

## Listeriosis in the Pregnant Guinea Pig: a Model of Vertical Transmission

Anna I. Bakardjiev,<sup>1\*</sup> Brian A. Stacy,<sup>2</sup> Susan J. Fisher,<sup>3,4,5</sup> and Daniel A. Portnoy<sup>1,6</sup>

Department of Molecular and Cell Biology<sup>1</sup> and School of Public Health,<sup>6</sup> University of California, Berkeley, Anatomic Pathology Service, Veterinary Medical Teaching Hospital, University of California, Davis,<sup>2</sup> and Departments of Stomatology,<sup>3</sup> Anatomy,<sup>4</sup> and Pharmaceutical Chemistry,<sup>5</sup> University of California, San Francisco, California

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**Feto-placental infections represent a major cause of pregnancy complications, and yet the underlying molecular and cellular mechanisms of vertical transmission are poorly understood. *Listeria monocytogenes*, a facultative intracellular pathogen, is one of a group of pathogens that are known to cause feto-placental infections in humans and other mammals. The purpose of this study was to evaluate possible mechanisms of vertical transmission of *L. monocytogenes*. Humans and guinea pigs have a hemochorial placenta, where a single layer of fetally derived trophoblasts separates maternal from fetal circulation. We characterized *L. monocytogenes* infection of the feto-placental unit in a pregnant guinea pig model and in primary human trophoblasts and trophoblast-derived cell lines. The clinical manifestations of listeriosis in the pregnant guinea pigs and the tropism of *L. monocytogenes* to the guinea pig placenta resembled those in humans. Trophoblast cell culture systems were permissive for listerial growth and cell-to-cell spread and revealed that *L. monocytogenes* deficient in internalin A, a virulence factor that mediates invasion of nonphagocytic cells, was 100-fold defective in invasion. However, crossing of the feto-placental barrier in the guinea pig model was independent of internalin A, suggesting a negligible role for internalin-mediated direct invasion of trophoblasts in vivo. Further understanding of vertical transmission of *L. monocytogenes* will help in designing more effective means of treatment and disease prevention.**

Mammalian reproduction poses an immunological paradox because fetal alloantigens encoded by genes inherited from the father should provoke cell-mediated immune responses from the mother, potentially leading to fetal loss. There is evidence that pregnancy leads to suppression of cell-mediated immunity in the mother in order to prevent fetal rejection (34, 46). However, cell-mediated immunity is necessary for the defense against intracellular pathogens, and therefore suppression of this arm of the immune response might predispose the fetus to infection with intracellular pathogens. Indeed, organisms which commonly cause feto-placental infections are usually intracellular, including viruses such as cytomegalovirus (CMV) (51, 52) and human immunodeficiency virus (7, 37, 38), intracellular protozoan parasites such as *Toxoplasma gondii* (17), and intracellular bacterial pathogens such as *Listeria monocytogenes* (29, 30, 36). Among this group of pathogens, *L. monocytogenes* is the most tractable and has been used for decades as a model pathogen to dissect basic aspects of intracellular pathogenesis as well as the host's innate and acquired cellular immune responses in the murine model of infection (42). Acquired immunity to *L. monocytogenes* is entirely cell mediated and largely dependent on cytotoxic CD8<sup>+</sup> T cells that recognize and lyse infected cells (23, 57).

*L. monocytogenes* is a ubiquitous gram-positive bacterium which has an unusually broad host range. Infection of humans and animals has been traced to contaminated foods. Ingestion

of *L. monocytogenes* by humans occurs relatively frequently. On average, healthy adults in the United States ingest 10<sup>5</sup> *L. monocytogenes* organisms four times per year (39). However, listeriosis is a rare disease, with an incidence of ~1,100 cases/year and high mortality of ~250 cases/year in the United States (53). Most individuals who develop listeriosis have predisposing factors which render them more susceptible to infection (29, 30). About two-thirds of the cases occur in immunocompromised individuals. The most common clinical manifestation in this group of patients is central nervous system infection. Almost all of the remaining cases occur in pregnant women and carry the risk of feto-placental infection (29, 30). Pregnant women usually develop a nonspecific febrile illness, which can lead to feto-placental infection with serious consequences for the fetus or newborn child (36). In one study, *L. monocytogenes* was found to be the cause of 3.9% of second-trimester miscarriages in humans (22), and infection of the mother around term may lead to severe neonatal disease, with neonatal mortality ranging between 22 and 47% (10, 54).

We sought to develop a pregnant animal model to study feto-placental transmission of *L. monocytogenes*. We decided to focus on pregnant guinea pigs because of significant similarities between the structures of human and guinea pig placentas. Humans and rodents have a hemochorial placenta, which means that fetally derived cells called trophoblasts invade the uterus and are in direct contact with maternal blood. Of all the rodent placentas, guinea pig placenta resembles the human placenta most closely. Both species have a discoidal placenta, trophoblasts invade the uterus into the endometrium and inner third of the myometrium, and the maternal blood spaces are lined by one layer of trophoblasts during the later

\* Corresponding author. Mailing address: Department of Molecular and Cell Biology, Portnoy Lab, 510 Barker Hall, University of California, Berkeley, CA 94720-3202. Phone: (510) 643-3926. Fax: (510) 643-6334. E-mail: annaib@uclink.berkeley.edu.

stages of pregnancy (19, 27). In addition to forming a physical barrier between maternal and fetal circulation, trophoblasts seem to play a role in the innate immune response to infection. Evidence comes from several studies which have shown that trophoblasts secrete cytokines, including interleukin-10 and the murine interleukin-8 analogs KC and macrophage inflammatory protein-2 (14, 45). Furthermore, trophoblasts have been shown to express the pattern recognition receptors Toll-like receptor-2 and Toll-like receptor-4 (15, 16).

We hypothesized that infection of trophoblasts is a critical step in the spread of infection from mother to fetus. In this study we focused on the importance of listerial virulence factors in crossing the physical barrier between maternal and fetal circulation. Trophoblasts might become infected with *L. monocytogenes* by either direct invasion or cell-to-cell spread. It has been shown previously that bacterial surface proteins, called internalins, mediate invasion of nonphagocytic cells by *L. monocytogenes* (3). The best characterized internalins are internalin A (InIA) (13) and internalin B (InIB) (3, 9), which bind to E-cadherin (35) and c-Met receptor tyrosine kinase (49), respectively. Similarities between human and guinea pig E-cadherins make the guinea pig a better animal model than the mouse to study the role of InIA in vivo (25, 26). Another possible mechanism by which trophoblasts can become infected is by cell-to-cell spread from infected maternal cells, specifically macrophages. Macrophages internalize *L. monocytogenes* by phagocytosis. Once *L. monocytogenes* has entered the host cell, it escapes into the cytoplasm, where the listerial ActA protein enables the bacterium to propel itself through the cytoplasm and spread from cell to cell without exposure to the extracellular environment (55).

