Growth of *Listeria monocytogenes* in the Guinea Pig Placenta and Role of Cell-to-Cell Spread in Fetal Infection

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*Listeria monocytogenes* causes foodborne outbreaks that lead to infection in human and other mammalian fetuses. To elucidate the molecular and cellular mechanisms involved in transplacental transmission, we characterized placental-fetal infection in pregnant guinea pigs inoculated with wild-type (wt) or mutant *L. monocytogenes* strains. The wt strain increased in number in the placenta by $>1000$-fold during the first 24 h after inoculation—an increase that was unparalleled in other maternal organs. The ActA$^{-}$/H11002 mutant, which is impaired in cell-to-cell spread and attenuated in maternal organs, increased in the placenta by a similar amount, although, in fetal infection, the number of ActA$^{-}$/H11002 mutant bacteria was 100-fold lower, compared with that of the wt strain. Furthermore, a mutant impaired in vacuolar escape was rapidly eliminated from maternal organs but persisted in the placenta. We concluded that cell-to-cell spread facilitates maternal-to-fetal transmission. Furthermore, the placenta provides a protective niche for growth of *L. monocytogenes*.

*Listeria monocytogenes* is a ubiquitous, rapidly growing, facultative, intracellular, gram-positive bacterium. Infection of humans and animals has been traced to contaminated foods and can lead to serious, often fatal disease. In humans, disease is most common in pregnant women, newborns, and immunocompromised individuals [1]. Pregnant women have an estimated 17-fold increased incidence of disease [2], compared with that in other individuals, and usually develop a nonspecific febrile illness, which can lead to a placental-fetal infection that results in spontaneous abortion, premature labor, stillbirth, or neonatal sepsis and meningitis [3, 4]. Despite significant fetal and neonatal morbidity and mortality due to vertical transmission of *L. monocytogenes*, little is known about its underlying molecular and cellular mechanisms. We previously developed a pregnant guinea pig model of listeriosis that mimics human disease [5]. The structural similarities between the guinea pig placenta and the human placenta make our model an attractive one for elucidation of the roles that important virulence factors play in vivo [6].

The biological processes of intracellular growth and the bacterial determinants of the pathogenicity of *L. monocytogenes* were extensively studied during the past decade [7, 8]. Bacterial cell-wall surface proteins called internalins (Inls) promote bacterial adherence and internalization into nonphagocytic host cells [9]. InlA and InlB are the best characterized Inls, and they bind to E-cadherin and c-Met-tyrosine kinase, respectively [10, 11]. After internalization, the bacterium escapes from the vacuole into the cytoplasm. This escape is largely mediated by a pore-forming virulence factor called listeriolysin O (LLO) [7, 8]. Once it is in the cytoplasm,
the bacterium multiplies rapidly. ActA, a bacterial surface protein, induces polymerization of host cell actin filaments, which enable \textit{L. monocytogenes} to migrate to the host cell periphery and into cell-wall protrusions. These pseudopodia are engulfed and ingested by neighboring cells, in which the life cycle begins anew [12–14]. Therefore, \textit{L. monocytogenes} can infect cells by 2 different mechanisms: direct invasion and cell-to-cell spread.

The roles that Inls play in virulence have yet to be fully explained. Although InlB is known to play a role in hepatic infection [15, 16], the mouse model, which has been used for decades in the study of listerial pathogenesis, is not a good model for use in the evaluation of the role of InlA, because there is a single amino-acid substitution in murine E-cadherin, compared with human E-cadherin. This structural difference significantly decreases the affinity of InlA to murine E-cadherin [17, 18]. Human and guinea pig E-cadherin interact equally well with InlA, and there is evidence that InlA plays a role in the crossing of the intestinal barrier in guinea pigs, humans, and transgenic mice that express human E-cadherin in the intestinal epithelium [19, 20].

