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Pregnancy reduces the genetic resistance of C57BL/6 mice to *Listeria monocytogenes* infection by intragastric inoculation

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Abstract

In this study, we compared genetically resistant C57BL/6 and susceptible A/J mice for their resistance to *L. monocytogenes* infection model during pregnancy. Intragastric infection with modest numbers of bacterial cells (10^5 CFU) caused reproducible fetal infection and abortion in both mouse strains. Bioluminescence imaging demonstrated dissemination of *L. monocytogenes* cells from maternal to fetal organs within 3 days of intragastric infection. Although non-pregnant C57BL/6 mice were significantly more resistant to infection than non-pregnant A/J mice, C57BL/6 and A/J mice had similar microbial loads (CFU) in maternal and fetal tissues during pregnancy. Inflammation and necrosis, however, were more severe in A/J mice as evaluated by semi-quantitative histopathology. Although the microbial load in fetal tissues was similar for all fetuses within a single uterus, inflammation and necrosis varied among individual fetuses and placentas. We also noted that the uterus is a target for *L. monocytogenes* infection in non-pregnant mice.

Keywords

Listeria monocytogenes; mouse; pregnancy; bioluminescence

1. Introduction

Listeria monocytogenes continues to be a significant cause of disease, especially in the industrialized nations of the world. Listeriosis results in an estimated 1600 cases (range 600-3200) and 250 deaths (range 0-700) annually in the United States [1]. The infectious dose for humans is uncertain and likely depends on the virulence characteristics of individual *L. monocytogenes* strains. Clinical disease can result from estimated ingestion of as few as 10^4 - 10^6 CFU/g in contaminated foodstuffs. However, the infectious dose is likely lower in immunocompromised or pregnant individuals [2, 3]. Pregnant women are estimated to be 20 times more likely than the average adult to acquire invasive listeriosis, and represent 16% of reported listeriosis cases in the USA [4, 5]. Most maternal *L. monocytogenes* infections occur in the 3rd trimester and are mild or asymptomatic. However, fetal infection can be severe with stillbirth and spontaneous abortion occurring in 20% of

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cases, and 68% of the surviving pregnancies resulting in neonatal infection [6]. Despite considerable knowledge of the pathogenesis of listeriosis, it is unclear why pregnant women do not successfully protect their fetus against intracellular pathogens such as *L. monocytogenes*.

Several different animal models have been developed to study *L. monocytogenes* infection during pregnancy. These differ in host species and strain of *L. monocytogenes* used, route of infection, dose of inoculum, and gestational length at inoculation, [7, 8]. It has been hypothesized that InlA and InlB mediate internalization of *L. monocytogenes* into non-phagocytic cells of the gastrointestinal system [8-12]. The receptors for InlA and InlB are E-cadherin and Met, respectively [13-15]. Importantly, InlA interacts with human and guinea pig E-cadherin but not that of mouse or rat [16]. On the other hand, InlB interacts with human and mouse Met but not that of the guinea pig [17]. Despite the poor ability of mouse E-cadherin to bind InlA, several previous studies used intravenous or intragastric infection with various strains and doses of *L. monocytogenes* to cause fetal infection and abortion in the mouse [18-22]. Pregnant mice exhibited increased severity of maternal disease after intragastric infection when compared to non-pregnant mice [18]. These studies suggest that virulence factors other than InlA allow translocation of *L. monocytogenes* cells across the intestinal epithelium in the mouse. In this paper, we sought to develop an intragastric (i.g.) infection model of fetal infection in pregnant mice, using a clinical isolate of *L. monocytogenes* from a human abortion outbreak, to more closely mimic natural infection in humans and domestic animals.