In order to investigate mechanisms of vertical transmission, we developed and characterized a pregnant guinea pig model of listeriosis which mimicked aspects of human disease. Invasion of isolated human trophoblasts, which were permissive for listerial growth and cell-to-cell spread, was facilitated by InIA. Furthermore, we evaluated the role of InIA in crossing the fetoplacental barrier in vivo.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The *L. monocytogenes* strains used in this study were derived from 10403S (2). All strains were propagated in brain heart infusion (BHI) (Becton Dickinson and Company, Sparks, Md.) agar and broth. For invasion assays and intracellular growth curves, *L. monocytogenes* strains were grown overnight in BHI broth to stationary phase. For animal infections, bacteria were grown in BHI broth to log phase, washed once with phosphate-buffered saline, and resuspended in fresh BHI broth. Aliquots were frozen and stored at  $-80^{\circ}\text{C}$ . On the day of infection, aliquots were thawed, diluted 1:5 in fresh BHI broth, and grown to log phase.

**Construction of deletion mutants.** (i) **Construction of  $\Delta$ inIA exchange plasmid.** Both 5' and 3' fragments of the *inIA* gene (5'*inIA* and 3'*inIA*) were generated by PCR with *L. monocytogenes* genomic DNA as a template. Primers CATTTTAAAGGTGGAATGACA and TCCGGATGTTACATTCGTTTTT CTAAGA (the *Bsp* EI site is underlined) were used to generate 5'*inIA*. Primers TCCGGAAATACCTGGAAGCGACACATCT (the *Bsp* EI site is underlined) and TCATTTGTGTCACTGCATCTG were used to generate 3'*inIA*. Klenow fragment was used to generate blunt-end PCR products, which were ligated into the *Stu* I site of pMTL20 (6) and then double digested with *Bam* HI and *Bsp* EI, leading to release of 3'*inIA*. 3'*inIA* was ligated into the vector containing 5'*inIA*, generating an *inIA* fragment ( $\Delta$ inIA) with deletion of bp 1734 to 3722 (*inIA*-*inIB* operon sequence from the National Center for Biotechnology Information; accession number AJ012346), which was confirmed by sequencing.  $\Delta$ inIA was then subcloned into pMTL23 (6), digested with *Aat* II, and inserted into pLMD1 (48), generating the  $\Delta$ inIA allelic exchange plasmid.

(ii) **Construction of  $\Delta$ inIB exchange plasmid.** A 3' fragment of the *inIB* gene (3'*inIB*) was generated by PCR with primers TCCGGAGGTACCTAACCTAC GAAAAAGGCTA (the *Kpn* I site is underlined) and TGGTCGTTATTAAG TGACTTAAGGC (the *Eco* RI site is underlined) with *L. monocytogenes* genomic DNA as a template. 3'*inIB* was digested with *Kpn* I and *Eco* RI and then cloned into pMTL23, which was then digested with *Xba* I. Klenow fragment was used to generate 3'*inIB* with blunt ends, which was subsequently digested with *Bam* HI. The vector pMTL20 containing 3'*inIA* was digested with *Bgl* II, and blunt ends were created with Klenow fragment and digested with *Bam* HI. The 3'*inIB* fragment was ligated into this vector, generating an *inIB* fragment ( $\Delta$ inIB), with deletion of bp 4240 to 5910, which was confirmed by sequencing. A 2.8-kb cassette from pE194ts (48) was introduced at the *Acc* I site of the  $\Delta$ inIB-containing plasmid, generating the  $\Delta$ inIB allelic exchange plasmid.

(iii) **Construction of *L. monocytogenes* InIA and InIB deletion mutants (DP-L4405 and DP-L4406, respectively).** The  $\Delta$ inIA and  $\Delta$ inIB allelic exchange plasmids were transformed into *L. monocytogenes* 10403S and selected in the presence of erythromycin (5). In-frame deletion of *inIA* or *inIB* was confirmed by Southern blot analysis. Western blot analysis was used to confirm the lack of InIA protein expression in selected mutants.

(iv) **Construction of *L. monocytogenes* InIA InIB double deletion mutant (DP-L4404).** A DNA fragment upstream of the 5' end of *inIA* was generated by PCR with primers GGGCTGCAGAGAGTTTTGGCGGTAAGAGTG and AGGTT TTCCGCTTTAGTCCAGTTTTCCCTAAGACCGTCTTCAT. A second DNA fragment of the 3' end of *inIB* was generated by PCR with primers CCCGAGC TCGCTGCTTTCGTTCCAACCAATG and ATGAAGACGGTCTTAGGAAA ACTGGACTAAAGCGGAAAACCT. These PCR products were then used as a template in a subsequent PCR with primers GGGCTGCAGAGAGTTTTGGC GGTAAGAGTG and CCCGAGCTCGTGCTTTTCGTTCCAACCAATG to generate  $\Delta$ inIAB, a DNA fragment with deletion of bp 1700 to 5389.  $\Delta$ inIAB was cloned into the *Sac* I and *Pst* I sites of plasmid pKSV7 (50). Inserts were verified by sequencing analysis and integrated into the *L. monocytogenes* 10403S genome. Southern blots were performed to confirm the in-frame deletion of *inIAB*.

**Construction of complementation strains.** DNA fragments containing the internalin A/B promoter and either *inIAB* or *inIA* were generated by PCR with wild-type *L. monocytogenes* strain 10403S chromosomal DNA as a template. Primer CGGGATCCACGAGCCAACCGTGG (the *Bam* HI site is underlined) annealed upstream of the internalin A/B promoter and was used in combination with primers CGGGATCCTTATTCTGTGCCCTAAATTAGC (the *Bam* HI site is underlined) and CGGGATCCTCTCCGCTTGTACTTT CGCC (the *Bam* HI site is underlined) to amplify DNA fragments that encode the internalin A/B promoter with either *inIAB* or *inIA*, respectively. Both PCR-generated DNA fragments were flanked by *Bam* HI sites and were cloned into the *Bam* HI site of the pPL2 site-specific shuttle integration vector (24). The inserts were verified by sequencing analysis. PPL2 containing the internalin A/B promoter with *inIA* or *inIAB* was integrated into DP-L4405 or DP-L4404, respectively, creating DP-L4454 and DP-L4455.

