Complications of Pregnancy and Transplacental Transmission of Relapsing-Fever Borreliosis

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Relapsing-fever borreliosis caused by Borrelia duttonii is a common cause of complications of pregnancy, miscarriage, and neonatal death in sub-Saharan Africa. We established a murine model of gestational relapsing fever infection for the study of the pathological development of these complications. We demonstrate that B. duttonii infection during pregnancy results in intrauterine growth retardation, as well as placental damage and inflammation, impaired fetal circulation, and decreased maternal hemoglobin levels. We show that spirochetes frequently cross the maternal-fetal barrier, resulting in congenital infection. Furthermore, we compared the severity of infection in pregnant and nonpregnant mice and show that pregnancy has a protective effect. This model closely parallels the consequences of human gestational infection, and our results provide insight into the mechanisms behind the complications of pregnancy that have been reported in human relapsing-fever infection.

Infectious disease is one of the most common causes of complications of pregnancy and infant death in developing countries. Approximately 25% of neonatal deaths in Africa result from severe infections [1]. Complications such as spontaneous abortion and perinatal death as a consequence of tickborne relapsing fever (RF) have been well documented, with the majority of cases occurring in sub-Saharan Africa [2–9], although reports from developed countries also exist [10–12]. In the present study, we developed a mouse model to elucidate the mechanisms behind complications of pregnancy caused by the sub-Saharan RF agent Borrelia duttonii.

Borrelia spirochetes are transmitted by arthropods and cause either Lyme disease or RF [13]. B. duttonii, the primary agent of RF in sub-Saharan Africa, is transmitted to humans by the tick Ornithodoros moubata [14]. The patient has a recurrent fever alternating with periods of relative well-being. The fever coincides with high numbers of borreliae in the blood. The relapsing pattern is due to antigenic variation of surface lipoproteins [15, 16]. Other symptoms include headache, abdominal pain, hemorrhage, hepatomegaly and splenomegaly, neurological manifestations, and weakness [17]. Unique to African tickborne Borrelia is the ability to form aggregates of erythrocytes and bacteria, called "rosetting" [18, 19], with subsequent disruption of the microcirculation [20]. Disease severity and mortality rates vary depending on the infecting strain and patient status but are generally highest in young children [17, 21].

As many as 6.4% of pregnant women admitted to a maternity ward in the Democratic Republic of the Congo received a diagnosis of RF [7]. Common complications of RF during pregnancy are low birth weight, preterm delivery, spontaneous abortion, and neonatal death [2–7, 9]. Two of the most severe consequences are pregnancy loss and neonatal death. B. duttonii causes preterm birth with a 30% risk of pregnancy loss, and rates of fetal/infant mortality of 15% [8] and 44%
a murine model of B. duttonii we demonstrate that B. duttonii understood.

the mechanism causing these complications remains poorly documented as well [23]. Despite numerous cases, [12], and the illness is not limited to humans—equine stillbirth has been documented as well [23]. Despite numerous cases, the mechanism causing these complications remains poorly understood.

To study pathogenesis of RF during pregnancy, we developed a murine model of B. duttonii infection. In the present article, we demonstrate that B. duttonii infection during pregnancy results in intrauterine growth retardation, placental damage and inflammation, impaired fetal and placental circulation, and decreased maternal hemoglobin (Hb) levels. We also show that B. duttonii can traverse the maternal-fetal barrier, causing congenital infection. Furthermore, we use this model to show a difference in RF outcome between pregnant and nonpregnant mice. Together, these findings suggest a mechanism for the complications of pregnancy associated with RF borreliosis.

MATERIALS AND METHODS

Mice and bacterial strains. Adult C3H/HeN mice purchased from B&K Universal were maintained in accordance with Swedish guidelines for animal welfare. B. duttonii 1120 (provided by Guy Baranton, Institute Pasteur, Paris, France) were passaged in mice, isolated from plasma, and frozen at −80°C in citrate buffer (0.136 mol/L sodium citrate dihydrate and 0.175 mol/L NaCl) with 20% glycerol.

