IMMUNOLOGICAL AND PATHOLOGICAL CHANGES AFTER MATERNAL

EXPOSURE WITH *LISTERIA MONOCYTOGENES* IN PREGNANT GUINEA PIGS

by

ELIZABETH ANN IRVIN

(Under the Direction of Mary Alice Smith)

ABSTRACT

Listeriosis has the highest case fatality rate of foodborne illnesses in the United States, approximately 30%. One-third of the 2500 cases involve the fetus or neonate. The mechanisms of fetal infection and death are largely unknown. The objectives of this study were to use pregnant guinea pigs 1) to determine effects of exposure to *L. monocytogenes* on apoptosis in the placenta, 2) to characterize maternal serum cytokine levels after exposure to *L. monocytogenes*, 3) to determine infection-related changes in placental cytokine expression, 4) to determine the immunoreactivity of a human anti E-cadherin antibody to intestinal and placental tissue. Oral inoculation of pregnant guinea pigs resulted in an increase in the number of placentas undergoing apoptosis at $\geq 10^6$ CFUs *L. monocytogenes* at 21 days post-treatment. Maternal serum TNF-α levels were significantly decreased 21 days post treatment at $\geq 10^4$ CFUs *L. monocytogenes*. After maternal treatment with 10^8 CFUs *L. monocytogenes*, placental inflammatory cytokine expression was altered as early as six days post-treatment. At 10^8 CFUs *L. monocytogenes*, an increasing trend of placental apoptosis was seen as infection proceeded. Guinea pig and rhesus monkey intestinal tissue was immunoreactive to a human anti E-cadherin antibody. In conclusion, the placental cytokine expression and apoptosis is affected after maternal infection with *L. monocytogenes*. TNF-α concentrations in maternal serum were significantly decreased after oral exposure to *L. monocytogenes*. A better understanding of mechanisms behind *Listeria*-induced stillbirths will allow for the development of biomarkers for infection and treatment capabilities.

INDEX WORDS: Listeriosis, *L. monocytogenes*, placenta, apoptosis, infection, cytokines, pregnancy, guinea pigs

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CHAPTER 1

INTRODUCTION

Listeriosis during pregnancy can result in spontaneous abortion or premature delivery of a stillborn fetus (Gray and Killinger, 1966; MacDonald et al, 2005). In 1985, the largest outbreak of listeriosis occurred with 93 pregnant women developing listeriosis after eating contaminated Mexican-style cheese (Linnan et al, 1988). Of the 93 perinatal cases, 20 cases ended with fetal death and 10 resulted in neonatal deaths (Linnan et al, 1988). While healthy individuals can develop gastroenteritis at high bacterial doses (Dalton et al, 1997), several subpopulations are susceptible to life-threatening listeriosis, including the elderly, immunocompromised persons, and the fetus or neonate (Farber and Peterkin, 1991; Enocksson et al, 1990). In the United States, approximately 2500 cases of listeriosis occur each year resulting in 500 deaths with one-third of the cases involving pregnant women (Mead et al, 1999; Linnan et al, 1988). The remaining two-thirds of cases of listeriosis are mainly in immunocompromised or elderly individuals where infection manifests as meningitis and/or encephalitis and sepsis (Notermans et al, 1998).

Ingestion of *L. monocytogenes* by pregnant women carries the risk of feto-placental transmission (Lorber, 1997). In these pregnancy-related cases, the mother may remain asymptomatic until the time of fetal distress which makes early diagnosis and treatment difficult (Gray and Killinger, 1966; MacDonald et al, 2005). Very little dose-response data are available from human outbreaks. Human studies to characterize dose response cannot be conducted because of the risk of severe illness or death. The

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mechanism of fetal infection and death is unknown. The maternal immune system is tightly regulated during pregnancy, and its disruption after exposure to *L. monocytogenes* could result in deleterious effects to the fetus.