Inbred mouse strains differ in their innate resistance to experimental infection with *L. monocytogenes*. Resistance is regulated principally by the *Hc* locus on chromosome 2 [23-25]. Mouse strains with the resistant allele at the *Hc* locus are significantly more resistant to i.v., i.p. or i.g. challenge with *L. monocytogenes* than strains with the susceptible allele [24-26]. Previous studies identified the C57BL/6 and A/J strains of mice as prototypic resistant and susceptible strains, respectively [24, 25]. Whether these mouse strains differ in resistance to *L. monocytogenes* infection during pregnancy is unknown. In this study, we compared pregnant C57BL/6 and A/J mice for their resistance to i.g. infection with a strain of *L. monocytogenes* isolated from a human listeriosis outbreak associated with abortion and fetal death [27]. To provide additional insights into the temporal response to *L. monocytogenes* infection in pregnant mice, we used bioluminescence imaging to visualize the progression of infection from maternal to fetal tissues. Our results show that pregnant C57BL/6 and A/J mice do not differ significantly in their microbial load in tissues. We also identify the uterus of non-pregnant mice as a site of *L. monocytogenes* infection and show that not all fetuses suffer the same degree of inflammation and necrosis during *L. monocytogenes* infection of the dam.

2. Results and discussion

2.1 Pregnancy alters the susceptibility of A/J and C57BL/6 mice to intragastric inoculation with *L. monocytogenes*

In our first experiments, we compared the susceptibility of non-pregnant and pregnant C57BL/6 and A/J mice (10-14 days of gestation) to an i.g. challenge with 10^5 CFU of *L. monocytogenes* strain LM2203. Mice were euthanized 72 hours post inoculation, a time point that represents the peak bacillary burden in the spleen and liver [28, 29]. As expected, significantly greater numbers of CFU were recovered from the spleens, livers, and uteruses of non-pregnant A/J mice than C57BL/6 mice (Figure 1A) [26]. These data confirm previous reports regarding the greater innate resistance of C57BL/6 vs. A/J mouse strains [26]. It should be noted that *L. monocytogenes* were recovered from the uteri of both

strains of non-pregnant mice. It is unknown whether colonizing uterine tissue is a general property of *L. monocytogenes*, or a unique attribute of strain LM2203.

We observed a trend for greater microbial loads in the spleen, liver, and fetoplacental units (FPU) of pregnant A/J mice when compared to C57BL/6 mice (Figure 1B). However, these differences are not statistically significant (spleen $P=0.16$, liver $P=0.093$, FPU $P=0.24$). We assessed mice at 72 hours of infection because this time produced the most consistent maternal and fetal infections following inoculation infections. Longer incubation times (96 hours) could not be used because some of the C57BL/6 and all of the A/J mice had developed severe clinical disease requiring euthanasia. We repeated these experiments using challenge inocula of 10^3 and 10^6 CFU of *L. monocytogenes* LM2203. The lower dose (10^3 CFU) resulted in consistent systemic infection of maternal but not fetal tissues (Figure 1C) and the higher dose (10^6 CFU) produced severe disease, which required euthanasia of all mice (data not shown). These findings suggest there is threshold dose ($>10^3$ CFU of LM2203) to cause fetal infection. This finding supports previous reports by Robbins et al. (2010), which suggest the placenta has multiple mechanisms to resist bacteremic spread of *L. monocytogenes* from maternal blood. As the challenge dose increases, these defenses are overcome, allowing fetal infection to occur. Transport of listerial cells from maternal to fetal tissues may not be unidirectional, as Bakardjiev et al. [30] reported trafficking of *L. monocytogenes* back to maternal organs, allowing the fetal tissues to become a source for re-infection of maternal tissues. We also examined the effect of *L. monocytogenes* infection in pregnant mice at different times of gestation (7-10 days and 14-17 days) and found that the 10-14 day gestation period resulted in the most reproducible infection of fetal tissues (data not shown). These data show that pregnancy reduces resistance to *L. monocytogenes* infection of C57BL/6 compared to A/J mice in terms of microbial load in maternal liver and spleen. Interestingly, despite similarities in microbial load in maternal and fetal tissues, the pregnant C57BL/6 mice displayed fewer clinical signs associated with septic listeriosis than pregnant A/J mice.

