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BASIC SCIENCE: OBSTETRICS

Hematogenous infection of Sprague-Dawley rats with *Mycoplasma pulmonis*: development of a model for maternal and fetal infection

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OBJECTIVES: The specific objective of this study was to conduct a dose response experiment with *Mycoplasma pulmonis* in Sprague-Dawley rats to develop a reproducible animal model of maternal and fetal infection that would provide a versatile mechanism to address the innate fetal immune response during intrauterine infection.

STUDY DESIGN: Pregnant rats were infected intravenously at gestation day 14 with 0 (control), 10^1, 10^3, 10^5, and 10^7 colony forming units of *M pulmonis* and necropsied at gestational day 18. Quantitative culture of maternal and fetal tissues as well as histopathologic examination of the placenta were performed.

RESULTS: We have characterized a rat model of maternal and fetal infection that can be manipulated by alteration of infectious dose. Colonization of Sprague-Dawley rat dam and fetal tissues by *M pulmonis* occurred in a dose-dependent manner after intravenous inoculation ($P < .001$). Placental lesion severity increased with infection dose ($P = .0001$). The minimum threshold dose required to establish infection of the dam and fetus was at least 10^3 colony forming units, with consistent colonization of maternal and fetal tissues achieved only with 10^7 colony forming units. In some instances, rat fetal tissues could be colonized in the absence of concomitant amniotic fluid colonization. Interestingly, there appeared to be a predilection for colonization of the reproductive tissues.

CONCLUSIONS: In the Sprague-Dawley rat, the infection rate of both the dam and fetus can be controlled by the inoculum dose. Our data support the concept that hematogenous spread of *M pulmonis* to the rat fetus can occur without amniotic fluid infection and suggest that the fetus itself can potentially seed the amniotic fluid with microorganisms. Importantly, manipulation of both the route of infection as well as infection dose provide a reproducible way to study both maternal and fetal immune response to infection during pregnancy.

Key words: animal model, fetal infection, intrauterine infection

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Microbial infections of the reproductive tract have devastating effects on pregnancy outcome and neonatal complications.[1-11] For the most part, intrauterine infections are clinically silent and are difficult to study, except with retrospective analysis. Therefore, development and characterization of appropriate animal models are critical to understand the causal relationship between infection and adverse outcomes. To elucidate the mechanisms and sequence of events by which bacterial pathogens cause adverse effects, a good animal model should use a natural disease that occurs in the model species, the infection should be easy to establish, the natural course of the disease should be similar to that in humans, and there should be a solid database of normal physiology.

Mycoplasmas are among the most common isolates from the amniotic cavity of women with preterm labor and intact membranes. Isolation of *Ureaplasma parvum/urealyticum* from the placenta is consistently correlated with disease when histologic evidence of chorioamnionitis is used as the criterion,[1,8-16] and adverse pregnancy outcomes (APO), including fetal infection have been correlated with the numbers of *U urealyticum* isolated from reproductive sites during pregnancy.[17] Importantly, antenatal intrauterine infection with ureaplasmas also has been linked to severe pulmonary and neurologic disorders in neonates.[2,7,9,18,19]

Mycoplasma pulmonis genital disease is the only naturally occurring reproductive disease in laboratory animals that is caused by a member of the class Mollicutes, and infection of the uterus can be accomplished by both intravaginal and intravenous (IV) routes.[20-23] Once infected, dams that have varying complications develop similar to ureaplasmal-associated reproductive disease of humans, including chorioamnionitis, fetal infection of the lung and central nervous system, low birthweight, and fetal and neonatal death.[20,22-26]

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The contribution of the fetal immune response to infection is now recognized as a key determinant of APO.[5,6] The specific objective of this study was to develop a reproducible animal model of fetal infection by manipulation of the infectious dose that would provide a versatile mechanism to address the innate fetal immune response without the confoundment of the adaptive maternal response, to correlate infection status among different maternal and fetal tissues, and to evaluate the placental pathology associated with the various infective doses.