In 2000, the Food and Agricultural Organization and the World Health Organization conducted a risk assessment for *L. monocytogenes* (FAO/WHO, 2001). Additionally, the United States Food and Drug Administration, United States Department of Agriculture, and the Centers for Disease Control and Prevention (USFDA, USDA, CDC) conducted a risk assessment of *L. monocytogenes* in ready-to-eat foods in 2001 with a final draft published in 2003 (USFDA/USDA/CDC, 2003). In both risk assessments, a data gap in animal dose-response data was identified. Previously, mice have been used to characterize the ability of *L. monocytogenes* to infect and invade tissues and to develop dose-response data (Le Monnier et al, 2006; Takeuchi et al, 2006). However, in human outbreaks the main route of exposure is though ingestion of contaminated foods, and generally mice are not susceptible to severe infection with *L. monocytogenes* after oral exposure (Lecuit et al, 1999). Like humans, guinea pigs are susceptible to *L. monocytogenes* after oral exposure and can be used to study *Listeria*-induced stillbirths.

We used pregnant guinea pigs to investigate the possible mechanisms by which *L. monocytogenes* induces stillbirths following oral exposure to the bacteria. In this study, pregnant guinea pigs were used to examine the maternal immune response and the placental immunological and pathological response following infection with *L. monocytogenes*. Additionally, we compared the immunoreactivity of guinea pig, rhesus monkey, human, and mouse tissues to a human anti E-cadherin primary antibody.

CHAPTER 2

LITERATURE REVIEW

Listeriosis

Listeriosis was first described as an illness affecting guinea pigs and rabbits in

1926 (Murray et al, 1926). Initially, listeriosis was considered to be of zoonotic origin;

however, in 1983 listeriosis was directly linked to consumption of contaminated foods

(Schlech et al, 1983). Several human subpopulations are susceptible to *L. monocytogenes*

infection resulting in severe illness. Fetuses and neonates are also susceptible to severe

infection with *L. monocytogenes* (Gray and Killinger, 1966). Recent studies suggest that

30% of human cases involve the fetus or neonate (Linnan et al, 1988; Mead et al, 1999).

The remaining two-thirds of cases are comprised of elderly, immunocompromised, or

immunosuppressed persons (Notermans et al, 1998). In immunocompromised or elderly

persons, exposure to *L. monocytogenes* can lead to meningitis, encephalitis, and/or sepsis

(Gray and Killinger, 1966; Olsen et al, 2005). In healthy individuals, febrile

gastroenteritis can occur after consumption of highly contaminated foods (Dalton et al,

1997).

Perinatal listeriosis in humans

Approximately 30% of the cases of listeriosis occur in pregnant women (Mead et

al, 1999). The incubation time for *L. monocytogenes* during pregnancy is relatively long,

5 to70 days, which makes diagnosis and treatment more difficult (Swaminathan and

Gerner-Smidt, 2007). While maternal bacteremia rarely causes illness, a nonspecific

febrile illness that is self-limiting can occur (Mead et al, 1999). In one outbreak of *L. monocytogenes*, 83% of infected pregnant women complained of chills, fever, or headache (MacDonald et al, 2005). After maternal exposure, transplacental migration of the bacteria to the fetus leads to spontaneous abortion and stillbirth (Farber and Peterkin, 1991). Fetal death has occurred as early as the first trimester; however, the second and third trimesters appear to be the most susceptible gestational times for fetal infection (Gray and Killinger, 1966; Cruikshank and Warenski, 1989; Swaminathan and Gerner-Smidt, 2007).

Second and third trimester infections can lead to premature delivery of a stillborn fetus (Farber and Peterkin, 1991; Enocksson et al, 1990). During later third trimester exposure, neonatal meningitis and/or sepsis can occur (Gray and Killinger, 1966; Ramaswamy et al, 2007). In one study of 11 pregnant women with listeriosis, two of the exposures to *L. monocytogenes* occurred during the second trimester and ended with spontaneous abortion of the infected fetus, while six women delivered premature infants with listeriosis (Mylonakis et al, 2002).