The above results are somewhat contrary to our previous report of the greater resistance to listeriosis in C57BL/6 vs. A/J non-pregnant mice [26]. The data suggest that the immunosuppressive effect of pregnancy diminishes the heritable resistance of C57BL/6 mice compared to A/J mice, which is linked to the *Hc* locus of chromosome 2 [23-25]. Reduced maternal resistance to *L. monocytogenes* in pregnant mice is consistent with listeriosis in pregnant women, who have a greater likelihood of contracting *L. monocytogenes* after ingesting contaminated food. There is an earlier report of decreased maternal resistance to i.g. *L. monocytogenes* infection in pregnant mice [18]. Those authors used two strains of *L. monocytogenes*, serotypes (1/2a and 4nonb), and found significant differences in microbial load in maternal livers and spleens of non-pregnant vs. pregnant mice at 120 hrs after inoculation. Hamrick et al.[18] also reported colonization of the conceptus 48 hours post inoculation, although the microbial load in fetal tissues was not quantified. Our data differ from those of Hamrick et al. by reporting a greater microbial load following infection with a serotype 4b strain of *L. monocytogenes*, and by quantifying microbial load in fetal tissues. The mechanism by which pregnancy increases susceptibility to listeriosis is unknown. Possibilities include pregnancy-related changes in cell-mediated immunity [31, 32], changing hormonal status in the peri-parturient period [33], and alterations in cytokines and chemokines present in amniotic and allantoic fluids [22]. Perhaps these and other changes during pregnancy influence resistance to *L. monocytogenes* infection.

2.2 Histopathological examination of lesions in maternal and fetal tissues from A/J and C57BL/6 mice

Severity of inflammation and necrosis in the spleen was scored on a 0-3 scale, with 0 defined as normal and 3 denoting severe inflammation (Figure 2a, 2c). All inflammation noted was of mixed cellular populations. Histopathology scores were significantly greater in pregnant A/J than C57BL/6 mice ($p=0.030$). Non-pregnant A/J mice also tended to have greater histopathology scores than non-pregnant C57BL/6 mice, however, in both strains the histopathological changes were less severe and the difference between strains did not achieve statistical significance ($p=0.08$). The clinical significance of semi-quantitative scoring of splenic tissue is difficult to interpret because of the heterogenous architecture of the spleen, especially during inflammation. Interpretation of liver pathology was more straightforward, as we quantified the number of focal inflammatory lesions per five 100 $\times$ magnification fields (Figure 2b). Both pregnant and non-pregnant A/J mice had significantly more liver lesions than pregnant ($p=0.02$) or non-pregnant ($p=0.02$) C57BL/6 mice (Figure 3). The reason for greater histopathological changes in A/J than C57BL/6 mice, despite recovery of similar numbers of CFU from the livers of the two mouse strains, is unclear. We also examined Gram-stained liver sections from C57BL/6 and A/J mice. Gram-positive rods were commonly observed in liver sections from A/J mice (pregnant and non-pregnant). However, bacteria were not seen in non-pregnant C57BL/6 mice and were rarely observed in liver sections from pregnant C57BL/6 mice. The former is likely due to the bacterial burden in non-pregnant C57BL/6 mice being near the minimum threshold for microscopic visualization of bacterial cells. The Gram stain and liver histopathology data are curious because they contradict somewhat the microbial load data for A/J and C57BL/6 mice illustrated in Figure 1. The histopathology data resemble more closely the previously reported greater resistance of C57BL/6 vs. A/J in non-pregnant mice [26]. The mechanism underlying the relatively high microbial load but low histopathology lesions in pregnant C57BL/6 mice is unknown and warrants further study.

Pregnant uterine horns were serial sectioned to visualize each fetus and placenta. Not all feti within a single uterus displayed the same degree of inflammation. In both A/J and C57BL/6 mice histopathology was variable within each pregnant uterus, ranging from normal viable feti to severe inflammation and autolysis of dead feti (Figure 2e, 2f). These findings are consistent with reports in the human clinical literature of twin human pregnancies in which culture positive *L. monocytogenes* infection was observed in only one fetus [34-36]. Human listeriosis infections also have variable inflammation and fetal mortality rates [34-36]. What is curious in the present study is that the CFU of *L. monocytogenes* recovered from the FPU of *L. monocytogenes*-infected pregnant mice are consistent, whereas the inflammation and necrosis in fetal tissues from a single pregnant mouse uterus were variable. We do not know whether the normal appearing fetuses would have resulted in normal deliveries and healthy pups. Our findings are contrary to reports in sheep and cattle, in which *L. monocytogenes* is a common cause of abortion. Similar to ruminants, which often have 2-3 developing fetuses per gestation, mice have multiple fetuses. However, published reports indicated that all fetal lambs and calves become infected and are aborted when a dam is infected with *L. monocytogenes* [37-39].