**Primary human trophoblasts.** Primary human trophoblasts were obtained from elective termination of pregnancy during the second trimester at the University of California, San Francisco (UCSF). Informed consent was obtained from all patients from whom tissue was collected. The UCSF Committee on Human Research approved the consent form. Placental and decidual tissues were collected within 1 h of isolation, washed in phosphate-buffered saline with antibiotics (penicillin, streptomycin, and gentamicin) (UCSF Cell Culture Facility), and placed on ice. Cells were isolated from human placentas by published methods (11, 28). Briefly, placentas were subjected to a series of enzymatic digests that detached cytotrophoblast stem cells from the underlying stromal core of the chorionic villus. Detached trophoblasts were purified over a Percoll gradient. To decrease residual macrophage contamination, the trophoblasts were further purified by incubation with anti-CD45-coated magnetic beads (Dynabeads M-450 CD 45; Dynal Biotech, Oslo, Norway). Trophoblasts were cultured overnight on Matrigel-coated 12-mm-diameter coverslips (BD Biosciences Discovery Labware, Bedford, Mass.) in Dulbecco's modified Eagle's medium-4.5 g of glucose per liter with 2% Nutridoma (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Immunofluorescence staining with monoclonal antibodies to CD45 (DakoCytomation, Carpinteria, Calif.) and cytokeratin confirmed that the cell population contained trophoblasts. Rat antitrophoblast monoclonal antibody to cytokeratin 7D3 was produced in the laboratory of S. J. Fisher (UCSF) according to previously described methods (8). Secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, Pa.) included rhodamine-conjugated goat anti-rat immunoglobulin G and fluorescein isothiocyanate-conjugated donkey anti-mouse immunoglobulin G.

**Cell lines.** The human choriocarcinoma cell line BeWo was obtained from the American Type Culture Collection (Manassas, Va.) (ATCC CCL-98) (41) and

grown in Ham's F12 medium with 2 mM L-glutamine, 1.5 g of sodium bicarbonate (Gibco, Grand Island, N.Y.) per liter and 10% fetal bovine serum (FBS) (Gemini, Woodland, Calif.). The guinea pig colorectal adenocarcinoma cell line GPC-16 was obtained from the American Type Culture Collection (ATCC CCL-242) (40) and grown in Eagle minimal essential medium with 2 mM L-glutamine and Earle's balanced salt solution adjusted to contain 1.5 g of sodium bicarbonate per liter, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate (Gibco), and 10% FBS (Gemini). The J774 mouse macrophage-like cell line (43) was grown in suspension in a spinner flask in Dulbecco's modified Eagle's medium supplemented with 10% FBS and 2 mM L-glutamine.

**Bacterial invasion and growth in primary cells and cell lines.** Invasion assays and growth curves were performed as previously described (18). Briefly, cells were plated onto 12-mm-diameter glass coverslips in their respective growth media without any antibiotics and grown overnight. The density of the plated cells was dependent on their size and rate of growth, because experiments were performed with a monolayer of cells. BeWo and J774 cells were plated at densities of  $3 \times 10^4$  and  $6 \times 10^4$  per coverslip, respectively, reflecting the larger size of BeWo cells in comparison to J774 cells. Primary human trophoblasts were plated at a density of  $5 \times 10^5$  per coverslip, because not all of the primary cells survived the preparation and attached to the coverslip. Cells were infected with wild-type or mutant *L. monocytogenes*. The multiplicity of infection (MOI) was estimated based on the number of bacteria added to the culture medium and the estimated number of cells at the time of infection. For J774 and BeWo cells, we estimated doubling of the cell numbers overnight. For the primary human trophoblasts, we used the number of cells plated to calculate the MOI, which did not take into account that some of the cells did not survive and attach to the coverslips. At 1 h after infection, gentamicin (Sigma, St. Louis, Mo.) was added at a final concentration of 50  $\mu\text{g/ml}$ . At specified times after infection (2 h for invasion assay and 2, 5, and 8 h for growth curves), coverslips were removed in triplicate and cells were lysed by vortexing each coverslip in 5 ml of sterile distilled water. Aliquots were plated onto Luria-Bertani (LB) agar plates (Becton Dickinson, Franklin Lakes, N.J.) and incubated overnight at 37°C. The average colony count was determined for each time point. A fourth coverslip was removed at each time point for fixation and staining of cells with Diff-Quik (Dade Behring, Dudingon, Switzerland).

**Histology.** Tissue was fixed in 10% paraformaldehyde (Electron Microscopy Sciences, Ft. Washington, Pa.) and processed by routine methods to provide paraffin wax sections (4  $\mu\text{m}$ ), which were stained with hematoxylin and eosin (Histology Laboratory, Veterinary Medical Teaching Hospital, University of California, Davis). Immunohistochemistry was performed at the California Animal Health and Food Safety Laboratory (Davis), using a rabbit anti-*Listeria* primary antibody (Difco Laboratories, Detroit, Mich.) and a peroxidase detection kit (Vector Laboratories, Burlingame, Calif.).

**Animal studies.** All animals 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 describing our studies. Pregnant female Hartley outbred guinea pigs between days 35 and 45 of gestation were purchased from Harlan Laboratories (Madison, Wis.) or Simonsen Laboratories (Gilroy, Calif.). Pregnant females were injected intravenously with *L. monocytogenes* into their ear veins. Animals were inoculated with  $1 \times 10^5$ ,  $1 \times 10^6$ , or  $2 \times 10^7$  bacteria. Inoculation was performed under general anesthesia with isoflurane (Baxter Healthcare Corporation, Deerfield, Ill.), and animals were premedicated with a subcutaneous injection of 0.05 mg of atropine (Phoenix Scientific, St. Joseph, Mo.) per kg. Animals were euthanized at 24 or 48 h after inoculation, and organs were harvested and homogenized in 0.2% NP-40 (Biosciences, Inc., La Jolla, Calif.). Serial dilutions were plated on LB agar plates (Becton Dickinson) and incubated overnight at 37°C. Bacteria per organ were enumerated.

Competitive index analysis was performed as previously described (1). Briefly, the erythromycin-resistant wild-type 10403S strain was compared to the erythromycin-sensitive mutant strains. The two strains were mixed in a 1:1 ratio. Guinea pigs were injected with a total infectious dose of  $1 \times 10^5$ ,  $1 \times 10^6$  or  $2 \times 10^7$ . Animals were sacrificed at 24 or 48 h postinoculation, and organs were harvested and homogenized in 0.2% NP-40. Serial dilutions were plated on LB agar plates and BHI agar plates containing erythromycin (Sigma) at a concentration of 2  $\mu\text{g/ml}$ . Agar plates were incubated overnight at 37°C, and erythromycin-sensitive and erythromycin-resistant colonies were enumerated. The competitive index was determined by calculation of the ratio between erythromycin-sensitive and erythromycin-resistant colonies.