Mating and infection of mice. Mice were mated and examined daily for the presence of vaginal plugs. Mated females were transferred to separate cages and randomly selected to be infected or uninfected. Fifteen pregnant mice at gestational day 9 (E9) and 11 nonpregnant mice were inoculated subcutaneously with 1 × 10^5 B. duttonii. Nine pregnant control mice were sham-infected with citrate buffer that contained 20% glycerol.

Blood was examined daily to determine the presence of spirochetemia. Mice were killed by decapitation and examined on E18.

An infectious dose of 1 × 10^7 bacteria administered intraperitoneally (ip) is commonly used [20]. In the present study a lower dose, 1 × 10^6 bacteria administered subcutaneously, was chosen to better mimic natural infection.

Hb levels. Tail blood was obtained on the day of infection and after 3, 6, and 9 days and was placed into EDTA anticoagulant. Hb was quantified in triplicate using a HemoCue photometer.

Histological and immunohistochemical analysis. Stainings were done on 2–3 placentas/mouse (8 control and 13 infected mice). Placentas were fixed in 5% paraformaldehyde, dehydrated, embedded in paraffin, cut into 8-μm sections, and stained with hematoxylin-eosin. Sections were viewed by light microscopy.

For immunohistochemical analysis, placentas were embedded in OCT compound (Sakura), cut into 8-μm-thick sections, and fixed in acetone. For the detection of spirochetes, sections were blocked with 5% goat serum (Gibco BRL) and incubated with rabbit antibody against B. duttonii flagellin (Agrisera). Thereafter, sections were incubated with a goat anti-rabbit Alexa 555–labeled antibody (Molecular Probes). Sections were counterstained with 4⃣,6⃣-diamidino-2-phenylindole (Molecular

Figure 1. Scatter plot of fetal weight vs. spirochete burden. The linear regression line shows a linear decrease in fetal weight as the total spirochetal burden increases during the 9 days of infection.

Figure 2. Maternal hemoglobin (Hb) level in peripheral blood. Hb levels were measured on gestational days (E) 9, 12, 15, and 18 in uninfected pregnant (white bars, n = 9), infected pregnant (striped bars, n = 18), and infected nonpregnant (hatched bars, n = 11) mice. In infected mice, these gestational days corresponded to infection days 0, 3, 6, and 9, respectively. On gestational day 9, the Hb level was obtained before infection. The horizontal line at 161 g/L represents the average Hb level in uninfected nonpregnant mice (n = 11). Each bar represents the spread of data points in the 25th–75th percentile. The horizontal line within each bar indicates the median. Vertical lines above and below the bars extend to the minimum and maximum values in the each data set. Outliers are shown as asterisks. On E15 and E18, the statistical significance between median Hb levels between infected and uninfected mice was P<.05. Of the infected mice, median Hb levels for nonpregnant mice were significantly different from those of pregnant mice (P<.05) on E15 and E18.
Probes) and fluorescein–5-isothiocyanate–conjugated wheat germ agglutinin (Vector Laboratories) and were analyzed by fluorescence microscopy. For the detection of leukocytes, sections were stained with anti-CD45 antibodies (Oxford Biotechnology); for the detection of macrophages, sections were stained with anti-F4/80 antibodies (Serotec). Sections were blocked with 3% H₂O₂, 20% rabbit serum (Gibco BRL), and avidin and biotin (Vector Laboratories). Sections were incubated with biotinylated rabbit anti-rat antibody (DAKO) and StreptABComplex/horseradish peroxidase (DAKO) and were then developed using 3,3′-diaminobenzidine (DAKO). Sections were counterstained with Mayer’s hematoxylin (Sigma Diagnostics) and examined by light microscopy.

**Mouse infectivity test (MIT).** To detect the transplacental transmission of spirochetes, fetal tissue was inoculated into naive mice. Three fetuses each from 10 infected mice were washed in 70% ethanol, rinsed in PBS, and aseptically dissected to avoid contamination by maternal body fluids. Fetal liver, spleen, lungs, and heart were homogenized and injected ip into naive mice. Blood was examined daily for the presence of spirochetemia until 14 days after infection. Transplacental transmission was confirmed by the development of spirochetemia.

**Spirochetemia.** Five microliters of tail blood was diluted in 45 μL of citrate buffer. Spirochetes were visualized microscopically and counted.