2.3 Bioluminescence imaging shows real-time progression of *L. monocytogenes* 10403S from maternal to fetal tissues in vivo

We next used bioluminescence imaging to visualize and quantify *L. monocytogenes* infection of pregnant mice. To do so we used a different strain of *L. monocytogenes* (LM10403S), which contains bacterial luciferase [40, 41]. The parent strain of *L. monocytogenes* is a serotype 1/2a strain, first isolated in 1968 from a human skin infection, [42] that is widely used to study the pathogenesis of listeriosis. This strain contains a

luciferase construct that emits light only when the bacterial cells are multiplying. It is important to note that this strain is a different serotype than LM2203 (serotype 4b) and requires a larger dose (10^6 CFU vs. 10^5 CFU) to cause fetal infection. To quantify the infection in pregnant mice, the signal was imaged and quantified with an IVIS® 200 Imaging System (Caliper Life Sciences, Hopkinton, MA).

A/J and C57BL/6 mice were inoculated i.g. with 10^6 CFU (based on pilot experiments with strain LM10403S) and imaged every 24 hours during a 72-hour infection period. This allowed us to monitor progression of the *L. monocytogenes* infection from maternal to fetal tissues (Figures 4a-c). Bioluminescence was quantified as photons/sec/cm² in regions of interest (ROI). After the last luminescence reading at 72 hours post infection, the mice were euthanized and tissues collected for microbial culture (Figure 4d). The CFU recovered from maternal and fetal tissues at the 72 hour timepoint correlated with the bioluminescent signals (ROI) calculated for the same tissues ($r^2 = 0.768$ and $p = 0.02$). These experiments show that following an i.g. infection, the maternal tissues are infected first, which leads to sepsis, fetal infection and abortion. Bioluminescence technology has been previously reported to image fetoplacental listeriosis following intravenous injection of *L. monocytogenes* into gerbils [43]. Here, we report use of bioluminescence imaging to visualize fetal infection following the physiologically relevant, intragastric route of inoculation. This more closely models how contaminated foodstuffs infect humans and their fetuses. We also show that infection can be quantified *in vivo* using ROI measurements. This imaging technology could be used in future studies to test microbial growth in tissues *in vivo* in respect to novel therapeutic or prophylactic regimens.

One disadvantage of this bioluminescence model is that *L. monocytogenes* strain LM10403S emits bacterial luciferase only during log phase growth. Thus, tissue can contain viable *L. monocytogenes* (as confirmed by CFU) but yield little or no luminescence signal at a given timepoint. An example of this is illustrated in Figure 4d, in which only one FPU is luminescent at 48 hours post i.g. infection despite the recovery of similar CFU/g from feti. However, these *in vivo* imaging data are consistent with our histopathology finding that not all fetuses within a single uterus suffer the same degree of inflammation and necrosis during *L. monocytogenes* infection.

3. Conclusions

Taken together, our infection model and bioluminescent imaging technology show that i.g. infection of pregnant mice with *L. monocytogenes* causes reproducible fetal infection and abortion that mimics several aspects of clinical listeriosis in human beings. In addition, we find that the superior resistance of C57BL/6 mice is muted when mice are infected during pregnancy. We also report that infection with *L. monocytogenes* strain LM2203 results in bacterial colonization of the uterus, even in non-pregnant mice. Perhaps this affinity for the uterus explains in part the predilection of *L. monocytogenes* to cause fetal infection and abortion.