MATERIALS AND METHODS

Mycoplasma preparation and culture

To ensure identical inocula for all experiments, a large-volume culture of *M pulmonis* strain X-1048 was grown to late logarithmic phase in Frey's medium, aliquoted, and frozen at −80°C. Immediately before infection, aliquots were thawed and diluted in sterile phosphate buffered saline (PBS) to obtain a concentration of 10^7 colony forming units (CFUs)/100 μL of medium. Ten-fold serial dilutions were made to obtain the additional infection doses. For each experiment, the number of CFUs in each inoculum was verified by culture. Unless otherwise noted, all cultures taken from animals at time of necropsy were serially diluted 10-fold in Frey's broth to 10^{-5} and processed as described previously[22-26] to determine number of CFUs. Selected cultures were confirmed as *M pulmonis* by polymerase chain reaction.[24]

Animals

All animals were handled in accordance with the University of Florida Institutional Animal Care and Use Committee approved protocols. Specific-pathogen-free (SPF) Sprague-Dawley (SD) timed-pregnant rats were purchased from a commercial vendor (Harlan Sprague-Dawley, Inc, Indianapolis, IN). Rats were monitored by the commercial vendor and were presumed SPF for the following: Sendai virus, H-1 virus, rat coronavirus, sialodacryoadenitis virus, reovirus type 3, Kilham rat virus, Hantaan virus, *M pulmonis*, respiratory and enteric pathogens, endoparasites, and ectoparasites.

Husbandry

Pregnant rats were shipped to arrive on gestational day (GD) 11 and housed in Microisolator (Lab Products, Inc, Maywood, NJ) cages. To maintain barrier conditions, all animals were handled within a laminar airflow hood. All food, water, and caging were autoclaved before use. Control rats were always handled first and were housed separately from inoculated rats.

Processing and experimental infection of rats

Previous studies have demonstrated that IV inoculation at GD 14 is the most efficient time point for inducing fetal infection.[20] Before inoculation, rats were killed on GD 14 with an intraperitoneal dose of ketamine (25 mg; Ketaject, Phoenix Pharmaceutical Inc, St. Joseph, MO) mixed with xylazine (0.375 mg; Xylazine-20, The Butler Co, Columbus, OH) to produce 30 minutes of anesthesia. Rats were ear-notched for identification and intravenously inoculated via the subclavian vein with 0 (control), 10^1, 10^3, 10^5, and10^7 CFU of *M pulmonis* in 0.1 mL of Frey's broth. To calculate the number of animals required to detect a true dose effect, a power analysis was performed and the desired significance level (α) was set at .05, power was set at 0.8, and the difference to detect between groups was set at 80%. By using a power chart for a variance of 1, a sample size of 6 falls between a power value of 0.8 to 0.92. By using these parameters, 5-6 animals per group would be the maximum required. A minimum of 5 animals was included in each treatment group, except the low- and high-dose groups. In accordance with the mandate of the Animal Welfare Act, we reduced the number of animals used at the highest and lowest infection dose. Because the results showed no colonization at the low dose ($n = 4$) and maximum colonization at the high dose ($n = 5$), no additional rats were infected at those doses.

Necropsy

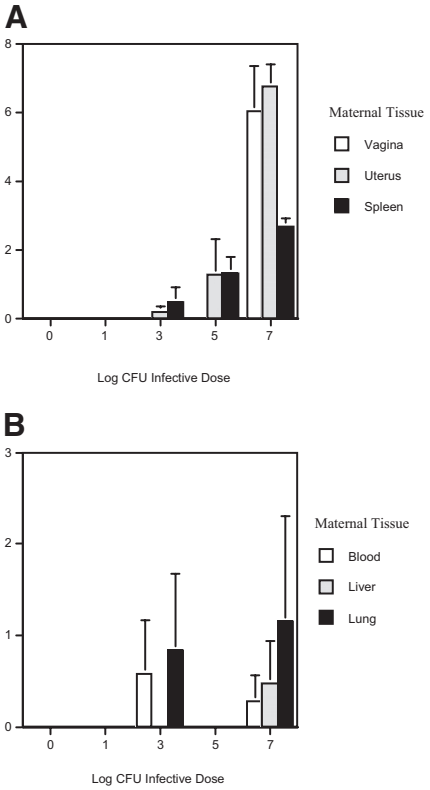
Necropsy procedures were performed as previously described.[22,25,26] Rats were killed with an overdose of sodium pentobarbital (180 mg; Veterinary Laboratories Inc, Lenexa, TX) injected intraperitoneally. The spleen, liver, trachea, lung, vagina, and uterus were aseptically removed from each dam and cultured for *M pulmonis*. Endometrial cultures were performed by aseptically opening the uterine cavity and swabbing the endometrium.