The placental-fetal barrier in the monohemochorial placenta of humans and guinea pigs consists of a single layer of fetal derived trophoblasts [6, 21]. We and others have shown elsewhere that the invasion of primary human trophoblasts in vitro is mediated by InlA [5, 22]. Furthermore, Lecuit et al. have shown that infection of syncytiotrophoblasts in human placental explants with a \textit{L. monocytogenes} strain deficient in InlA reduced the number of bacteria by 10-fold, compared with that in infection with wild-type (wt) \textit{L. monocytogenes} [22]. In humans, epidemiological data have shown that 100% of the \textit{L. monocytogenes} strains isolated in pregnancy-associated and focal infections, 98% of the strains isolated in central nervous system (CNS) infections, and 93% of the strains isolated in bloodstream infections expressed full-length InlA. In contrast, only 65% of the strains isolated from food products expressed full-length InlA [20]. These data are consistent with experimental evidence suggesting that InlA plays a role in the crossing of the intestinal barrier [19]. Because the crossing of the intestinal barrier is a prerequisite for placental infection in naturally occurring listeriosis, it cannot be concluded from the available epidemiological data that InlA mediates the crossing of the placental-fetal barrier in humans. Furthermore, we have shown elsewhere that InlA is not important for the crossing of the placental-fetal barrier in the pregnant guinea pig model of listeriosis [5].

We therefore decided to examine the role that cell-to-cell spread plays in trophoblast infection and vertical transmission. The ActA\textsuperscript{−} mutant, a \textit{L. monocytogenes} strain deficient in ActA, is impaired in its ability to spread from cell to cell. In vitro studies have shown that a single J774 cell initially infected with a single ActA\textsuperscript{−} bacterium harbors 250–500 bacteria at 8 h after infection [23]. Subsequent bacterial growth results in lysis of the host cell and necrotic death. In an animal model of infection, cell lysis would result in the exposure of extracellular bacteria to host defense mechanisms—specifically neutrophils, macrophages, and the humoral immune response. Indeed, cell-to-cell spread has been shown to be important for virulence. In the murine model of listeriosis, an ActA\textsuperscript{−} mutant strain of \textit{L. monocytogenes} is 1000-fold less virulent than is the wt strain [23]. In the present study, we compare placental-fetal infection with the ActA\textsuperscript{−} mutant and wt \textit{L. monocytogenes} in the pregnant guinea pig model of listeriosis. We show that cell-to-cell spread facilitates maternal-to-fetal transmission. Although it is attenuated in maternal organs, the ActA\textsuperscript{−} mutant seems to grow well in the placenta. Furthermore, a \textit{L. monocytogenes} strain deficient in vascular escape is rapidly eliminated in maternal organs but is able to persist, and even to grow, in the placenta. Our results demonstrate that the mammalian placenta provides a unique protective environment for \textit{L. monocytogenes}.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** \textit{L. monocytogenes} 10403S (erythromycin-sensitive wt) [24] and the following strains derived from it were used: DP-L3903 (erythromycin-resistant wt) [25], DP-L3078 (ActA\textsuperscript{−}) [26], DP-L2161 (erythromycin-sensitive LLO\textsuperscript{−}) [27], and DP-L4694 (erythromycin-resistant ActA\textsuperscript{−}). DP-L4694 was generated from strain DP-L3078 by transduction of the gene for erythromycin resistance from strain DP-L3903 by use of phage U153, as described elsewhere [25, 28, 29]. All strains were propagated in brain-heart infusion (BHI) agar and broth (Becton Dickinson). For guinea pig studies, bacteria were grown in BHI broth to log phase, washed once with PBS, and resuspended in fresh BHI. Aliquots were frozen and stored at −80°C. On the day of infection, aliquots were thawed, diluted to a concentration of 1:5 in fresh BHI, and grown to log phase.