**Statistical analysis.** The statistical significance of placental and fetal abnormality data was analyzed by an approximation to the central-limit theorem. All other calculations were done using Student’s *t* test.

**RESULTS**

**Murine model of RF borreliosis during pregnancy.** The pathogenesis of African RF has been studied in several animal models [20, 24, 25]; however to our knowledge, this is the first model of the disease during pregnancy. Mice were chosen because RF is well documented in mice, and their relatively short gestational period allows a reasonable experimental time frame. Furthermore, cell types and anatomical regions are similar to those of the human placenta [26], although humans have a hemochorial placenta, whereas mice have hemotrichorial placentas [27] and, thus, more layers of tissue separating the murine fetus from maternal blood.

In this model, the outcome was comparable to that of reported human infection, with spirochetemia, anemia, and neonatal spirochetemia [10, 12, 14, 17]. After infection on the ninth day of pregnancy, the first spirochetes appeared in maternal blood on day 3 or 4 after infection, and mice had one relapse after the initial bacteremic peak (data not shown). In initial attempts to establish this model, mice were inoculated on the day when the presence of the vaginal plug was confirmed, which almost exclusively resulted in no pregnancies (1/13). Subsequently, to study the consequences of infection during the course of pregnancy and in the developing fetus, infection on E9 was determined to be the optimal time point, because a preexisting RF infection appeared to be detrimental to the implantation process.

**Correlation of fetal weight with maternal spirochetemia.** Low birth weight is the major determinant of infant mortality and is a common complication of maternal infection during pregnancy [28]. To examine the effect of maternal RF on fetal growth, numbers of spirochetes in maternal blood were monitored daily and were plotted against fetal weight on E18. In uninfected mice, the average fetal weight was 1.17 ± 0.12 g (*n* = 67), whereas fetuses of infected mice weighed 0.96 ± 0.18 g (*n* = 124), which was 18% lower and was a statistically significant difference (*P* < .001). The total maternal spirochete burden over the course of infection correlated with fetal weight in an inverse linear manner (figure 1), which suggests that damage to the mouse, placenta, and/or the fetuses themselves as a consequence of *B. duttonii* exposure contributes to growth retardation. Although fetuses from infected dams were generally

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**Figure 3.** A murine fetus (gestational day 18) from an uninfected (A) and an infected (B) dam. The fetus in panel B has recently died in utero. Note the abdominal hemorrhage and lack of blood in vessels. The bar represents 1 mm.

**Figure 4.** Mouse placentas (gestational day 18) from an uninfected (A) and an infected (B) dam. Note the lack of circulation in the placenta, paws, and tail, as well as the placental hemorrhage in panel B. The bar represents 1 mm.
Figure 5. Histological analysis of placentas from *Borrelia duttonii*–infected mice. Slides shown are representative results. Hematoxylin-eosin staining shows a clot with neutrophils in a maternal vessel in the decidua basalis from an infected mouse (A) and an uninfected control mouse (B). The bars in panels A and B represent 10 μm. Staining for CD45 shows leukocytes partly colocalized with and lining necrotic lesions in tissue from an infected mouse (C), whereas leukocytes are only sparsely scattered in an uninfected placenta (D). Hematoxylin-eosin and staining of the same area as in panel C shows the necrotic lesions after a focal infarct in the maternal parts of the placenta (E). F, Hematoxylin-eosin staining of an uninfected control mouse in the same area as that shown in panel D. The bars in panels C–F represent 100 μm.

smaller, the percentage of live fetuses per pregnancy was similar in both groups (data not shown). There was no evidence of increased fetal death or resorption in infected mice, probably because of the late time of infection and short time of exposure to the spirochetes.

**Lower Hb levels during infection.** Because maternal health is a predictor of fetal growth, we wanted to determine which health parameters were affected by RF infection. In developing countries, anemia during pregnancy is an important cause of maternal mortality and morbidity and of intrauterine growth retardation and preterm labor, which leads to high perinatal mortality [1]. To assess the impact of infection on maternal Hb levels, an indicator of blood iron levels and an important factor for general health, Hb levels in peripheral blood from uninfected pregnant, infected pregnant, and infected nonpregnant mice were measured every third day from the day of infection, E9 (figure 2).

