in 7 of 68 gestational sacs (10%) in eight infected mice, compared to 0 of 65 gestational sacs in eight control mice (P < 0.001). The proportion of fetal deaths was similar to that in acutely infected C3H/HeN females mated with C57BL/6 males. Although C3H mice develop more severe arthritis when infected with *B. burgdorferi* than C57BL/6 mice, our data show that these strains do not differ in their paternal contribution to susceptibility to *B. burgdorferi*-induced fetal death.

TABLE 2. Fetal outcome after chronic infection with *B. burgdorferi* 3 weeks prior to mating

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mice</th>
<th>No. of gestational sacs</th>
<th>% of live fetuses</th>
<th>% of fetal deaths</th>
<th>% of fetal resorptions</th>
<th>No. of affected mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (controls)</td>
<td>5</td>
<td>38</td>
<td>36 (95)</td>
<td>2 (5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. burgdorferi</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^6</td>
<td>6</td>
<td>50</td>
<td>50 (100)</td>
<td>0</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>10^7</td>
<td>6</td>
<td>43</td>
<td>41 (95)</td>
<td>2 (5)</td>
<td></td>
<td>3 (30)</td>
</tr>
<tr>
<td>2 × 10^7</td>
<td>6</td>
<td>37</td>
<td>35 (95)</td>
<td>2 (5)</td>
<td></td>
<td>2 (5)</td>
</tr>
<tr>
<td>All doses</td>
<td>18</td>
<td>130</td>
<td>126 (97)</td>
<td>4 (3)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^a In this analysis, there were no fetal deaths and no affected mice.
We also studied the maternal contribution of inbred strains of mice to susceptibility of \textit{B. burgdorferi}-induced fetal death. BALB/c female mice were infected with $2 \times 10^5$ \textit{B. burgdorferi} cells 4 days after mating with BALB/c males. BALB/c mice treated with both \textit{B. burgdorferi} and vehicle suffered high rates of fetal deaths. This mating was quite sensitive to the handling required for intradermal injection, and the high rate of background fetal wastage precluded any conclusions about the rate of \textit{B. burgdorferi}-induced fetal death in BALB/c mothers.

Detection of \textit{B. burgdorferi} DNA in murine tissues. PCR using primers specific for OspA was used to determine the presence of spirochete DNA in intrauterine tissues. Figure 1 shows a representative PCR performed with DNA taken from C3H/HeN female mice mated with C57BL/6 males and infected with \textit{B. burgdorferi} on day 4 of gestation. These mice were sacrificed 10 to 12 days later. \textit{B. burgdorferi} DNA was detected in all uteri taken from eight infected mice but was not detected in any of four control animals. In addition, \textit{B. burgdorferi} DNA was not detected in any of 10 fetuses (including 2 fetuses that were dead at the time of sacrifice) and was only faintly detected in one of eight placentas from infected animals. OspA sequences were not detected in fetuses even after PCR amplification for 35 cycles. These data indicate that transplacental transmission of \textit{B. burgdorferi} is not required for fetal death in association with murine Lyme disease.

Similar results were obtained with DNA taken from C3H/HeN female mice mated with C3H/HeN males and infected with \textit{B. burgdorferi} on day 4 of gestation. OspA sequences were detected in the uteri but not the placentas or fetuses of infected mice.

We amplified DNA from intrauterine tissues in the presence of both a competitive target and primers specific for OspA, to be certain that the absence of detectable OspA sequences in fetal DNA was not a result of a PCR inhibitor (24). The competitive target was readily amplified in the presence of fetal DNA taken from infected mice (Fig. 2). This finding implies that the lack of detectable OspA sequences in fetal tissues is due to the absence of spirochetes, as opposed to an inhibitor of the PCR. There was some variability in the intensity of the signal from the competitive target among samples. This was likely due to differences in exposure times, freshness of $^{32}$P, and slight day-to-day assay variability. However, in all cases, the competitive target was easily detected to a level of 40 copies.

This method can also be used to quantify the number of copies of a gene sequence in tissue (24) and has been used to determine the number of OspA gene sequences in infected tissues in murine Lyme disease (35). We estimate that there are $10^7$ copies of the \textit{ospA} gene sequence in the uteri of acutely infected mice (Fig. 2).

We considered the possibility that sacrifice of animals by day 16 of gestation might cause us to miss fetal \textit{B. burgdorferi} transmission in acutely infected mothers. Fetuses could be infected transplacentally either later in gestation or during the process of labor and delivery. In an additional experiment, fetuses were allowed to deliver spontaneously from four C3H/HeN mice mated with C57BL/6 males and infected with $2 \times 10^5$ \textit{B. burgdorferi} cells on day 4 of gestation. No \textit{B. burgdorferi} DNA was found in any of eight of these fetuses (Fig. 3). These data, taken together with the absence of spirochete DNA in fetuses from acutely infected animals that were sacrificed, indicate that fetal infection does not routinely occur under these conditions.

However, fetal infection may sometimes occur. We detected a faint band of \textit{ospA} DNA in one of three fetuses and one of two placentas taken from mice that were infected with \textit{B. burgdorferi} 5 days prior to mating and sacrificed on day 14 of gestation. It is noteworthy that the detection of spirochete
DNA in the fetus did not correlate with fetal outcome. The fetus with detectable *B. burgdorferi* sequences was alive, while one of the fetuses without detectable ospA DNA was dead at the time of sacrifice.

In contrast to uteri harvested from acutely infected animals, no *B. burgdorferi* DNA was detected in two uteri taken from pregnant mice chronically infected with *B. burgdorferi* 5 to 6 weeks prior to sacrifice (Fig. 4). Amplification of the competitive target was not inhibited by the presence of DNA from the uteri of these animals (Fig. 2).