**Statistical analysis.** Statistical analysis was performed with the Mann-Whitney confidence interval test with the student edition of Minitab release 12.

## RESULTS

**Pregnant guinea pig model.** Pregnant guinea pigs were infected intravenously by injection into their ear veins with doses of  $1 \times 10^5$ ,  $1 \times 10^6$ , or  $2 \times 10^7$  *L. monocytogenes* organisms during the late second and early third trimester (days 42 to 52 of gestation). A dose of  $10^5$  did not lead to fetoplacental infection and did not cause any significant infection in maternal spleen and liver (data not shown). The lowest possible dose causing maternal and fetoplacental infection in all animals was  $10^6$  (data not shown). In all of our subsequent experiments we used either  $1 \times 10^6$  or  $2 \times 10^7$  bacteria per animal. The pregnant guinea pigs developed spontaneous abortions but appeared otherwise well after intravenous inoculation of *L. monocytogenes*. In one experiment, five out of seven guinea pigs had spontaneous abortions by 48 h postinoculation with  $2 \times 10^7$  bacteria. The guinea pig mothers seemed to recover without further problems after miscarriage.

At 24 h after intravenous inoculation of  $2 \times 10^7$  bacteria, all animals had placental infection, with bacterial numbers per placenta similar to those in maternal liver and spleen. (Fig. 1A, C, and D). At this time, three out of four fetuses were infected (Fig. 1B). At 48 h postinoculation, the numbers of *L. monocytogenes* had increased in all tested organs, but most of the increase was observed in the fetoplacental unit. There was almost a 2-log-unit increase in bacteria per placenta (Fig. 1A). In contrast, the bacterial numbers in maternal liver and spleen had increased only slightly, by less than 1 log unit (Fig. 1C and D). All of the fetal livers were infected (Fig. 1B). Neither amniotic fluid nor fetal or maternal brain contained detectable bacteria (data not shown).

Histological examination was performed on placental sections taken from guinea pigs at 24 h postinoculation with  $2 \times 10^7$  *L. monocytogenes* bacteria. All examined placentas contained multiple foci of inflammation, comprised mostly of neutrophils and small numbers of macrophages. Lesions were multifocally distributed within the labyrinth portion and along maternal arteries of the placenta (Fig. 2A). Immunohistochemistry for *L. monocytogenes* revealed large numbers of immunoreactive bacteria within the inflammatory infiltrate. The bacteria appeared to be mostly extracellular or inside macrophages. Small numbers of bacteria appeared to be within the cytoplasm of trophoblasts (Fig. 2B). Because a single trophoblast layer separates fetal from maternal circulation in the hemochorial placenta of humans and guinea pigs, this was of particular interest to us and suggested that trophoblast infection is a critical step in transmission of *L. monocytogenes* infection from mother to fetus.

**Infection of BeWo cells, a human trophoblast-derived cell line.** The BeWo cell line is a human choriocarcinoma cell line derived from trophoblasts. We found that BeWo cells were readily infected by wild-type *L. monocytogenes* (Fig. 3, right panel). At 1 h after infection we added gentamicin to the culture medium in order to observe the intracellular growth of *L. monocytogenes* without contamination by extracellular bacteria. Infection of BeWo cells at an MOI of 1:8 led to infection of 1 to 2% of the cells at 2 h postinoculation, which was followed by a 20-fold increase in bacterial numbers over the next 6 h (Fig. 4). The doubling time of *L. monocytogenes* in BeWo cells was 63 min between 2 and 5 h postinoculation. We