In cases of premature delivery of stillborn fetuses, microabscesses and granulomas on the fetal organs, including liver, spleen, lungs, and skin, have been noted at autopsy (Gray and Killinger, 1966; Spencer, 1987). Additionally, placental histology reveals microabscesses (Spencer, 1987; Topalovski et al, 1993; Mylonakis et al, 2002). The intervillous and intravillous microabscesses may be associated with areas of inflammation or fibrin deposits (Benshushan et al, 2002). In most cases, the abscesses have an area of central necrosis with polymorphonuclear leukocyte infiltration, and the bacteria are present on the amnionic surface (Benirschke et al, 2006).

Central Nervous System Infection

   L. monocytogenes can cross the blood-brain barrier and cause severe central nervous system illness, including meningitis. Meningitis is usually seen in individuals with underlying conditions, such as a weakened immune system due to aging or disease and suppressed immune systems (including HIV/AIDS patients, transplant patients, and chemotherapy patients) and in neonatal infants. Even with the capability for antibiotic treatment (including ampicillin and gentamicin), listeric meningitis can be fatal (Temple and Nahata, 2000). *L. monocytogenes*-associated meningitis is among the most common causes of bacterial meningitis with a mortality rate of approximately 25 to 30%, even with antibiotic treatment (Skogberg et al, 1992; Mylonakis et al, 1998). The mortality rate for listeric infection, including meningitis, encephalitis and sepsis, was 20% in patients over 70 years of age and 24% in patients with an underlying condition (Gerner-Smidt et al, 2005).

L. monocytogenes in food

   It was not until 1983 that *L. monocytogenes* was discovered in contaminated food (Schlech et al, 1983). Some commonly contaminated foods that have been associated with outbreaks are Mexican-style cheese, hot dogs, and deli-meat (Fleming et al, 1985; Dalton et al, 1997; CDC, 1999; Kathariou et al, 2006; Olsen et al, 2005). While the U.S. FDA has a zero-tolerance policy for food products contaminated with *L. monocytogenes*, outbreaks of listeriosis still occur. Listeriosis is the fourth most common food-related illness; however, the case-fatality rate of listeriosis, 27.6%, is the highest of all food-borne pathogens (Mead et al, 1999). In recent years, several large outbreaks with at least 25% of perinatal cases resulting in fetal death have occurred (Table 2.1). The recent

outbreaks have been associated with Mexican-style cheese and deli meats or hotdogs with the case rate for fetal mortality ranging from 24% to 46%. For these selected outbreaks, there was a total of 347 cases including 137 perinatal cases which resulted in 45 fetal deaths, an average of 12% fetal mortality. As of 2008, listeriosis continues to be a problem in the United States despite the zero-tolerance policy adopted by U.S. FDA. In January 2008, an outbreak of invasive listeriosis was traced back to the consumption of contaminated milk in Massachusetts (Gerner-Smidt, 2008). By March 2008, five deaths, including one fetal death had been reported (Gerner-Smidt, 2008).

Table 2.1: Selected outbreaks of listeriosis in the United States (Adapted from Swaminathan and Gerner-Smidt, 2007)

Year	No. cases	No. perinatal cases	No. perinatal deaths (% mortality)	Location	Contaminated food product	Source of contamination	Reference
1985	142	93	30 (32%)	California	Mexican-style cheese	Unknown	Linnan et al, 1988
1998	108	13	4 (31%)	Multistate	Hotdogs	Refrigeration unit removal	Mead et al, 2006
2000	30	8	3 (38%)	Multistate	Turkey deli meat	Unknown	Olsen et al, 2005
2000	13	11	5 (46%)	North Carolina	Mexican-style cheese	Unknown	MacDonald et al, 2005
2002	54	12	3 (25%)	Multistate	Turkey deli meat	Unknown	Gottlieb et al, 2005

L. monocytogenes has the ability to grow at refrigerated temperatures, low pH values, and high salt environments, which makes eradication difficult (Gray and Killinger, 1966; Farber and Peterkin, 1991). The mechanisms of infection in the susceptible populations are still undefined. Research is needed to investigate possible biomarkers for infection, such as evaluation of inflammatory cytokine production or neutrophils CD64 measurements.