**Guinea pig studies.** All guinea pigs were housed and handled in accordance with federal and institutional guidelines. The animal use committee at the University of California, Berkeley, approved the animal use protocol. Pregnant female Hartley outbred guinea pigs between days 25 and 45 of gestation were purchased from Simonsen Laboratories or Elm Hill Breeding Labs. Pregnant females were injected with \textit{L. monocytogenes} into their foot veins between days 42 and 52 of gestation. Guinea pigs were premedicated with a subcutaneous injection of 0.05 mg/kg atropine (Phoenix Scientific) and then anesthetized with isoflurane (Baxter Healthcare Corporation) before inoculation with either 1 × 10\textsuperscript{8} or 1 × 10\textsuperscript{9} cfu of bacteria. The dose of 1 × 10\textsuperscript{9} cfu was used when histologic examination was planned. Such a high dose was necessary for sufficient numbers of bacteria to be detected by immunohistochemistry. Because this dose caused clinical illness in the guinea pigs at
24 h after inoculation, a dose of $1 \times 10^8 \text{ cfu}$ was used for experiments with later time points—in particular, the competitive index analysis. Guinea pigs were euthanized at specified time points after inoculation, and the organs were harvested and homogenized in 0.2% NP-40 (Biosciences). Serial dilutions were plated on Luria-Bertani (LB) agar plates (Becton Dickinson) and were incubated overnight at 37°C. The number of bacteria per organ was counted.

A competitive index analysis was performed as described elsewhere [25]. Briefly, the erythromycin-sensitive wt 10403S strain was compared with the erythromycin-resistant ActA<sup>−</sup> strain DP-L4694. The strains were mixed at a 1:1 ratio. Guinea pigs were injected with a total infectious dose of $1 \times 10^8 \text{ cfu}$ of bacteria. Guinea pigs were euthanized at 33 h after inoculation, and the organs were harvested and homogenized in 0.2% NP-40. Serial dilutions were plated on LB agar plates and on BHI agar plates containing erythromycin (Sigma) at a concentration of 2 µg/mL. The plates were incubated overnight at 37°C, and erythromycin-sensitive and erythromycin-resistant colonies were counted. The competitive index was determined by calculation of the ratio of erythromycin-resistant to erythromycin-sensitive colonies.

**Histologic analysis.** Tissue was fixed in 10% buffered formalin phosphate (Fisher Scientific) and processed by routine methods to provide paraffin wax sections (4 µm), which were stained with hematoxylin-eosin. Immunohistochemistry was performed at the California Animal Health and Food Safety Laboratory in Davis, California, using a rabbit anti-*Listeria* primary antibody (Difco Laboratories) and a peroxidase detection kit (Vector Laboratories). Examination of stained placental sections was performed by microscopy, and examiners were blinded to the identity of the infecting strains.

## RESULTS

### Early events during *L. monocytogenes* infection in the pregnant guinea pig

We intravenously (iv) inoculated pregnant guinea pigs between days 42 and 52 of gestation with $1 \times 10^7 \text{ cfu}$ of the wt strain. This gestational age corresponds with the late second/early third trimester of pregnancy. For histologic analysis, a sufficient number of bacteria had to be present in the placenta to allow for localization of organisms during the early stages of infection, specifically the first 24 h after inoculation. Thus, it was necessary to use a relatively high inoculum, which typically causes lethal disease in 2–3 days.

At 30 min after inoculation, the number of bacteria in the placenta was between 1000-fold and 10,000-fold lower than that in the maternal liver and spleen and between 10-fold and 100-fold lower than that in the maternal lungs and kidneys (figure 1). We concluded that *L. monocytogenes* seeds the placenta initially with fewer bacteria than it does any of the other maternal organs. During the subsequent 24 h after inoculation, we observed a >1000-fold increase in the number of *L. monocytogenes* in the placenta—an increase unparalleled in any other maternal organ we tested (figure 1). This increase in the number of bacteria in the placenta could be due to bacterial growth or influx. Influx could occur in the form of extracellular bacteria or infected maternal cells—specifically, macrophages. If the increase was solely due to bacterial growth, the predicted generation time of *L. monocytogenes* in the guinea pig placenta at 6–10 h after inoculation would be ~70 min (figure 1A).