The opened uterus was examined for evidence of fetal resorption, maceration, or obvious fetal abnormalities. Resorbed fetuses were those that had obviously implanted in the uterine wall but had no remaining recognizable fetal structure compared with other fetal units at the same stage of gestation. In early resorptions, some evidence of an amniotic sac remained. Six fetal units were chosen at random for culture, and the remaining fetal units were placed in buffered formalin (1:10; Biochemical Sciences, Inc, Swedesboro, NJ) for histopathologic evaluation. The intact fetal unit (placenta, chorionic and amniotic membranes, amniotic fluid [AF], and fetus) with uterine tissues attached on either side of the placenta was removed. Each fetal unit and its corresponding tissues were labeled such that the fetal unit of origin could be identified. The placenta was separated from the endometrium and fetal membranes before culture. AF from each fetal unit was obtained by puncturing the chorioallantoic membrane with a sterile needle.[22,25,26] The fetus was disinfected with 70% ethanol before aseptic dissection and the fetal brain, lung, and spleen/liver was removed and minced separately for culture.

Histopathology and lesion scoring of placental tissues

At least 3 placentas per dam per treatment group were randomly selected for histopathologic evaluation. After fixation in buffered formalin, the amniotic sac was punctured and the fetus removed. The endometrium with attached placenta and amniotic membranes was

FIGURE 1
Number of *M pulmonis* isolated from dam tissues at gestational day 18



Rats were inoculated IV at GD 14 with 0 (n = 5), 10^1 (n = 4), 10^3 (n = 6), 10^5 (n = 6), or 10^7 (n = 5) CFU *M pulmonis* diluted in sterile Frey's broth. Results are expressed as mean log CFU + standard error (SE) of *M pulmonis* isolated from each site. **A**, Vagina, uterus, and spleen. There were significant increases in number of *M pulmonis* colonies isolated from the vagina ($P = .0001$), uterus ($P = .0001$), spleen ($P = .0005$) at 10^7 dose when compared with all other doses. **B**, Blood, liver, and lung. No significant differences were detected in blood ($P = .62$), liver ($P = .27$), or lung ($P = .60$).

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transected so that a cross-sectional view of endometrium, decidua/labyrinth, and chorioamnion would be present on each section. Tissues were processed routinely, and stained with hematoxylin-eosin (H&E). Each placental section was randomly assigned a number to 4 observers blinded to the treatment of each tissue. The lesion scoring system was based on the degree of polymorphonuclear neutrophil (PMN) infiltration, presence of mononuclear cells, and cellular degradation in order to measure the degree of deciduitis.[23] Numerical scores for grading the severity of deciduitis were as follows: 1 for absence of deciduitis, 2 for moderate deciduitis, and 3 for severe deciduitis.

Statistical analysis

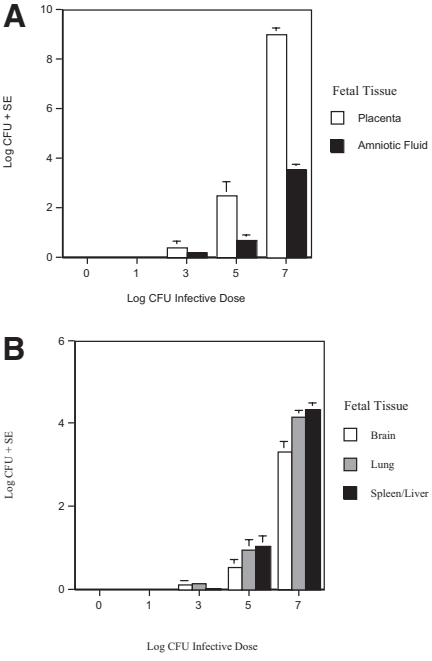
Data were examined for potential differences in litter size, number of resorptions, and log CFU/mL by least-squares analysis of variance (ANOVA). When significant dose effects ($P < .05$) were detected, individual means were separated by the Student-Newman-Keuls test. Linear regression analysis was also performed on CFU data from cultured dam and fetal tissues. Categorical responses for culture status were compared by the χ^2 test. Kruskal–Wallis 1-way nonparametric ANOVA was used to compare factor levels for ordinal response variables of histology slide scores (Statistix Analytical Software, Tallahassee, FL). Differences were considered significant at $P < .05$.