Pregnancy itself leads to decreased Hb levels [29], which was clear when levels were compared in pregnant and nonpregnant mice before infection on E9 (figure 2). On E12, the same pattern was even more prominent, with Hb levels decreasing further in both sets of pregnant mice but remaining unchanged in infected nonpregnant mice. By E15, 6 days after infection, which approximately coincided with the first peak in spirocheteemia, infected mice in both groups had significantly lower blood Hb levels than uninfected mice (*P* < .05), and this pattern continued on E18 (*P* < .05). Infected nonpregnant mice were more anemic than infected pregnant mice on both E15 and E18, with a statistically significant difference (*P* < .05) on E15. Over the time course monitored, median Hb levels decreased from 140 to 115 g/L in uninfected pregnant mice, from 140 to 90 g/L in infected pregnant mice, and from 159 to 78 g/L in infected nonpregnant mice. Thus, uninfected pregnant mice exhibited an 18% decrease in Hb levels, compared with 36% and 51% in infected pregnant and infected nonpregnant mice, respectively. Rather than acting synergistically, as one might
expect, pregnancy and RF infection seem to be antagonistic, with nonpregnant mice tending toward more-severe anemia than their pregnant counterparts.

**Tissue damage and inflammation caused by B. duttonii infection.** In addition to measuring weight, we analyzed fetuses for morphological and physiological abnormalities—signs of impaired development in utero. To assess the degree of macroscopic damage, all fetuses were classified as normal or abnormal. Normal fetuses appeared pink, healthy, and had translucent skin with visible blood in the vessels (figure 3A). Those classified as abnormal had a range of defects, such as ashen skin, blotchy complexion, the absence of visible blood in vessels, and/or the presence of apparent internal hemorrhages (figure 3B). For the most part, all fetuses had normal bodies morphologically. The types of defects observed in abnormal fetuses indicated restricted blood flow, decreased red blood cells, and/or internal tissue damage. The percentage of abnormalities in fetuses from infected and uninfected mice was 25% (21/83) and 10% (5/50), respectively, which indicated that gestational *B. duttonii* infection can impede normal fetal development \( P < .05 \).

Because the placenta is the interface between mother and fetus, its function is critical to fetal growth and development. To investigate whether the fetal abnormalities (figure 3B) and intrauterine growth retardation (figure 1) might have been due to placental damage, placentas were analyzed for gross abnormalities, defined as blotchiness, apparent hemorrhaging, and/or impaired circulation. Sixty-six percent (55/83) of placentas from infected mice were graded as having abnormalities, compared with only 10% (5/50) of those from uninfected mice. The difference was statistically significant \( P < .001 \). Hemorrhages and pale areas indicating tissue necrosis and poor blood distribution were apparent in abnormal placentas (figure 4).

Placentas were further examined microscopically, in a blinded fashion, to determine the extent of tissue damage and inflammatory cell infiltration. Nine of 10 placentas from infected mice showed pathological signs indicative of inflammation that appeared to start on the maternal side and ascend to the fetal side (data not shown). The most consistent pathological sign was the presence of fibrin deposits and leukocytes within and outside maternal vessels in the decidua basalis (figure 5A), whereas no such pathological sign was visible in tissues from uninfected control mice (figure 5B). Inflammation was also detected by immunohistochemical analysis as assessed by the presence of CD45-positive cells, predominantly neutrophils (figure 5C). CD45-positive cells were sparse in placentas from uninfected mice (figure 5D). Numbers of macrophages were approximately the same in placentas from infected and uninfected mice (data not shown). Necrotic lesions were commonly colocalized with the immune-cell infiltrations (figure 5E), which indicated tissue damage in areas close to maternal vessels. By contrast, of 8 placentas obtained from uninfected mice, none showed any evidence of inflammation (figure 5F). Six of 10 placentas from infected mice had reduced amounts of blood in fetal vessels inside the labyrinths, which suggests vessel damage and/or impaired blood flow, whereas all labyrinths from control mice were uniformly filled with blood (not shown).