**L. monocytogenes impaired in cell-to-cell spread.** We iv inoculated pregnant guinea pigs with $1 \times 10^8 \text{ cfu}$ of the ActA<sup>−</sup> mutant. In maternal organs, the ActA<sup>−</sup> mutant was attenuated, compared with the wt strain (figure 1B–E). The ActA<sup>−</sup> mutant was either unable to grow (in maternal lungs [figure 1D] and kidneys [figure 1E]) or began to decrease in number at 10 h after inoculation (in maternal liver [figure 1B] and spleen [1C]). In the placenta, in contrast, the ActA<sup>−</sup> mutant increased in number by ~1000-fold during the first 24 h after inoculation, which was similar to the growth of the wt strain (figure 1A).

**Histologic examination of placental sections.** Histologic examination was performed on placental sections taken from guinea pigs at 0.5, 6, 10, and 24 h after inoculation with $1 \times 10^7 \text{ cfu}$ of either the wt strain or the ActA<sup>−</sup> mutant. Fewer than 5 bacteria per placental section were observed at 0.5, 6, and 10 h after inoculation. At these early time points, no difference in the number of bacteria was detected between guinea pigs inoculated with the wt strain and those inoculated with the ActA<sup>−</sup> mutant. Placental lesions consisting of neutrophils, macrophages, and necrotic trophoblasts were observed in both groups of guinea pigs at 24 h after inoculation. Lesions were most frequent and severe in the ascending and radial maternal arterial main lacunae. These vascular structures are lined by endovascular trophoblasts and are the primary arterial blood supply to the placenta from the maternal circulation. Bacteria were present in variable numbers and appeared to be extracellular, inside of neutrophils and macrophages, and occasionally inside of trophoblasts (figure 2). In one-half of the guinea pigs infected with the ActA<sup>−</sup> mutant, we found, in the labyrinth region, small clusters of trophoblasts that were filled with bacteria (figure 2B). This visually demonstrates the effect that impaired cell-to-cell spread has and shows that *L. monocytogenes* grows well inside of trophoblasts in vivo.

**Cell-to-cell spread and transmission of *L. monocytogenes*.** We compared transmission of *L. monocytogenes* from mother to fetus in pregnant guinea pigs iv inoculated with $1 \times 10^7 \text{ cfu}$ of either the wt strain or the ActA<sup>−</sup> mutant. At 10 h after inoculation, fetal infection with *L. monocytogenes* was not detectable (data not shown). At 24 h after inoculation, infected livers were found in all of the fetuses in pregnant guinea pigs inoculated with the wt strain but in only 3 of the fetuses in pregnant guinea pigs inoculated with the ActA<sup>−</sup> mutant (figure...
Figure 1. Listeriosis in pregnant guinea pigs. The number of bacteria per placenta (A), maternal liver (B), maternal spleen (C), maternal lungs (D), and maternal kidneys (E) was counted at 0.5, 6, 10, and 24 h after intravenous inoculation of pregnant guinea pigs with $1 \times 10^6$ cfu of wild-type (wt; black circles) or ActA (white circles) Listeria monocytogenes. Solid and broken lines connect mean values of wt and ActA strains, respectively.

3A). To further quantify the virulence defect of the ActA− mutant in fetal infection, we performed a competitive index analysis with a 1:1 ratio of the erythromycin-sensitive wt strain to the erythromycin-resistant ActA− mutant. At 33 h after inoculation, we observed a 100-fold decrease in virulence of the ActA− mutant in fetal liver (figure 3B). In contrast, we observed a <10-fold decrease in virulence of the ActA− mutant in placenta, maternal liver, and maternal spleen. This indicates that cell-to-cell spread contributes to the crossing of the placental-fetal barrier.