RESULTS

Colonization of maternal tissues

Not surprisingly, the numbers of *M pulmonis* recovered from the tissues were dependent on the initial inoculation dose (Figure 1). Consistent colonization of the dam tissues occurred only in the high-dose (10^7) group ($P < .001$). Dams inoculated with 10^7 CFU inoculation had significantly higher numbers of *M pulmonis* colonies isolated from the vagina ($P = .0001$), uterus ($P = .0001$), and spleen ($P = .0005$) than any of the other inoculation groups (Figure 1A). However, no significant differences were detected in the number of *M pulmonis* isolated from blood ($P = .62$), lung ($P = .60$), and liver ($P = .27$) (Figure 1B). Interestingly, there appeared to be a predilection for colonization of the reproductive tissues rather than respiratory sites in the pregnant rat. At the lower infection doses, isolation of *M pulmonis* was variable. No dams were culture positive for *M pulmonis* in the trachea at any dose.

FIGURE 2
Number of *M pulmonis* isolated from fetal tissues at gestational day 18



Dams were inoculated IV at GD 14 with (n = 5), 10^1 (n = 4), 10^3 (n = 6), 10^5 (n = 6), or 10^7 (n = 5) CFU *M pulmonis* diluted in sterile Frey's broth. Six fetal units were cultured per dam. Results are expressed as mean log CFU + standard error (SE) of *M pulmonis* isolated from each site. **A**, Placenta and amniotic fluid. There was an increase in number of CFU isolated from AF ($P = .0001$), and placenta ($P = .0001$), at 10^7 compared to all other doses. **B**, Brain, lung, and spleen/liver. Similar results were found for brain ($P = .0001$), lung ($P = .0001$), and spleen/liver ($P = .0001$).

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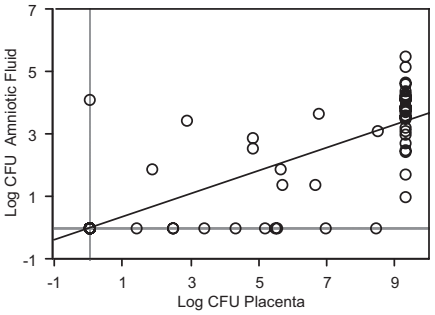
Fetal infection and APO

There was no significant effect of *M pulmonis* infection (data not shown) on either litter size ($P = .4739$) or the number of resorptions ($P = .2029$). The fetal tissues were colonized by *M pulmonis* in a similar way as the maternal tissues. A significant difference among dose groups ($P < .0001$) was observed in log CFU of *M pulmonis* isolated from fetal tissues, and the numbers of *M pulmonis* recovered from fetal tissues were directly correlated with the dose group (Figure 2). At the highest dose (10^7), *M pulmonis* was

FIGURE 3
Correlation between colonization of placenta and amniotic fluid



CFU determinations for 126 matched samples were compared by regression analysis (correlation R = .864, R^2 = .754, $P < .0001$). Overlapping points occurred if CFU were identical for samples. Ten placentas were colonized by *M pulmonis* in the absence of concomitant colonization of the paired amniotic fluid. One amniotic fluid was colonized by *M pulmonis* in the absence of concomitant colonization of the paired placenta.