**Histology.** Histologic features of gestational sacs associated with dead fetuses were nonspecific. These postmortem changes included decidual necrosis, calcifications, and collapse of the fetal circulation, representing typical changes in decidual and placental tissue after murine fetal death of any cause (25a) (Fig. 5). The labyrinthine zones of the placentas of live fetuses from mothers infected with *B. burgdorferi* exhibited more edema and vacuolar changes than controls. However, these changes were also nonspecific, were not associated with an inflammatory response, and were found in a few control animals. Fetal tissues of dead animals showed nonspecific autolytic changes, and tissues from live fetuses of infected mothers showed intact visceral histology without diagnostic pathology. Polymorphonuclear infiltrates were not consistently noted in the gestational tissues of animals infected with *B. burgdorferi*.

**DISCUSSION**

Acute infection with *B. burgdorferi* early in gestation is associated with fetal death in C3H mice, but chronic infection is not. These data are consistent with observations in human pregnancy. Sporadic cases of fetal loss associated with Lyme disease have been reported, and all were characterized by acute infection during pregnancy (14, 15, 17, 26, 32). In contrast, perinatal outcome in previously infected women in areas of high endemicity are similar to outcomes in the general population (30, 34). As with our chronically infected mice, previous or chronic infection with *B. burgdorferi* is not associated with perinatal loss in humans. Adverse perinatal outcomes have also been noted in a canine model of *B. burgdorferi* infection (10). Gustafson and colleagues noted higher rates of perinatal loss, increased fetal resorptions, and shorter gestations in beagles that were acutely infected with *B. burgdorferi* during pregnancy than in controls (10).

In our model, transplacental transmission of *B. burgdorferi* was not required for fetal death. Spirochete DNA was not demonstrated in any fetuses, including several that suffered in utero death, taken from or born to mothers infected on day 4 of gestation. We used a very sensitive and specific PCR technique that can detect as few as three spirochetes in 150 ng of tissue DNA (36). This technique was chosen because PCR is a more sensitive method than culture or immunohistology for the detection of *B. burgdorferi* in synovial fluid and tissues (12, 22). Also, we have not been able to reliably culture *B. burgdorferi* from gestational tissues. Spirochetes that were added to uterine, gestational, and fetal tissues were not recovered by culture in BSK-II medium but were detected by PCR (unpublished observations). Furthermore, we ruled out the possibility of a PCR inhibitor as the reason for inability to detect OspA sequences by amplifying a competitive internal control in the presence of fetal DNA.

Transplacental transmission of the spirochete did not routinely occur in our mouse model. This finding is consistent with the observations that vertical transmission of *B. burgdorferi* does not occur in either Lewis rats (21) or outbred white-footed mice (*Peromyscus leucopus*) (18), as assayed by spirochete culture and infectivity. However, we cannot exclude the possibility of occasional murine transplacental transmission of *B. burgdorferi* under these or other conditions. In fact, we detected a faint band of *B. burgdorferi* DNA in one fetal sample taken from an animal that was infected 5 days prior to mating. This could represent contamination during tissue handling. However, this is unlikely since no OspA sequences were detected in fetuses from animals infected on gestational day 4 or in any control animals that were sacrificed and processed along with infected mice. Alternatively, spirochete load to the uterus may be higher in mice infected 5 days prior to as opposed to 4 days after fertilization, increasing the chances for fetal infection. Tissues in C3H mice are most heavily infected 2 weeks postinoculation (3, 36).

Spirochete DNA was detected in all uteri tested from acutely infected animals but was not present in uteri taken from chronically infected animals. Thus, *B. burgdorferi* was present in the uterus at time points associated with fetal death but absent at time points associated with normal reproductive outcome. This finding, as well as the lack of association between transplacental transmission of the spirochete and fetal death, implies that fetal death is due to a maternal response to infection rather than fetal infection. 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 (5, 9), and the administration of lipopolysaccharide to pregnant mice causes fetal death via a maternal response as opposed to a direct fetal effect (28).

The mechanism(s) by which a maternal response to *B. burgdorferi* causes fetal death is unknown. We speculate that intrauterine production of inflammatory mediators may play a role...
in the pathogenesis of fetal loss. *B. burgdorferi*, as well as OspA and OspB, can stimulate the production of interleukin-1 (4, 11, 13, 19, 20) and tumor necrosis factor alpha (8, 13, 25) in vivo and in vitro. Maternal production of these inflammatory cytokines has been implicated as a cause of murine fetal death (27, 28). Other compounds induced by *B. burgdorferi*-specific lipoproteins that could potentially cause fetal harm include nitric oxide, interleukin-6, and gamma interferon (13). Alternatively, maternal inflammatory mediators may be nonspecifically induced by tissue destruction secondary to spirochete invasion in the uterus. The mechanism of fetal loss appears to be different from that of arthritis, since fetal death is not associated with an infiltrate of polymorphonuclear cells.

The presence of uterine spirochetes did not inevitably cause fetal death. It is unclear why some fetuses die in utero while others appear unaffected by maternal infection with *B. burgdorferi*. Spirochete inoculum was not the explanation for this phenomenon, since increasing doses of *B. burgdorferi* did not cause increased rates of fetal death. However, it is possible that lower inocula would have exhibited dose dependence.

Infection of C3H/HeN mice with *B. burgdorferi* provides an excellent model for the study of Lyme disease during pregnancy, although murine Lyme disease may differ from human pregnancy with regards to the rate of fetal infection. We are currently using this model to better characterize the maternal intrauterine response to infection with *B. burgdorferi* and to explore the mechanisms of *B. burgdorferi*-induced fetal death. This model may also provide insight into the pathophysiology of insidious intrauterine infection during pregnancy.

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**REFERENCES**