**Penetration of the maternal-fetal barrier by B. duttonii.** To determine whether *spirochetes* reside in placenta, potentially causing the extensive tissue damage and inflammation (figures 4 and 5), placentas from infected mice were examined for the presence of *B. duttonii*. Numerous *spirochetes* were detected in the labyrinth area of the placenta by immunofluorescence microscopy (figure 6A).

Furthermore, fetuses from infected mice were themselves analyzed for the presence of live *B. duttonii* by microscopy and MIT. In several cases, *spirochetes* were numerous enough to be visualized directly in fetal abdominal fluid or organ homogenates by microscopy (figure 6B). MIT was performed to detect the presence of small numbers of live bacteria; fetal organ homogenates were inoculated into naive mice that were examined daily for the presence of *spirochetes*. Using this method,
we confirmed infection in utero by transplacental transmission in 22 (73%) of 30 fetuses and thereby demonstrated that RF borreliosis is frequently transferred congenitally.

**Milder disease during pregnancy.** We used our model to compare the severity of RF between pregnant and nonpregnant mice. It may seem logical to predict that infection would be more severe during pregnancy, given that pregnancy increases host susceptibility to many infectious agents because parts of the immune system are down-regulated to prevent rejection of the fetus. Contrary to what we expected, levels of spirochetemia were significantly lower and symptoms were markedly less severe in pregnant than in nonpregnant mice. We monitored the time course and severity of spirochetemia. The median peak was 5.8 times higher in nonpregnant than in pregnant mice (figure 7). Furthermore, nonpregnant mice had significantly higher levels of spirochetemia on days 5 and 6 after infection ($P = .02$ and $P = .01$, respectively), despite a great deal of variability among mice. The resolution of spirochetemia was also slower in this group, occurring on day 7 after infection, as opposed to day 6 in pregnant mice (figure 7), which suggests that the pregnant host is better able to control the infection. The total average spirochete burden during the 9 days of infection was $1.5 \times 10^8$ (SD, $1.4 \times 10^8$) in nonpregnant mice, compared with $1.2 \times 10^8$ (SD, $6.8 \times 10^7$) in pregnant mice ($P < .05$). Therefore, nonpregnant mice sustained a 12.5-fold greater total spirochete burden over the course of the 9 days, compared with pregnant mice. Furthermore, nonpregnant mice appeared to be dehydrated, had ruffled fur, and were generally less active than their pregnant counterparts. These results indicate that pregnancy has a protective effect on the severity of RF borreliosis, perhaps because of higher interleukin-10 levels during pregnancy in both mice [30] and humans [31], which drives the Th2 response that is necessary to clear *Borrelia* infections [32].

**DISCUSSION**

Infectious diseases are important determinants of complications of pregnancy and perinatal death worldwide. RF infection has been documented to pose an enormous risk to pregnant women and their fetuses, especially in areas of Africa where it is endemic [7], making this illness a particular threat to women in a part of the world where their well-being is already compromised. Several studies of the risks during gestational RF have listed infant death, preterm delivery and spontaneous abortion [7, 8], low birth weight, and perinatal disease [4, 6, 9], which likely arise from the overall deterioration in health of the mother, as well as potential bacterial spread to the fetus. Reports where a tick bite or infection at birth can be excluded have suggested that spirochetes can be transmitted in utero [10, 12, 23]; however, such transmission has been difficult to prove definitively. To understand the mechanisms of complications of pregnancy during RF, we developed an animal model using *B. duttonii*.

We have presented a model resembling manifestations reported in human infection [2–12], in that mice exhibited a similar frequency and duration of spirochetemia and anemia. We established the inoculation time point such that fetuses would survive the gestational period, to study the pathogenesis of infection on near-full-term fetuses and placentas. The timing of infection during murine pregnancy greatly influences fetal death rates in both Lyme disease [33] and RF (data not shown). The 9 gestational days of murine infection correspond to 18 weeks (second trimester) in a human pregnancy—a time when fetuses are fairly well developed. With the chosen time point of infection, no difference in fetal death or resorption rates was observed between infected and uninfected mice (data not shown), although more fetuses from infected mice exhibited gross abnormalities (figure 3) that would likely have contributed to their death shortly after birth. Indeed, in preliminary experiments, few pups from infected dams survived after birth (data not shown).
We observed a correlation between spirochete exposure and low gestational weight (figure 1), a serious condition in developing countries that is tightly linked to infant mortality [28]. This correlation supports the findings of Melkert [8], who noted that patients with high levels of spirochetaemia were more likely to have severe complications of pregnancy than were patients with milder disease. The lower fetal weight may be explained by intrauterine growth retardation caused by poor gas exchange and nutrient transport. Maternal Hb levels were lower in infected mice (figure 2), which limited the availability of iron to those fetuses. Hb levels naturally decrease during the course of a murine pregnancy [29], but, concomitant with B. duttonii infection, this decrease was even more pronounced late during gestation.