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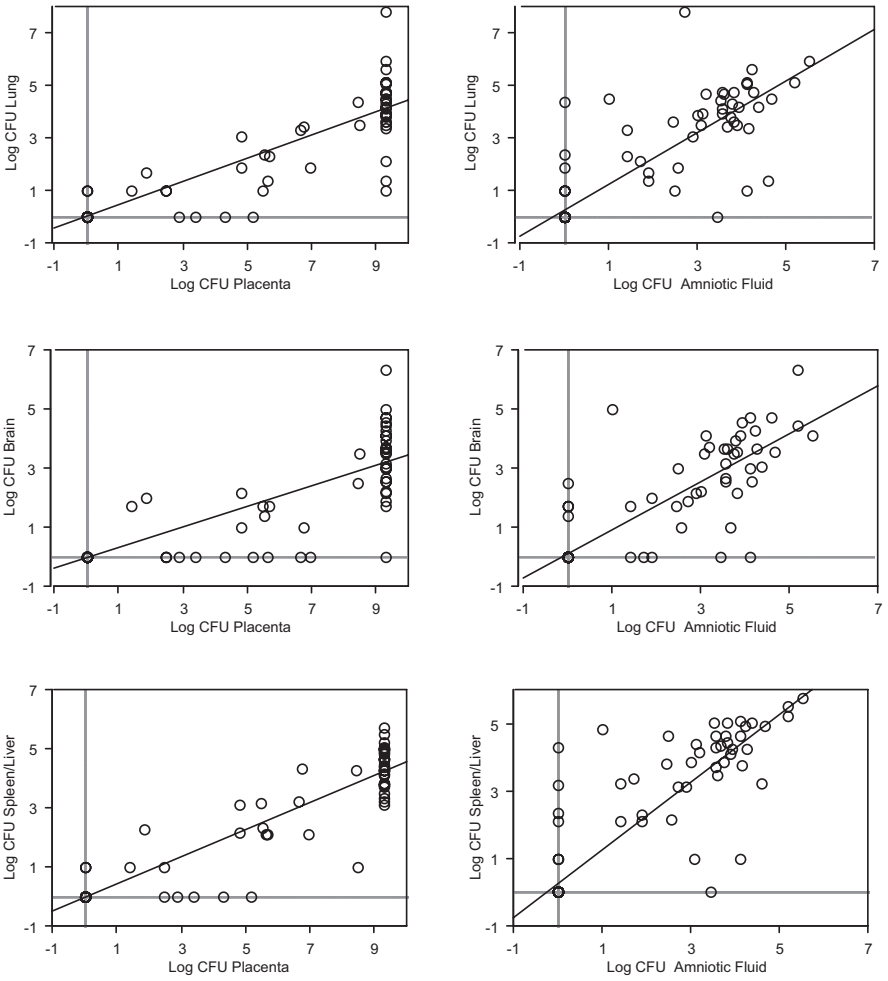
recovered from brain (28/30) and all other fetal tissues (30/30). Perhaps the most informative data were obtained from the 10^5 inoculation dose group. *M pulmonis* was recovered from placenta (16/36), AF (8/36), spleen/liver (15/36), lung (15/36), and brain (10/36). At 10^5 inoculation dose group, both negative and positive fetal units could be identified within a litter. *M pulmonis* was not recovered from either control or the low-dose (10^1) group.

Correlation of mycoplasmal load among tissues

The generally accepted mechanism of spread of infectious agents is that placental colonization precedes infection of AF. Fetal tissues are then infected via the AF. Because matched samples were available, the numbers of *M pulmonis* isolated from 126 fetal units were compared by regression analysis (Figures 3 and 4). Not unexpectedly, a high correlation was found for all tissues ($P < .0001$), with the greatest correlation found between the placenta and the fetal spleen/liver (correlation 0.95). There were a number of

FIGURE 4
Correlation between colonization of placenta or amniotic fluid and fetal tissues



CFU determinations for 126 matched samples were compared by regression analysis; overlapping points occurred if CFU were identical for samples. Correlations with placental CFU are shown on left and correlations with amniotic fluid CFU are shown on right. CFU of *M pulmonis* recovered from fetal lung (top) was correlated with recovery from the placenta (R = .923, R^2 = .85, $P < .0001$) and amniotic fluid (R = 0.863, R^2 = .743, $P < .0001$). *M pulmonis* was recovered from 2 fetal lungs in the absence of concomitant colonization of the paired placenta and from 8 fetal lungs in the absence of concomitant colonization of the paired amniotic fluid. CFU of *M pulmonis* recovered from fetal brain (middle) was correlated with recovery from the placenta (R = .923, R^2 = .85, $P < .0001$) and amniotic fluid (R = .863, R^2 = .743, $P < .0001$). *M pulmonis* was not recovered from any fetal brain in the absence of concomitant colonization of the paired placenta but was recovered from four fetal brains in the absence of concomitant colonization of the paired amniotic fluid. CFU of *M pulmonis* recovered from fetal spleen/liver (bottom) was correlated with recovery from the placenta (R = .95, R^2 = .901, $P < .0001$) and amniotic fluid (R = .884, R^2 = .78, $P < .0001$). *M pulmonis* was recovered from 2 fetal spleen/livers in the absence of concomitant colonization of the paired placenta and from 7 fetal spleen/livers in the absence of concomitant colonization of the paired amniotic fluid.