Animal models

Consumption of foods contaminated with L. monocytogenes is still a problem in the United States and in other countries, such as France and the United Kingdom. The 2003 revised risk assessment for L. monocytogenes in ready-to-eat foods conducted by U.S. FDA relied on animal studies conducted with mice. However, appropriate animal models are necessary for the development of dose-response curves and approximate lethal dose values for 50% mortality (LD50), including LD50 values for fetal mortality, after oral exposure to L. monocytogenes.

Mice

Mice have been the primary animal model used to study L. monocytogenes since the early 1980's. Interestingly, listeriosis is not known to occur naturally in mice (Lecuit, 2007). In laboratory studies, oral exposure of mice with L. monocytogenes rarely causes severe systemic infection (Marco et al, 1997). However, a few studies showed infection in mice after oral exposure to L. monocytogenes. In 1970, Miller and Burns used Albany strain mice as an animal model for 24-hour oral exposure to L. monocytogenes-contaminated drinking water (Miller and Burns, 1970). Histopathology showed microgranuloma lesions on the liver of the exposed nonpregnant and pregnant animals

sacrificed at post-treatment day seven; however, the lesions were more severe in pregnant mice (Miller and Burns, 1970). At 14 days post-treatment, no lesions were present in nonpregnant mice, where as the lesions in pregnant mice were more numerous and extensive than reported seven days earlier. A more recent study in nonpregnant mice showed no bacteria present in the liver or spleen seven days post-treatment (Park et al, 2004).

The use of an anesthetic, sodium pentobarbital, prior to intragastric treatment with *L. monocytogenes* in mice appears to enhance the ability of the bacteria to invade and infect the liver (Czuprynski et al, 2003; Park et al, 2004). The bacterial counts in the liver and spleen of mice treated with sodium pentobarbital prior to *L. monocytogenes* dosing were significantly higher than non-anesthetized mice receiving the same *L. monocytogenes* dose (Czuprynski et al, 2003). However, the effects of sodium pentobarbital on susceptibility to *L. monocytogenes* decreased if anesthetic treatment occurred greater than 2 hours prior to dosing with *L. monocytogenes* (Czuprynski et al, 2003). Based on further treatments with loperamide (for a reduction in gastric motility) and sodium bicarbonate (to neutralize gastric acid), it is unlikely that decreased gastric motility or a reduction in gastric acidity result in an increased susceptibility to *L. monocytogenes* infection (Czuprynski et al, 2003).

Previously, mice have been used to determine effects of *L. monocytogenes* after intravenous (IV) or intraperitoneal (IP) inoculation at various doses. Resistant and susceptible mice have been used to develop LD50 values after exposure to *L. monocytogenes* with LD50 values ranging from 10^2 to 10^7 CFUs *L. monocytogenes* (Cheers et al, 1978; Takeuchi et al, 2006). Additionally, for listeriosis studies during

pregnancy, IV inoculation is often used as a route of exposure. After IV inoculation of pregnant mice, bacterial titers are higher in the liver than in nonpregnant mice (Abram et al, 2002). The pregnant mice develop severe necrotizing hepatitis while nonpregnant mice are able to clear the bacteria from the liver prior to seven days post-inoculation (Abram et al, 2002). Additionally, a significant increase in pregnancy loss was noted at days three and six post-treatment compared to non-infected mice (Abram et al, 2002). However, human exposure to *L. monocytogenes* is through consumption of contaminated foods, not IV exposure.

Several recent studies of listeriosis in mice have shown that mice are not an appropriate animal model for oral exposure to *L. monocytogenes* (Marco et al, 1992; Marco et al, 1997; Lecuit, 2007). Mice are not susceptible to infection with *L. monocytogenes* through oral ingestion due to a difference in the active site of their E-cadherin, the protein receptor necessary for intestinal transmigration (Lecuit et al, 1999). Because of this difference in the amino acid sequence of E-cadherin in mice, other animal models are needed to study the effects of infection after oral exposure to *L. monocytogenes*.