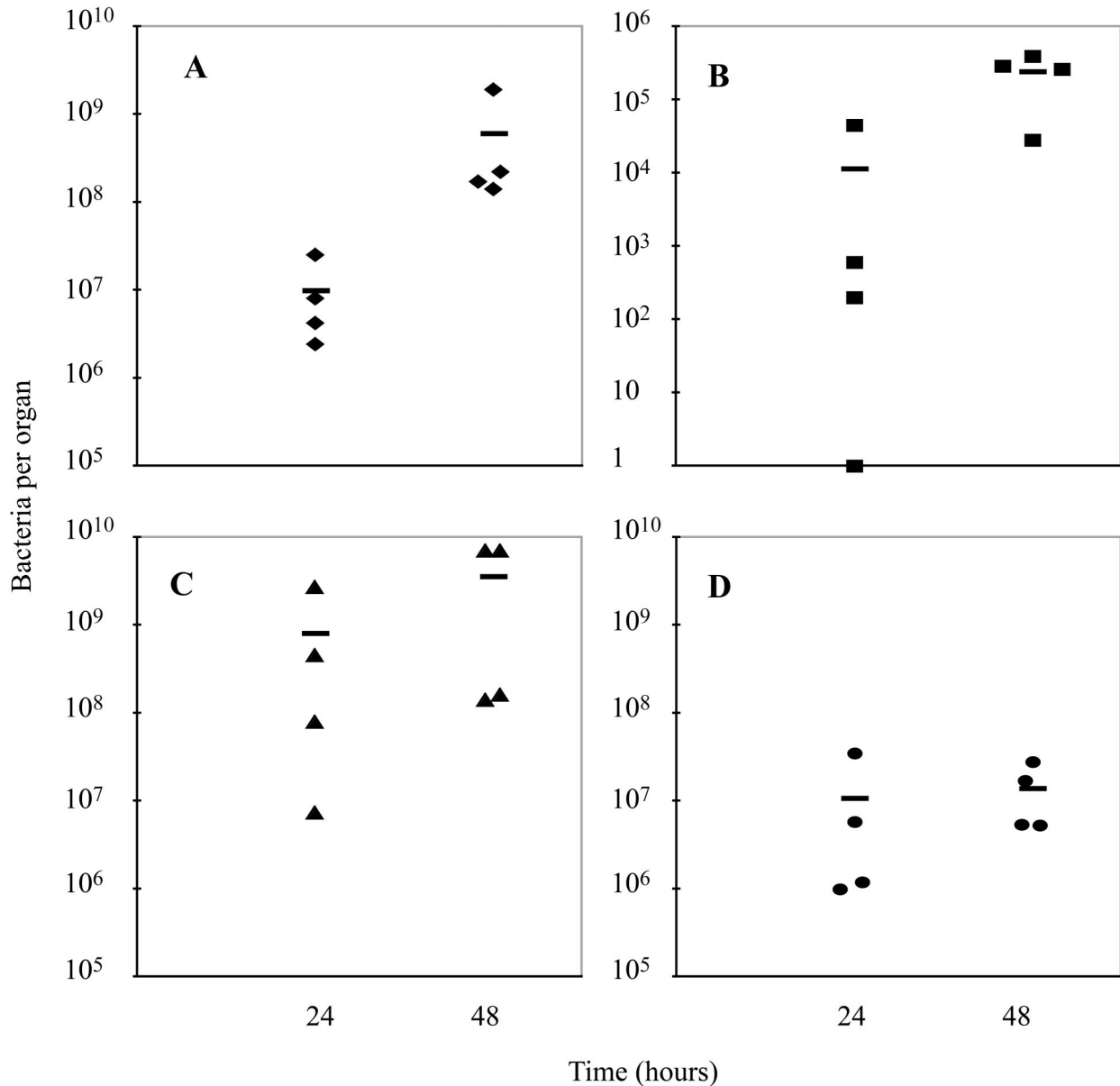


FIG. 1. Listeriosis in pregnant guinea pigs between days 42 and 52 of gestation. Bacteria per placenta (A), fetal liver (B), maternal liver (C), and maternal spleen (D) were enumerated at 24 or 48 h after intravenous inoculation of animals with  $2 \times 10^7$  wild-type *L. monocytogenes* organisms. The bars represent means.

compared the results for BeWo cells with *L. monocytogenes* infection of the macrophage-like cell line J774, which is easily infected by *L. monocytogenes* (Fig. 4) (18). We found that the rates of internalization in the two cell lines were comparable. The rate of growth in BeWo cells was  $\sim 0.5$ -fold the rate of growth in J774 cells. *L. monocytogenes* appeared to spread normally from cell to cell in BeWo cells. BeWo cells grow in islet formations and at early time points of infection bacteria were observed in peripherally located cells, from where they seemed to have spread at later time points (data not shown).

The *L. monocytogenes* virulence factors InlA and InlB interact with E-cadherin and c-Met-tyrosine kinase, respectively, to facilitate invasion of nonphagocytic cells (35, 49). InlA has

been shown to be important for invasion of the human intestinal cell line Caco-2 and to facilitate crossing of the intestinal barrier in guinea pigs and transgenic mice expressing human E-cadherin under control of an intestinal specific promoter (26). We infected the guinea pig colorectal adenocarcinoma cell line GP-16 with an InlA deletion mutant of *L. monocytogenes* and found a 2-log-unit reduction in invasion (data not shown). We subsequently tested whether InlA or InlB facilitated invasion of trophoblasts, in particular because trophoblasts express E-cadherin and c-Met-tyrosine kinase (12, 21). Infection of BeWo cells with an InlA deletion mutant of *L. monocytogenes* led to a 2-log-unit reduction in invasion in comparison to wild type infection (Fig. 5A). Infection with an InlB



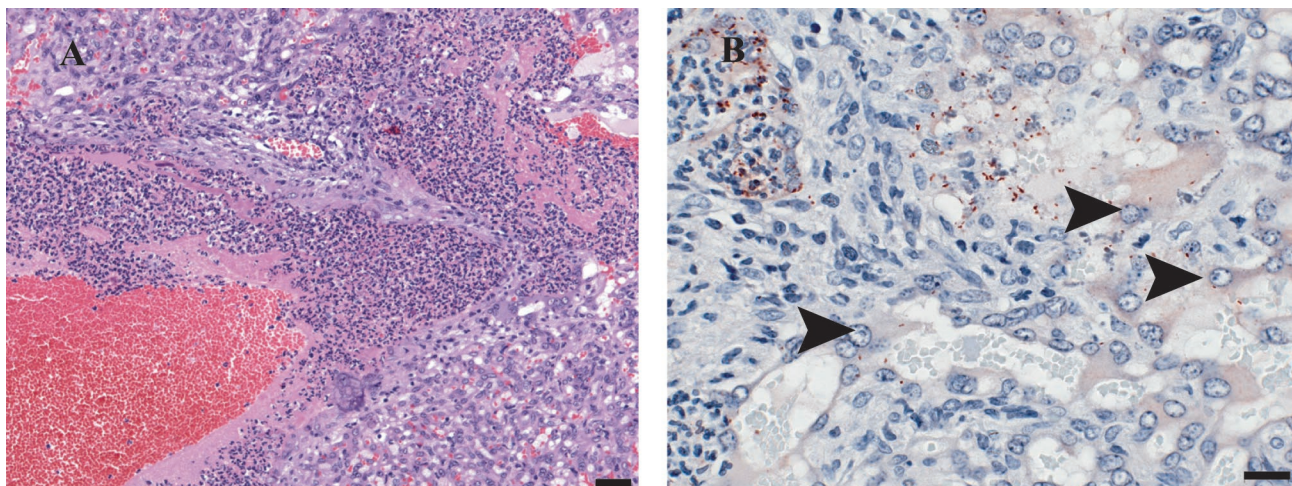


FIG. 2. Histological examination of guinea pig placental sections. Pregnant guinea pigs between days 42 and 52 of gestation were inoculated intravenously with  $2 \times 10^7$  wild-type *L. monocytogenes* bacteria. (A) Hematoxylin-eosin staining of placental section, showing the labyrinthine region at 24 h postinoculation. Moderate numbers of neutrophils and very small numbers of macrophages infiltrate the central region of the lobe. There is fibrin deposition associated within this infiltrate. Bar, 50  $\mu$ m. (B) Immunohistochemistry for *L. monocytogenes* reveals large numbers of immunoreactive bacteria within the inflammatory infiltrate. Some bacteria appear to be inside trophoblasts (arrowheads). Bar, 20  $\mu$ m.

deletion mutant had the same phenotype as wild-type *L. monocytogenes* infection (Fig. 5A). The InlA InlB double deletion mutant had the same phenotype in BeWo cells as the InlA single deletion mutant (Fig. 5A). Complementation of InlA single and InlA InlB double deletion mutants with the *inlA* gene or the *inlA* and *inlB* genes, respectively, led to complete restoration of the wild-type phenotype (Fig. 5B). These results indicated that *L. monocytogenes* directly infects BeWo cells in an InlA-dependant manner. However, even without InlA there was a low rate of infection, indicating that there might be other mechanisms by which *L. monocytogenes* can directly invade BeWo cells, although not very efficiently. The InlA deletion mutant had no defect in growth or cell-to-cell spread, despite the 100-fold decrease in entry (data not shown).