4. Materials and Methods

4.1 Strains of *L. monocytogenes*

L. monocytogenes strain 2203 (serotype 4b) was generously donated by Dr. Sophia Kathariou (Raleigh, NC). This is a clinical isolate from a food-borne disease outbreak that caused disease in 13 people, 11 of whom were pregnant. Five of the pregnant women experienced stillbirth, 3 were induced into premature labor, and 3 births resulted in neonatal infections [27]. Bioluminescent *L. monocytogenes* strain 10403S was originally obtained

from Dr. Christopher Contag (Stanford, CA) and made bioluminescent by transformation with the plasmid pAUL-A Tn4001 *luxABCDE Km^r* [40, 41].

4.2 Preparation of *L. monocytogenes*

L. monocytogenes cells were stored at -20°C on Cryobank™ Cryobeads (Copan Diagnostics, Inc., Corana, CA). For each experiment, a bead was placed into 5 ml of brain heart infusion (BHI) broth and incubated overnight with shaking at 37°C. Bacterial cells were harvested by centrifugation (3,500 × g for 5 minutes), washed three times in phosphate buffered saline and kept on ice prior to inoculating mice. The bacterial suspensions were diluted to the desired concentration, and numbers of viable *L. monocytogenes* confirmed by plating serial dilutions onto tryptic soy agar with 5% sheep blood (BD® Biosciences).

4.3 Inoculation of mice

Female inbred A/J and C57BL/6 mice were obtained from the Jackson Laboratories (Bar Harbor, Maine) at 6 weeks of age and housed under microisolator caps at the School of Veterinary Medicine animal care facility. For bioluminescence studies, albino C57BL/6 (Jackson Laboratories) were obtained at 6 weeks of age and housed under microisolator caps at the UW-Madison Microbial Sciences animal care facility. Mice were acclimated for 1 week in these facilities prior to being paired with a breeding male. Female mice were allowed to reach 7-10 days of gestation prior to use in an experiment. Mice received food and water ad libitum until 5 hours prior to an intragastric inoculation experiment, at which time food was removed from the cage. This was done to minimize the risk of delivery of the bacterial inoculum into stomachs that were engorged with mouse chow, which could lead to aspiration of the inoculum into the lungs. Mice were anesthetized by i.p. injection of sodium pentobarbital (40 mg/kg). When the mice were sedated, the listerial inoculum was introduced (as a total volume of 0.1 ml) via a 1.5 in.-long, 24 gauge, stainless steel oral esophageal tube attached to a 1-ml syringe.

4.4 Bioluminescence imaging

Bioluminescence imaging was performed using an IVIS® 200 Imaging System (Caliper Life Sciences, Hopkinton, MA) as instructed by the manufacturer. Mice were anesthetized with Isoflurane and bioluminescence was recorded for 3 minutes at a pixel binning of 8. Bioluminescence was measured as total photon flux (photons/sec/cm²) by the Living Image® software package (Caliper Life Sciences, Hopkinton, MA).

4.5 Recovery of *L. monocytogenes* from the tissues of infected mice

At the desired time points, mice were humanely euthanized by asphyxiation with CO₂ followed by exsanguination and cervical dislocation. Blood was collected into a syringe containing sodium citrate as an anticoagulant. The blood was then serially diluted in sterile saline, plated (0.1 ml) on blood agar, and the plates incubated at 37°C. The abdominal cavity was then aseptically opened and portions of the spleen, liver, and fetoplacental units (FPU) (3 FPU per pregnant mouse) were removed. These tissues were weighed in sterile weigh boats and placed into separate sterile tissue grinders that contained 1 ml of cold, sterile saline. The tissues were homogenized with sterilized Teflon tissue grinders, diluted in sterile saline, and plated on blood agar. The plates were allowed to dry and then incubated at 37°C for 48 hours. Colonies were counted and the data expressed as mean ± standard error of the mean (SEM) log₁₀ CFU of *L. monocytogenes* per gram of tissue (wet weight). Mice that died or were euthanized due to systemic disease were assigned a value of 10⁸ CFU/g for the spleen, liver, and FPU, based on bacterial burden typically observed in previous experiments [24, 26, 44].