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TABLE
Histology of placenta after IV inoculation with *M pulmonis*

	Number of placenta	Deciduitis mean rank	Mean lesion ± SD
Control	13	26.654[a]	1.54 ± 0.22
10^1 dose	11	33.591[b]	1.82 ± 0.23
10^3 dose	17	32.088[b]	1.77 ± 0.16
10^5 dose	14	36.607[b]	1.93 ± 0.22
10^7 dose	25	58.64[c]	2.76 ± 0.12

Dams were inoculated at GD 14 and necropsied on GD 18. Dams were inoculated IV with 0, 10^1, 10^3, 10^5, or 10^7 CFU infective dose of *M pulmonis* diluted in sterile Frey's broth. Placentas from at least 3 fetal units per dam were randomly selected from each dose. Tissue sections containing decidua, chorion and amnion were processed and stained with hematoxylin and eosin (H&E). Tissues were scored for deciduitis based on (1) no neutrophilic infiltration, (2) moderate neutrophilic infiltration, (3) severe neutrophilic infiltration, neutrophil degradation and presence of mononuclear cells. There was a significant difference in mean ranks for deciduitis among doses ($P = .0001$).

[a,b,c] Mean ranks with the same symbol are not significantly different from each other.

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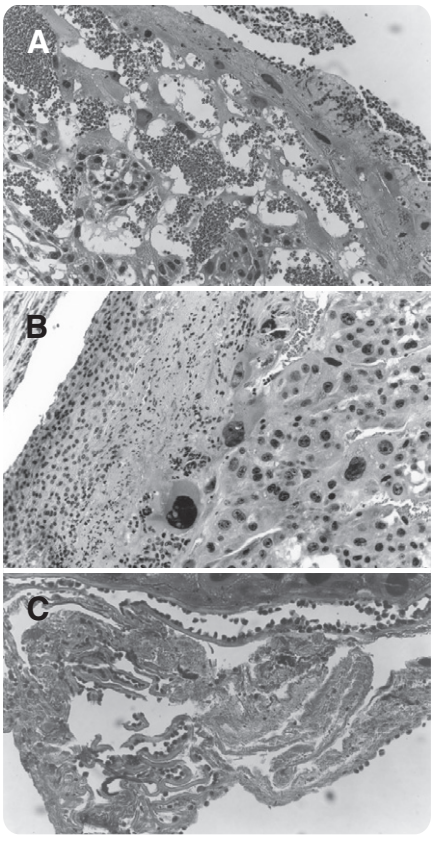
FIGURE 5
Representative placental lesions



A, Severe inflammatory infiltrate indicative of deciduitis. **B**, Moderate inflammatory infiltrate indicative of deciduitis. **C**, Normal placenta with absence of significant inflammatory infiltrate. (Hematoxylin-eosin; original magnification, ×20.)

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AFs that were culture negative, even when the corresponding placentas had mycoplasmas isolated at log CFU greater than 5 (Figure 3). Conversely, there were isolated cases in which the AF was infected without placental infection. The discrepancies between AF colonization and colonization levels in fetal lung and brain, in which mycoplasmal log CFU greater than 2 could be obtained in the absence of infected AF (Figure 4) are also intriguing. Colonization of the fetal spleen/liver also occurred in the absence of AF infection, and in some instances the number of mycoplasmas recovered from spleen/liver were several logs higher than the corresponding isolations from AF. Taken together, these data suggest that in at least some cases, fetal colonization preceded AF colonization.

Histopathologic lesions

Differences in mean ranks for deciduitis (Table) were significantly different in some treatment groups ($P = .0001$); dams that received 10^7 CFU had the highest deciduitis rank. As would be expected, 62% of control tissues showed normal placental degeneration (Table 1, Figure 5, **C**). At GD 18, this normal degradation is identified by only small, scattered necrotic cells and occasional PMN[27] and received a lesion score of 1. Moderate inflammation (lesion score of 2) was seen in placentas from the 10^1, 10^3, and 10^5 dose groups (Figure 5, **B**). Severe inflammatory infiltrate of PMN, mononuclear cells, and cellular degradation (lesion score of 3) was found in 84% of placentas at the 10^7 dose (Figure 5, **B**).

