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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 $10^5$ 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

Mycobacterial infections of the reproductive tract have devastating effects on pregnancy outcome and neonatal complications. 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, and adverse pregnancy outcomes (APO), including fetal infection have been correlated with the numbers of *U urealyticum* isolated from reproductive sites during pregnancy. Importantly, antenatal intrauterine infection with ureaplasmas also has been linked to severe pulmonary and neurologic disorders in neonates.

*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. 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.
The contribution of the fetal immune response to infection is now recognized as a key determinant of APO.\textsuperscript{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 \(-80^\circ\text{C}\). 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 \(\mu\text{L}\) 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\textsuperscript{22-26} to determine number of CFUs. Selected cultures were confirmed as *M pulmonis* by polymerase chain reaction.\textsuperscript{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 coronoavirus, sialodacryoadenitis virus, reovirus type 3, Kilham rat virus, Han-tan virus, *M pulmonis*, respiratory and enteric pathogens, endoparasites, and ectoparasites.

**Necropsy**

Necropsy procedures were performed as previously described.\textsuperscript{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.\textsuperscript{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...
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. 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 $\chi^2$ 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 ($P = .0001$), spleen ($P = .0005$) than any of the other inoculation groups (Figure 1A). However, no significant differences were detected in the numbers 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.

**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
CFU determinations for 126 matched samples were compared by regression analysis (correlation R = .864, R² = .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.

Riggs. Hematogenous infection of Sprague-Dawley rats with Mycoplasma pulmonis. *Am J Obstet Gynecol* 2008.

recovered from brain (28/30) and all other fetal tissues (30/30). Perhaps the most informative data were obtained from the 10⁵ 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 10⁵ 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 (10¹) 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. Correlation of mycoplasmal load among 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, R² = .85, P < .0001) and amniotic fluid (R = .863, R² = .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, R² = .85, P < .0001) and amniotic fluid (R = .863, R² = .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, R² = .901, P < .0001) and amniotic fluid (R = .884, R² = .78, P < .0001). *M. pulmonis* was recovered from 2 fetal spleen/livers in the absence of concomitant colonization of the paired amniotic fluid.

Riggs. Hematogenous infection of Sprague-Dawley rats with Mycoplasma pulmonis. *Am J Obstet Gynecol* 2008.
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 study by Riggs et al. (2008) showed that the lesion score of deciduitis in the placenta was significantly different among the different doses of M. pulmonis. The lesion score of 0 was found in 26.65% of placenta from the control group, while the lesion score of 1 was found in 33.59% of placenta from the 10^3 dose group, and the lesion score of 2 was found in 32.08% of placenta from the 10^5 dose group. The lesion score of 3 was found in 14.97% of placenta from the 10^7 dose group. These results indicate that the lesion score of deciduitis increased with the dose of M. pulmonis.

**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. 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_{SD}, 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 frequently 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 findings 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. 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.1,29-31 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 model20 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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