Nonhuman primates

Listeriosis in nonhuman primates is similar to the clinical manifestations of infection in humans. McClure et al (1986) described *L. monocytogenes* as endemic in a group of outdoor-housed colony reared macaques, causing stillborn fetuses and neonatal death under natural feeding conditions (McClure et al, 1986). Other research centers have reported stillbirths and neonatal illness due to *L. monocytogenes* infection in outdoor housed primate populations (Paul-Murphy et al, 1990). Additionally, case

reports of neonatal death due to infection of nonhuman primates with *L. monocytogenes* are available. One specific case reported the death of a 3-day old *Macaca fascicularis* resulting from septicemia and meningoencephalitis (Chalifoux and Hajema, 1981). The infant had *L. monocytogenes* present in the liver and brain as shown by Gram stain and positive culture; however, there was no evidence of infection in the mother (Chalifoux and Hajema, 1981). Similarly, a *Macaca niger* delivered a stillborn infant with no evidence of maternal illness (McClure and Strozier, 1975). Gram stain revealed small, gram positive rods in various fetal organs, including the placenta and liver, and *L. monocytogenes* was cultured from the fetal liver (McClure and Strozier, 1975).

Nonhuman primates have rarely been used in the past as an animal model for human listeriosis. However, some studies have used nonpregnant and pregnant primates to investigate the dose related effects of infection with *L. monocytogenes*. At lower doses in healthy nonhuman primates, no adverse effects were noted (Farber et al, 1991); this is similar to what is seen in humans (Gray and Killinger, 1966). Silverman et al (1963) used nonhuman primates to investigate the effects of a series of animal passages on the virulence of *L. monocytogenes*. Doses of 10^7 to 10^8 CFUs *L. monocytogenes* were injected into the spine of monkeys up to 22 times. The numerous passages of the bacteria did not affect virulence of *L. monocytogenes* in the nonhuman primate (Silverman et al, 1963).

One investigation into the effects of infection with *L. monocytogenes* in pregnant nonhuman primates has been done. Ten pregnant *Macaca mulatto* (rhesus monkeys) were inoculated via a nasogastric tube with *L. monocytogenes* during the last trimester (Smith et al, 2003). Forty percent of the pregnancies resulted in the delivery of stillborn

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fetuses. Additionally, a subsequent dose-response study was conducted using a total of 23 pregnant rhesus monkeys (Smith et al, 2008). Combining the original nonhuman primate study and the subsequent study, a log-logistic model was used to determine the approximate LD50 for fetal mortality, which was calculated to be 8.45 x 10^7 CFUs *L. monocytogenes* (Smith et al, 2008). Similar to humans, no gastrointestinal disturbances were noted in any of the treated pregnant animals (Smith et al, 2003; Smith et al, 2008).

Guinea pigs

In 1926, Murray et al isolated *L. monocytogenes*, then called *Bacterium monocytogenes*, from a colony of infected guinea pigs and rabbits (Murray et al, 1926; Gray and Killinger, 1966). It was not until the 1960's that guinea pigs were used to investigate the effects of *L. monocytogenes*. Silverman et al (1963) used guinea pigs to determine the effects of animal passage on *L. monocytogenes* virulence. There appeared to be a decrease in *L. monocytogenes* virulence after numerous IP passages in guinea pigs (Silverman et al, 1963). Additionally, guinea pigs were used as an animal model to determine LD50 values using different routes of exposure. In one study, the LD50 value after IP exposure was greater than 10^7 cells of *L. monocytogenes* (Kautter et al, 1963). Dustoor et al (1977), used guinea pigs to determine the effects of infection with *L. monocytogenes* after different routes of exposure. Intracardial (IC), IV, and IP inoculations were investigated. At the highest dose, 1 x 10^8 CFUs *L. monocytogenes* via IV inoculation, 100% mortality occurred (Dustoor et al, 1977). The LD50 for IV inoculation appears to be 100 times higher (approximately 1.2 x 10^7 CFUs *L. monocytogenes*) than the LD50 for IC inoculation (1.2 x 10^5 CFUs *L. monocytogenes*). However, after IC inoculation with doses greater than the LD50, an increase in deaths

was not seen, suggesting there is not a good correlation between the IC inoculation dose and the number of deaths (Dustoor et al, 1977).