Comment

We have characterized a rat model of maternal and fetal infection that can be manipulated by alteration of infectious dose (ID). Colonization of SD rat dam and fetal tissues by *M pulmonis* occurred in a dose-dependent manner after IV inoculation, similar to colonization results seen previously in vaginally and IV-infected rats.[20,22,25,26] The minimum threshold dose required to establish infection of the dam and fetus is at least 10^3 CFU, with consistent colonization of all tissues achieved only with 10^7 CFU. For rat fetal units, the 10^5 dose may be too low to achieve ID_{50}, but this dose would allow comparisons to be made between infected and uninfected fetal tissues within the same dam. The 10^5 dose may be a valuable tool to determine whether the maternal response to infection, without the confounding presence of infection in the fetus, influences fetal growth and development. At the 10^7 dose, consistent infection of fetal and placental tissues can be achieved for use in collecting infection data from an entire litter.

Interestingly, in the SD rat model, the respiratory sites were colonized infrequently during pregnancy. Conversely, the uterus and vagina were colonized by *M pulmonis* in all dams at the 10^7 dose. This strongly suggests that there is an affinity for infection of the reproductive tract vs the respiratory tract after IV inoculation of pregnant rats. One explanation for this result may be the increased vascularization of the reproductive tract during gestation. Increased vascularization may more easily allow the microorganism to access and adhere in these regions. These tissues also may be enriched for nutrients that would favor the growth of *M pulmonis*. The tropism of *M pulmonis* for the reproductive tissues appears to be pregnancy-specific; in a previous study *M pulmonis* was recovered from the trachea in 35 of 36 naturally infected nonpregnant rats but was isolated from the uterus of only 17 of these same rats.[21] Further, in rats experimentally infected

in the vagina before breeding, the reproductive tissues were more commonly colonized than the trachea once pregnancy was established.[22,25,26]

The current paradigm is that infectious agents colonize the placenta, breach the placental barrier, and infect the AF, thereby gaining access to the fetus directly or via hematogenous spread from the placenta.[28,30] Our results strongly support the concept that the SD rat fetuses may have been directly infected via the labyrinthian circulation. We observed that hematogenous spread of *M pulmonis* from the infected placenta to the fetus can occur without AF infection, and that, in some cases, the fetus itself could potentially seed the AF with microorganisms. This is consistent with some models proposed for human fetal infections that suggest the most severe consequences of intrauterine infection occur when the infectious agent colonizes the placenta and then is transported to the AF and fetus via hematogenous spread from the placenta.[1,29-31]

One drawback of the IV model is that establishment of infection is not the same as in naturally occurring disease. Although the vaginal model of infection is a more natural method of causing intrauterine infection, it also has limitations such as increased time to establish infection and uncertainty of level of infection. In addition, significant intrauterine infection is seen only in animals infected before breeding rather than at time points throughout gestation.[20-22] The IV model is more useful for studying acute, temporal effects of infection, whereas the vaginal infection model is better suited for chronic situations. Studies focused on mechanisms of low birthweight would be more appropriate for the vaginal model[20] as acute infection established in IV-infected rats did not result in low birthweight. An additional limitation of the model is that the use of *M pulmonis* rather than *U parvum/urealyticum* precludes addressing microbe-specific factors that may be involved in pathogenesis. This is somewhat analogous to other models, for example, that use purified LPS rather than live Gram-negative microbes for infection studies or that use of in vitro cell lines rather than whole animal studies; these models still provide critical insights into pathogenesis.

The IV rodent model described here has provided important insights into lesion formation, mechanisms of fetal colonization, and fetal and maternal immune response. Although no animal model can be directly applicable to human disease, the similarities between human and rodent mycoplasmal genital infection (inflammatory cytokines present in AF, placental lesions, and the clinical presentation of poor pregnancy outcome) argue that the rodent model is appropriate. Further, manipulation of both the route of infection as well as infection dose provide a reproducible way to study both maternal and fetal immune response to infection during pregnancy. ■

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