Nutrient and oxygen acquisition were also hindered by placental tissue damage in infected mice that exhibited inflammation, red blood cell depletion, and hemorrhaging (figure 4). In more than one-half of placentas from infected mice, erythrocytes were depleted in the labyrinth, an area where maternal-fetal exchange takes place. Such depletion may have been due to low hematocrit levels, which is a known consequence of RF infection [25], and/or restricted blood flow resulting from rosetting, the erythrocyte clustering that leads to reduced microcirculation and blood clots [24]. The examination of fetuses revealed abnormalities consistent with poor blood circulation and tissue necrosis (figure 3), which likely resulted from placental damage limiting nutrient and oxygen transfer. With prolonged oxygen deprivation, tissue necrosis and even fetal death are inevitable; thus, infection at an earlier time point may have induced a higher incidence of fetal mortality. In Lyme disease during pregnancy, fetal death is determined by acute infection early during gestation [33].

Although case reports have provided evidence of transplacental transmission in humans [10, 12, 23], infection after birth through umbilical cord severance or while the infant traverses the birth canal could never definitively be ruled out, and its incidence remains unclear. We have confirmed the presence of congenital infection in our model by microscopy (figure 6B) and the fulfillment of Koch’s postulates. We have shown that RF borreliae reside in placenta (figure 6A) and infect the fetus (figure 6B), with transplacental transmission occurring at an incidence of 74%, which is consistent with incidences of other spirochetal infections, Lyme disease, and syphilis, which can also be transmitted congenitally [33, 34].

We have used this model to study the role that RF plays in gestational outcome. During pregnancy, immunological competence is altered to prevent rejection of the fetus. Several diseases—for instance, lysteriosis [35] and malaria [36]—are more severe during pregnancy. It is tempting to assume that pregnancy would also lead to increased susceptibility to infection and more-severe disease in RF. On the contrary, we have shown a 12.5-fold decrease in total spirochete burden over the course of 9 days of infection, faster clearance from the blood (figure 7), and less-pronounced signs of illness in pregnant than in nonpregnant mice. These milder symptoms are consistent with the less-severe anemia during infection in pregnant than in nonpregnant mice (figure 2). Apparently, pregnancy confers protection against RF borreliosis, perhaps because of the gestation-induced shift toward a Th2 response. Mice already exhibiting a Th2 pattern because of pregnancy might respond with faster antibody production and clear borreliae more efficiently [32]. This milder RF is also consistent with reports of less-severe Lyme arthritis during pregnancy [37, 38]. Alternatively, other immunological and physiological factors that are altered during pregnancy, including hormonal fluctuations, may also contribute to our observed outcome. Although the mother is partially protected, gestational RF clearly has detrimental consequences for the fetus, as demonstrated by the intrauterine growth retardation (figure 1), increased risk of fetal abnormalities (figure 3), and transplacental transmission. From a bacterial evolution viewpoint, less-debilitating maternal illness increases the possibility that she will survive the infection and transfer the borreliae congenitally to some offspring.

The pathophysiological characteristics of RF during pregnancy are multifactorial and involve placental damage, anemia, and transplacental infection, which together provide a mechanism for intrauterine growth retardation and, potentially, perinatal death. In addition, we have demonstrated that gestation in mice is protective against B. duttonii infection. Studies are ongoing to elucidate the factors involved in this intriguing outcome. The model that we have presented provides a system not only for the study of RF but for further investigations to understand the gestational immune response to infection.

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