Recently, guinea pigs have been used to model human listeriosis during pregnancy. Bakardjiev et al (2004) used pregnant guinea pigs to investigate the effects of IV inoculation with *L. monocytogenes* on pregnancy. Interestingly, infection of the maternal liver and spleen and placenta occurred only one day after maternal inoculation (Bakardjiev et al, 2004). After maternal IV inoculation with 2×10^7 CFUs *L. monocytogenes*, 71% of dams had spontaneous abortions by 2 days post-treatment (Bakardjiev et al, 2004). In a subsequent study, Bakardjiev et al (2006) used a mathematical model to study replication and clearance of *L. monocytogenes* after IV treatment in the pregnant guinea pig. The results of the mathematical model suggest that the placenta acts as a niche for the bacteria and is used to traffic bacteria to and from maternal organs (Bakardjiev et al, 2006). However, the most common route of exposure is through ingestion of contaminated foods. Therefore, an animal model mimicking human exposure, oral consumption of the bacteria, should produce a more appropriate understanding of dissemination of the bacteria in humans. In studies using oral exposures to *L. monocytogenes* in pregnant guinea pigs, when *L. monocytogenes* was cultured from an infected placenta or fetus, the maternal liver was always infected (Williams et al, 2007). In some cases, *L. monocytogenes* was isolated from the maternal liver without isolation from the placenta (Williams et al, 2007)

Until recently, no small animal model was available for oral exposure to *L. monocytogenes* during pregnancy that adequately describes pregnancy-associated human listeriosis. In 2007, Williams et al published a study describing the guinea pig as an

animal model for human listeriosis during pregnancy following ingestion of *L. monocytogenes* (Williams et al, 2007). Pregnant guinea pigs were used to develop a dose response curve and a LD50 for fetal mortality following maternal treatment with *L. monocytogenes* (Williams et al, 2007). After maternal treatment, the pregnancy was allowed to proceed normally for 21 days or until premature delivery. At the highest dose, 10^8 CFUs *L. monocytogenes*, 75% of dams delivered stillborn fetuses. The resulting LD50 for fetal mortality was calculated as approximately 2 x 10^7 CFUs *L. monocytogenes*. A subsequent study was done to determine effects of three different strains of *L. monocytogenes* on feto-placental invasion in pregnant guinea pigs (Jensen et al, 2008). Similar to humans, the dams were asymptomatic until the time of premature delivery (Williams et al, 2007; Jensen et al, 2008). The pregnant guinea pig model appears to be a potential model for investigating the mechanisms of *L. monocytogenes*-induced stillbirths in humans after oral exposure.

L. monocytogenes and pathogenesis

L. monocytogenes are gram-positive bacteria that have the ability to replicate within eukaryotic cells. There are five species of *Listeria* with *L. monocytogenes* being pathogenic to both humans and animals. *L. monocytogenes* was discovered in 1926 and in 1936 cultured from a human aborted fetus (Murray et al, 1926; Burn, 1936). This intracellular pathogen has the ability to cross the intestinal, blood-brain, and placental barriers. Two of the important aspects of human infection are the ability of *L. monocytogenes* to cross the intestinal barrier after consumption of contaminated foods, and the ability of *L. monocytogenes* to replicate intracellularly.

L. monocytogenes contains two surface proteins which are used for cellular bacterial invasion, internalin A (InlA) and internalin B (InlB) (Gaillard et al, 1991). Interestingly, InlB does not appear to contribute to the intestinal transmigration, and the role of InlB in human infection is relatively unknown (Khelef et al, 2006). InlA is the surface protein expressed by L. monocytogenes that allows for bacterial internalization into non-phagocytic cells (Gaillard and Finlay, 1996). The bacterium binds to a surface receptor on human epithelial cells, E-cadherin, which allows for cellular internalization (Mengaud et al, 1996).