L. monocytogenes impaired in vacuolar escape. To evaluate whether the placental environment is particularly per-
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Figure 2. Immunohistochemical examination of guinea pig placental sections. Pregnant guinea pigs were intravenously inoculated with $1 \times 10^9$ cfu of wild-type (wt; A) or ActA$^{-}$ (B) Listeria monocytogenes. In guinea pigs infected with the wt strain (A), abundant intracellular and extracellular bacteria were present in the inflammatory infiltrate, and a small number of bacteria appeared to be inside of trophoblasts (arrowheads). In comparison, in guinea pigs infected with the ActA$^{-}$ mutant (B), a large number of bacteria filled trophoblasts (arrowheads). Bar, 26 μm.

Discussion

The results of the present study show that L. monocytogenes does not preferentially seed the guinea pig placenta in an iv model of inoculation. However, there was a 1000-fold increase in the number of bacteria in the placenta during the first 24 h after inoculation. Such an increase was not observed in any other maternal organ. In addition, L. monocytogenes strains that were impaired in their ability to spread from cell to cell or to escape the vacuole were able to persist in the placenta and even exhibited a slight increase in number (figure 4B). Most important, these results indicate that L. monocytogenes cannot be effectively eliminated from the placental environment.

A disadvantage of our pregnant guinea pig model is that we infected the animals by iv inoculation, which led to an initial seeding of the placenta by extracellular bacteria. We used this route because oral infection of pregnant guinea pigs with $1 \times 10^7$ cfu of the wt strain did not lead to placental infection (data not shown).

In our pregnant guinea pig model, we observed a 100-fold decrease in the number of bacteria in fetal infection with the
ActA− mutant, compared with that in fetal infection with the wt strain. These results suggest that cell-to-cell spread plays a role in vertical transmission. A potential explanation for this finding is the large size of the syncytiotrophoblasts, compared with that of macrophages. In pregnant guinea pigs infected with the wt strain, the trophoblasts contained only a small number of bacteria. In contrast, the ActA− mutant appeared to reach high densities inside the trophoblasts before being able to infect neighboring cells (figure 2). This could decrease the efficiency of spread to fetal tissues.

Once the placenta was infected, we found a >1000-fold increase in the number of bacteria, which could be due to growth or influx of bacteria. The generation time of L. monocytogenes in a variety of guinea pig, murine, and human cell lines and primary cells is ∼60 min [30]. In comparison, if the increase of L. monocytogenes in the guinea pig placenta is solely due to bacterial growth, the generation time would be ∼70 min. This would lead to the conclusion that L. monocytogenes proliferates virtually unrestricted in the placental environment. In addition, in the placenta, we observed an increase in the number of LLO−

Figure 3. Fetal infection with wild-type (wt) or ActA− Listeria monocytogenes strains. A, The number of bacteria per fetal liver was counted at 24 h after intravenous (iv) inoculation of pregnant guinea pigs with 1 × 10^8 cfu of wt (black rectangles) or ActA− (white rectangles) L. monocytogenes. ND represents <100 bacteria per organ, which is below the level of detection. Black bars represent mean values. B, Competitive index analysis of ActA− and wt strains. Pregnant guinea pigs were iv inoculated with a 1:1 mixture of the 2 strains at a total dose of 1 × 10^8 cfu of bacteria. Ratios between erythromycin-resistant (ActA−) and erythromycin-sensitive (wt) colonies were calculated for placenta (PL), maternal liver (ML), maternal spleen (MS), and fetal liver (FL). Black bars represent mean indices (MI).

Figure 4. Phenotype of the listeriolsin O− (LLO−) mutant strain of Listeria monocytogenes in pregnant guinea pigs. The number of bacteria per maternal liver, spleen, kidneys, lungs (A), and placenta (B) was counted at 0.5, 6, and 24 h after intravenous inoculation of pregnant guinea pigs with 1 × 10^8 cfu of the LLO− mutant. Solid and broken lines connect mean values.
mutant, which could be due to extracellular or intracellular bacterial growth. The latter could occur if the LLO\textsuperscript{−} mutant could escape from the phagolysosome because of the activity of phospholipases, which contribute to vacuolar escape in some cell types [39]. Consistent with this hypothesis is evidence that the LLO\textsuperscript{−} mutant escapes to a limited degree from the vacuole in the human choriocarcinoma cell line BeWo (data not shown). Our observations that L. monocytogenes mutants that are attenuated in maternal organs are able to increase in number or persist in the placenta suggest that growth, at least, contributes to the observed increase in the number of bacteria in the placenta.