**Infection of primary human trophoblasts.** We wanted to confirm our results for the BeWo cell line with primary cells. Primary human trophoblasts, derived from second-trimester placentas, resemble BeWo cells morphologically. We infected the primary trophoblasts with a higher MOI than that used for infection of BeWo cells, because we were able to assess *L.*

*monocytogenes* infection at only one time point due to limited availability of primary trophoblasts. The MOI needed to be high enough not only for quantification of intracellular wild-type bacteria but also for quantification of the intracellular

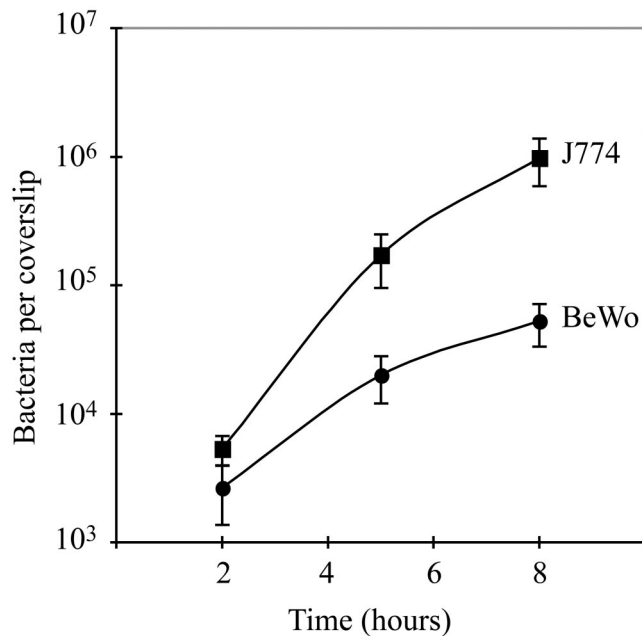


FIG. 4. Growth of *L. monocytogenes* in BeWo cells and J774 murine macrophages. BeWo cells ( $3 \times 10^4$ ) and J774 cells ( $6 \times 10^4$ ) were plated on glass coverslips and grown overnight, leading to formation of monolayers. The difference in the number of plated BeWo and J774 cells reflects the difference in size between these two cell types. Cells were infected with a 1:20,000 dilution of an overnight culture of wild-type *L. monocytogenes*, resulting in MOIs of 1:8 for BeWo cells and 1:15 for J774 cells. At the specified times after infection, cells were lysed, and the number of bacteria per coverslip was determined in triplicate. In each case, gentamicin (50  $\mu$ g/ml) was added 1 h after infection. Each growth curve represents the means and standard deviations from three separate experiments.

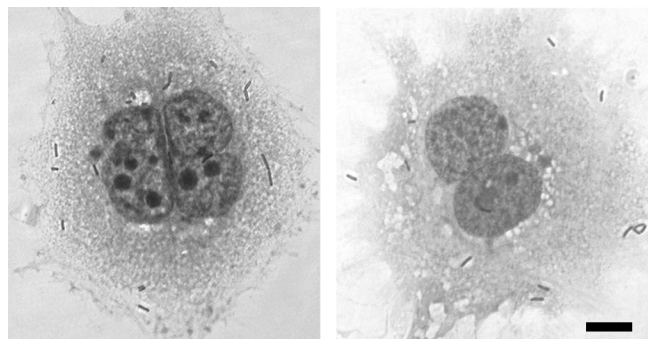


FIG. 3. Light micrographs of Diff-Quik stains of trophoblasts 2 h after infection with wild-type *L. monocytogenes*. (Left panel) Primary human trophoblasts. (Right panel) BeWo cells. In each case, gentamicin (50  $\mu$ g/ml) was added 1 h after infection. Bar, 10  $\mu$ m.

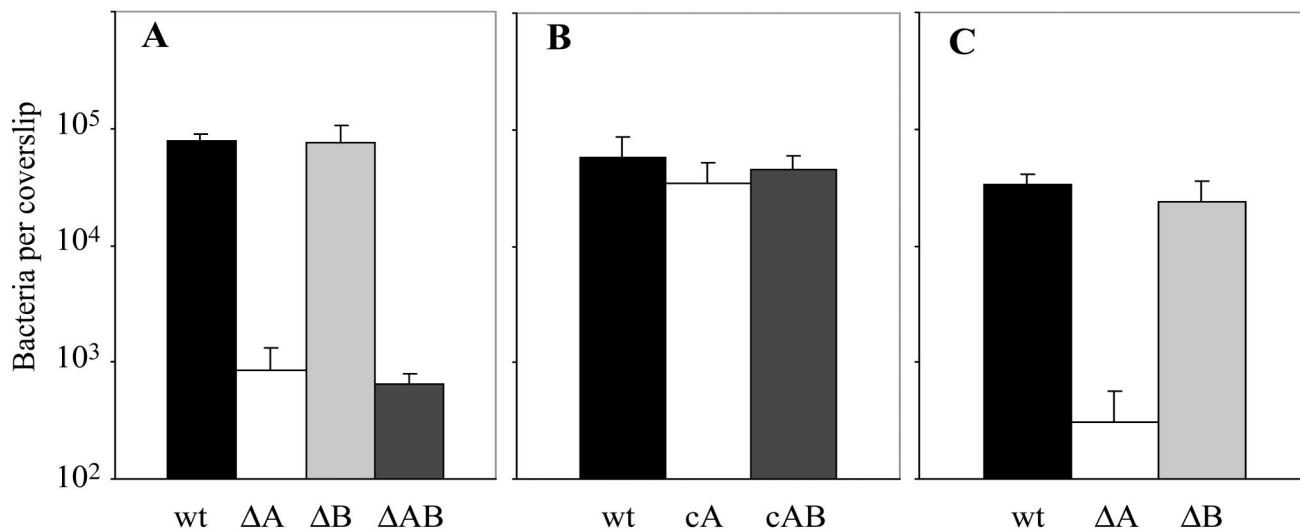


FIG. 5. Invasion of trophoblasts by *L. monocytogenes*. Monolayers of cells were grown on glass coverslips. Trophoblasts were infected with *L. monocytogenes* 10403S (wild type [wt]) and the InlA deletion mutant ( $\Delta A$ ), the InlB deletion mutant ( $\Delta B$ ), the InlA InlB double deletion mutant ( $\Delta AB$ ), the InlA complementation mutant (cA), and the InlA InlB complementation mutant (cAB). BeWo cells were infected at an MOI of 1:1 (A and B), and primary human trophoblasts were infected at an estimated MOI of 20:1 (C). In each case, gentamicin (50  $\mu\text{g/ml}$ ) was added 1 h after infection. At 2 h after infection, monolayers were lysed and the number of bacteria per coverslip was determined in triplicate. All bacterial numbers are the averages and standard deviations from three separate experiments.