4.6 Histopathology

At the time of necropsy, portions of the spleen, liver, and fetoplacental units were removed, placed in plastic cassettes, and fixed in 10% buffered formalin. Following fixation and embedding into paraffin, the tissues were serial sectioned, mounted on glass slides, and stained with hematoxylin and eosin or a tissue gram stain. The sections were coded and evaluated by a veterinary pathologist who is board certified by the American College of Veterinary Pathologists (H.S.). Pathological changes in spleen samples were scored on a 0-3 scale with 0 defined as no lesions present, 1 mild to moderate inflammation, 2 moderate to severe inflammation, and 3 severe inflammation and necrosis. Liver sections were scored based on number of focal inflammatory lesions per five -100× fields. Bacteria seen in tissues was scored on a 0-3 scale, with 0 defined as no bacteria and 3 denoting large bacterial numbers in spleen and liver.

4.7 Statistical analysis

Non-parametric analysis was done using Wilcoxon Rank Sum Test due to bimodal distribution, and variability of intragastric infection (SAS program Version 9.2 Cary, NC). The statistical significance for all comparisons was set at $P < 0.05$.

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Fig. 1. *Listeria monocytogenes*

(CFU/g) recovered from maternal and fetal tissues. Non-pregnant (A) and pregnant (B) C57BL/6 and A/J mice were anesthetized with 40 mg/kg of sodium pentobarbital (i.p.) and inoculated i.g. with 10^5 CFU (A and B) or 10^3 CFU (C) of *L. monocytogenes* strain LM2203. Seventy-two hours later, the mice were euthanized and log₁₀ CFU/g determined for spleen, liver, uterus, and fetoplacental units as described in the Materials and Methods. Data are presented as the mean $\pm$ SEM log₁₀CFU/g of tissue. The experimental groups in (A) consisted of 13 C57BL/6 and 11 A/J non-pregnant mice, in (B) of 15 C57BL/6 and 14 A/J pregnant mice, and in (C) of 8 C57BL/6 and 6 A/J pregnant mice. (*) $P < 0.05$ A/J vs. C57BL/6 mice.

Fig. 2.

Inflammation and necrosis of maternal and fetal tissues assessed by histopathology. H&E staining (100×) of maternal spleen (A) and liver (B) from uninfected mice show normal tissue. Spleen inflammation was scored on a 0-3 scale, as described in the Materials and Methods. Panel (C) is an example of severe spleen inflammation (Score=3) from an infected pregnant mouse. Liver sections were evaluated by counting focal inflammatory lesions per five 100× magnification fields. Panel (D) is an example of multiple focal inflammatory lesions (arrows) in a liver section from an infected mouse. H&E staining (100×) of endometrium (Endo) and fetuses from different FPU from the same uterine horn of a C57BL/6 mouse are labeled in (E) and (F). The FPU in (E) is histopathologically normal, whereas the FPU in (F) shows severe inflammation and necrosis (arrows). Bacterial evaluation of these same tissues yielded an average of 4.1 log₁₀ CFU/g.

Fig. 3.

Liver inflammation is more severe in *L. monocytogenes* infected A/J than C57BL/6 mice. Tissue sections were stained with H&E and the number of focal inflammatory lesions per five 100× magnification fields quantified. All lesions contained mixed cellular populations. Pregnant and non-pregnant A/J mice had significantly more liver lesions than pregnant (* $p = 0.02$) or non-pregnant (** $p = 0.02$) C57BL/6 mice.

Fig. 4.

Bioluminescence imaging of *L. monocytogenes* infection in living mice. All mice were inoculated i.g. with 10^6 CFU of LM10403S at 10-14 days of gestation. Pregnant mice infected with LM10403S demonstrate progression of infection from maternal to fetal tissues at (A) 24 hours, (B) 48 hours, and (C) 72 hours post infection. Panel B illustrates bioluminescence in the liver and one fetus. Panel C illustrates more severe maternal infection with intense bioluminescence in maternal liver and throughout the pregnant uterus. Panel D illustrates 4 pregnant mice at 72 hours after inoculation. Bioluminescence quantification (ROI) of actively growing *Listeria* cells (quantified as total photon flux in photons/sec/cm²) correlated with log₁₀ CFU/g for the same tissues in these mice ($r^2=0.768$ and $p=0.02$).