The unique immunological condition of pregnancy is a possible explanation for why the placenta provides a protective environment for the growth of L. monocytogenes. The maternal immune system faces the double task of preventing the rejection of the semi-allogeneic fetus and protecting the mother and the fetus against infection. How these tasks are accomplished simultaneously remains to be fully explained. Suppression of maternal T cell–mediated immunity—for example, by induction of apoptosis in T cells via secretion of HLA-G and Fas ligand [40, 41] and by starvation of T cells for the essential amino acid tryptophan via production of indoleamine 2,3-dioxygenase [42]—most likely contributes to maternal immunological tolerance of the fetus. Changes in maternal cell-mediated immunity may increase the importance of the innate immune response for the defense against infection [43]—especially against intracellular pathogens. Indeed, cells of the innate immune response are strongly represented at the maternal-fetal interface. The predominant population of leukocytes in the hemochorial placenta of humans and rodents consists of non–antigen-specific NK cells and macrophages [44–46]. The interaction between macrophages and NK cells is critical during the early stages of listeriosis [47, 48]. However, the roles that uterine macrophages and NK cells play in protection against vertical transmission of L. monocytogenes are unknown.

In human tissues, uterine NK cells comprise 30%–40% of the total cells in first trimester decidua [44]. Notably, interleukin-15–deficient mice, which are depleted of NK cells, are not more susceptible to placental infection with L. monocytogenes than are wt mice [49], which suggests a negligible role for NK cells in the defense against L. monocytogenes, at least in murine placental tissues.

Macrophages act as primary host cells for L. monocytogenes and critical effector cells that are essential for the defense against listeriosis [47]. Colony-stimulating factor 1 (CSF-1) is the major regulator of cells of the mononuclear phagocytic lineage [50, 51]. CSF-1 is synthesized to very high concentrations at the maternal-fetal interface by the uterine epithelium [52–54]. In mouse peritoneal macrophages, CSF-1 stimulates macrophage phagocytic activity and intracellular growth of L. monocytogenes but not bactericidal activity [55, 56]. High concentrations of CSF-1 in the placenta could, therefore, shift the role of macrophages away from that of immune effector cells and toward that of primary host cells for L. monocytogenes. Contrary to this explanation are the results of a study on the effect that CSF-1 has on placental-fetal infection in a pregnant mouse model of listeriosis [57, 58]. The osteopetrosic mouse (Csfr\textsuperscript{−}/Csfr\textsuperscript{−}), which carries a null mutation in the CSF-1 gene, had higher levels of L. monocytogenes in placental-fetal tissues than did the heterozygous control mouse (+/Csfr\textsuperscript{−}), because of a failure to recruit neutrophils to the placenta [58]. However, results in a pregnant mouse model of listeriosis might not readily reflect the situation in the pregnant guinea pig model. For unknown reasons, the placental-fetal unit of guinea pigs—and humans—is much more susceptible to infection than is that of mice. Differences in susceptibility are apparent for infection with a variety of pathogens, such as cytomegalovirus [59, 60] and T. gondii [61, 62], in addition to L. monocytogenes. Furthermore, the time course of infection may be another important factor. The present study focused on the first 24 h after inoculation, whereas increased susceptibility of the Csfr\textsuperscript{−}/Csfr\textsuperscript{−} mouse to placental infection started to become apparent at 24 h and was significant at 72 h after inoculation.

In summary, we show evidence that the placenta provides a protective niche for L. monocytogenes and that cell-to-cell spread facilitates transplacental fetal infection. Regardless of how the bacteria travel to the placenta, the results of the present study clearly show that the placenta is uniquely permissive of listerial growth.

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References

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