InlA deletion mutant at 2 h postinfection. We therefore chose to infect the primary trophoblasts at an estimated MOI of 20:1. Multiple bacteria per cell were observed 2 h after infection with wild-type *L. monocytogenes* (Fig. 3, left panel). Because of the presence of syncytium, it was difficult to estimate the percentage of infected cells. Like in BeWo cells, infection of primary human trophoblasts with the InlA deletion mutant led to 2-log-unit reduction in invasion (Fig. 5C). The InlB deletion mutant had the same phenotype as wild-type *L. monocytogenes* (Fig. 5C), and the InlA InlB double deletion mutant had the same phenotype as InlA single deletion mutant (data not shown).

**Evaluation of the role of InlA in crossing of the placental barrier in vivo.** Based on in vitro results, we hypothesized that InlA-dependent invasion of trophoblasts plays a role in fetoplacental transmission of *L. monocytogenes*. We infected pregnant guinea pigs with  $10^6$  *L. monocytogenes* wild-type or InlA deletion mutant bacteria. Bacteria per organ were enumerated at 48 h postinoculation. We found no statistically significant differences between wild-type and mutant bacterial numbers in placenta, fetal liver, or maternal organs (Fig. 6). Microscopic examination of placental sections showed no difference in the placental lesions caused by the InlA deletion mutant and those caused by the wild type. Immunohistochemical staining for *L. monocytogenes* showed small numbers of bacteria, which appeared to be in the cytoplasm of trophoblasts in both cases (Fig. 2B and data not shown).

Competitive index analysis with a 1:1 ratio of erythromycin-sensitive InlA deletion mutant and erythromycin-resistant wild-type *L. monocytogenes* was performed in the pregnant guinea pig model. At 24 h after inoculation we found no statistically significant differences between the mean competitive indices in placenta and maternal organs, which were all close to 1 (Fig. 7). Furthermore, competitive index analysis with wild-

type *L. monocytogenes* versus the InlA InlB double deletion mutant was performed under the same conditions except that the bacterial numbers per organ were enumerated at 48 h postinoculation. Again, the mean competitive indices were close to 1 and were not statistically different in fetal liver, placenta, and tested maternal organs (data not shown). These results indicate that InlA and InlA InlB deletion mutants and wild-type *L. monocytogenes* strains proliferate equally well in all tested organs.

## DISCUSSION

*L. monocytogenes* is one of a group of pathogens that are known to be vertically transmitted in humans and other mammals. In this study we developed a pregnant guinea pig model of listeriosis. Like in humans, there seemed to be a tropism of *L. monocytogenes* to the guinea pig placenta, because the clinical manifestations in both species were similar. Subsequent to intravenous infection, guinea pigs developed spontaneous abortions without appearing to be systemically ill. Only 1 day after inoculation, the guinea pig placenta was consistently infected and comparable numbers of bacteria were found in placenta, maternal liver, and spleen. Infection of the fetus occurred slightly later than infection of the placenta, indicating that the infection spread from the placenta to the fetus. The ability of *L. monocytogenes* to proliferate in fetoplacental tissues exceeded that in other organs, possibly due to impaired cell-mediated immune responses in the fetoplacental unit.

A single trophoblast layer separates fetal from maternal circulation, suggesting that infection of trophoblasts might be a critical step in crossing the fetoplacental barrier. Indeed, *L. monocytogenes* appeared to be in the cytosol of some trophoblasts. Trophoblast infection could occur by two possible mechanisms in vivo: direct invasion by extracellular bacteria or

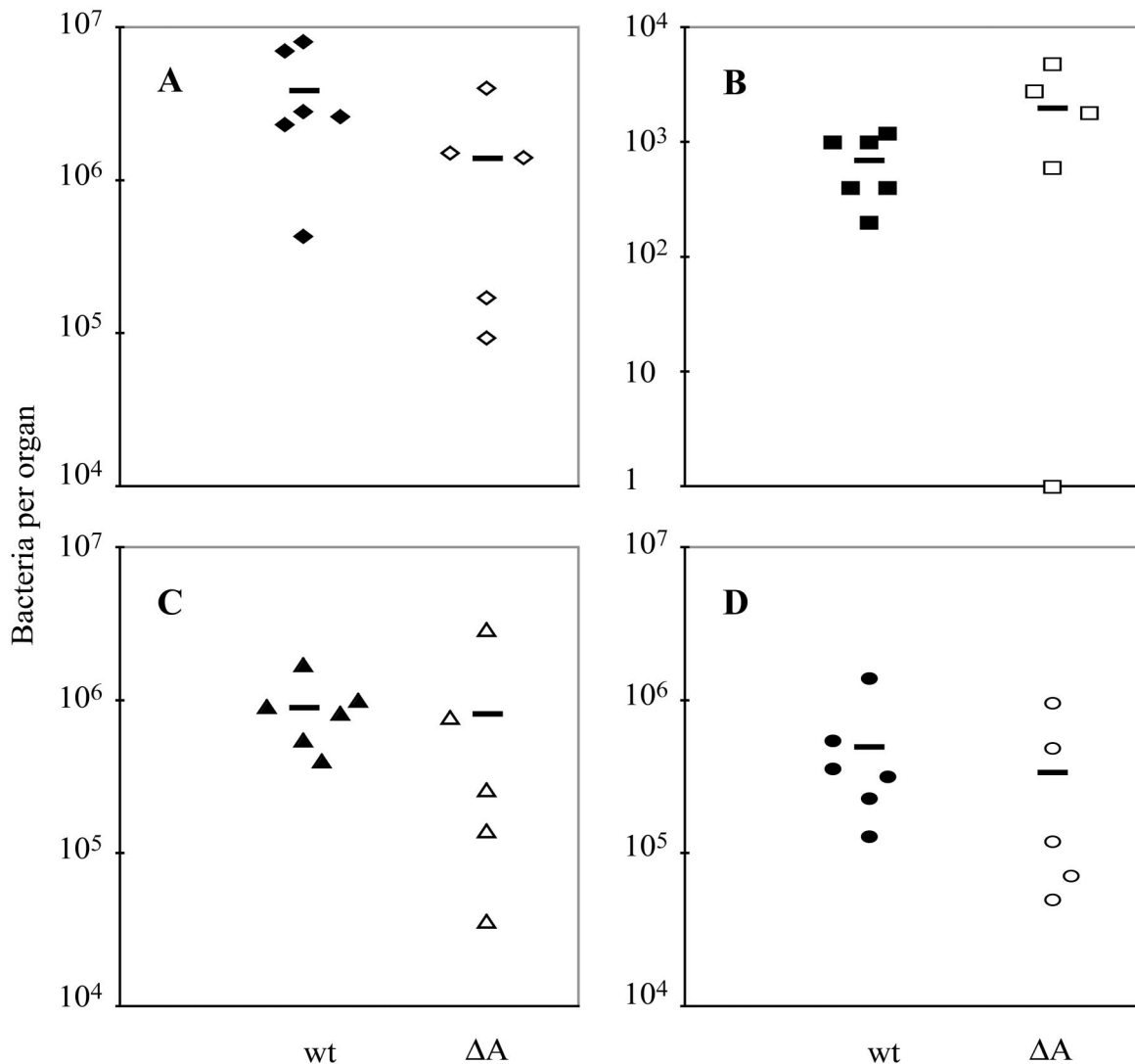


FIG. 6. Infection of pregnant guinea pigs with wild-type *L. monocytogenes* (*wt*) or the InIA deletion mutant ( $\Delta A$ ). Groups of one to three pregnant guinea pigs between days 42 and 52 of gestation were inoculated intravenously with  $10^6$  bacteria. Bacteria per placenta (A), fetal liver (B), maternal liver (C), and maternal spleen (D) were enumerated at 48 h postinoculation. Bars represent means.

cell-to-cell spread from infected maternal cells, specifically macrophages. Internalins would play a role in direct invasion but not in cell-to-cell spread. Our in vitro results demonstrated that invasion of isolated primary human trophoblasts and BeWo cells was greatly enhanced by the presence of InIA. However, we found no difference in fetoplacental infection with wild-type *L. monocytogenes* or the InIA deletion mutant in the pregnant guinea pig model of infection, indicating that InIA does not play a significant role in crossing the fetoplacental barrier in vivo. One possible explanation for our findings is that trophoblast infection occurs via cell-to-cell spread in vivo. There are indications that cell-to-cell spread is an important mechanism of trophoblast infection by other pathogens. Studies on mechanisms of fetoplacental transmission of CMV by using a coculture system of polarized uterine microvascular endothelial cells and trophoblasts showed that neutrophils transmitted CMV infection to uterine microvascular endothe-

lial cells, which in turn infected trophoblasts (32). Comparison between the placental structures of different mammals also suggests that cell-to-cell spread might be an important mechanism for trophoblast infection. *L. monocytogenes* is the cause of spontaneous abortions in cows, sheep, and goats (20, 31, 56). Ruminants have a synepitheliochorial placenta, which means that the trophoblast layer is attached to an almost intact uterine mucosa (27). It seems that in these species, cell-to-cell spread from infected endothelial cells or migrating tissue macrophages would be more important than direct invasion for trophoblast infection.

Another explanation for our findings could be that direct invasion of trophoblasts in vivo is mediated by other internalins. Twenty proteins or open reading frames encoding for putative proteins with homology to InIA have been identified (4). Their roles in fetoplacental transmission are unknown. We found a low level of infection with *L. monocytogenes* that



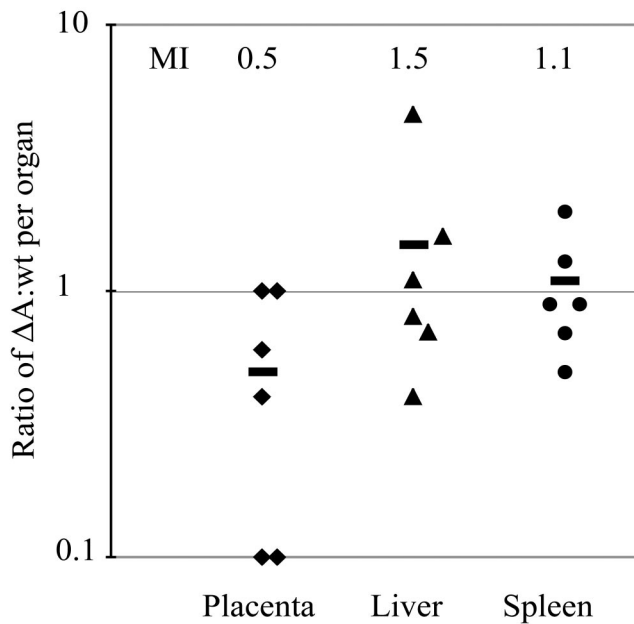


FIG. 7. Competitive index analysis of InlA deletion mutant ( $\Delta A$ ) and wild-type (wt) *L. monocytogenes*. Groups of three pregnant guinea pigs were inoculated intravenously with a 1:1 mixture of the two strains at a total dose of  $2 \times 10^7$ . Bacteria per organ were enumerated at 24 h postinoculation. Ratios between erythromycin-sensitive ( $\Delta A$ ) and erythromycin-resistant (wt) colonies were calculated for placenta, maternal liver, and spleen. Bars represent mean indices (MI).

was independent of InlA or InlB in primary human trophoblasts and BeWo cells. This suggests the presence of a mechanism of internalization not mediated by InlA or InlB, although this mechanism seems to be inefficient.

The nonpregnant mouse has been used extensively as a model host for listeriosis. There have been few studies of fetoplacental infection in mice with *L. monocytogenes*. In contrast to our findings with the pregnant guinea pig model, only a small number of pregnant mice had fetoplacental infection 18 or 24 h after intravenous inoculation, despite high bacterial burdens in maternal liver and spleen (reference 44 and our unpublished results). Interestingly, the pregnant guinea pig is also better than the mouse for studies of vertical transmission of CMV (33, 47). The reasons for the differences in susceptibility of the fetoplacental unit to infection in the mouse and guinea pig are unknown. Structural differences in the fetoplacental barrier could play a role. Both mice and guinea pigs have a hemochorial placenta, but mice have three layers of trophoblasts instead of one to separate maternal and fetal circulation. This might make the transmission of *L. monocytogenes* to the fetus less efficient. Another possible explanation is that the placental immunological environment might be more permissive for growth of *L. monocytogenes* in guinea pigs than in mice.

In our study we used intravenous inoculation of *L. monocytogenes* as the route of infection. In the setting of natural infection, *L. monocytogenes* is a food-borne disease and is thought to spread to the placenta from the gastrointestinal tract by hematogenous dissemination. Bypassing the gastroin-

testinal tract could lead to differences in dissemination, and we are in the process of developing an oral infection model in the pregnant guinea pig.

In summary, we demonstrate that human trophoblasts are permissive to listerial growth and we describe a pregnant guinea pig model to study fetoplacental transmission of *L. monocytogenes* in vivo. Despite the importance of InlA for invasion of isolated human trophoblasts, we could not find a role for InlA in crossing of the fetoplacental barrier in vivo. This underlines the importance of a good animal model for evaluating the significance of in vitro findings. Further studies will focus on the molecular and cellular mechanisms of listerial fetoplacental transmission. This understanding is fundamental to the development of preventive measures against fetal infection.

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