E-cadherin is expressed on the apical surface of the epithelial cell (Pentecost et al, 2006). The L. monocytogenes surface protein, InlA, binds to the first of five extracellular cadherin domains (EC1) of E-cadherin (Mengaud et al, 1996) at multi-cellular junctions with no apparent disruption of the junction (Pentecost et al, 2006). The C-terminus of the EC1 domain contains an active site of 35 amino acids (Lecuit et al, 2000). The InlA-E-cadherin receptor interaction is species-specific due to a proline residue at amino acid site 16 in the EC1 domain active site of E-cadherin (Lecuit et al, 1999). Humans and guinea pigs have identical active sites for E-cadherin, while mice have a glutamic acid substituted at amino acid site 16 (Lecuit et al, 1999). Using several mouse cells lines that express E-cadherin (NMe, Els8, Elβl, L KB, WCE2, and CHO), Lecuit et al (1999) showed that L. monocytogenes does not interact with mouse E-cadherin. Due to the lack of InlA-E-cadherin interaction, mice are not naturally susceptible to L. monocytogenes infection after oral exposure (Lecuit et al, 1999). The L. monocytogenes surface protein, InlA, contains a hydrophobic pocket into which the proline on E-cadherin is inserted (Schubert et al, 2002). The adhesion of the internalinA-E-cadherin complex to the host

cell epithelium initiates host cell invasion (Schubert et al, 2002). While the internalin-dependent crossing of the host cell cytoplasmic membrane is not fully understood, interaction with the host cell actin cytoskeleton and subsequent membrane remodeling are necessary (Hamon et al, 2006). After the bacteria have been internalized into the host cell, a series of intracellular events occur which allow for the multiplication and subsequent invasion of *L. monocytogenes* into surrounding cells.

As a result of cellular internalization, *L. monocytogenes* is surrounded by a membrane. Listeriolysin O (LLO), a pore-forming hemolytic factor, is produced by the bacteria and is necessary for the bacteria to escape the host vacuole (Gaillard et al, 1987). LLO causes the dissipation of the pH gradient of the vacuole, and is necessary for the intracellular spread of the bacteria (Gedde et al, 2000).

Once the bacteria have escaped the vacuole, intracellular multiplication can occur. Portnoy et al (1988) showed that the intracellular doubling time for the bacteria is approximately one hour. After the bacteria divide, they become surrounded by actin filaments. The ability of *Listeria* to polymerize host cell actin is mediated by a bacterial protein, ActA (Kocks et al, 1992), and the polymerization assists with bacterial movement (Smith et al, 1996). The bacteria then use the actin tail to move through the cell towards the cell surface (Tilney and Portnoy, 1989). In cells treated with Cytochalasin D, an actin filament growth inhibitor, *Listeria* are incapable of cell-to-cell spread and appear to have a decreased ability to migrate to the cell surface (Dabiri et al, 1990; Tilney and Portnoy, 1989).

After bacterial migration to the cell surface, *L. monocytogenes* form a cell extension which allows for transfer of a bacterial cell from the initial infected host cell to

a new uninfected neighboring cell. The newly formed filopod, containing *Listeria* and some of the actin filaments, is ingested by a neighboring cell via an unknown mechanism. The new host cell contains a double-membrane encapsulated *Listeria* (Tilney and Portnoy, 1989).

For extracellular replication to proceed, the bacteria must escape the double membrane vacuole. For the dissolution of the double-membrane vacuole, *Listeria* secretes two extracellular products, phosphatidylinositol phospholipase C (PI-PLC) and phosphatidylcholine phospholipase C (PC-PLC), which act in coordination to assist with vacuolar lysis (Grundling et al, 2003; Schwan et al, 1994). Only pathogenic *Listeria* species secrete both extracellular products; *L. monocytogenes* secretes both PI-PLC and PC-PLC and also expresses LLO (Leimeister-Wächter et al, 1991). PC-PLC and PI-PLC facilitate the lysis of the inner membrane while LLO causes the dissipation of the outer membrane, resulting in release from the vacuole (Alberti-Segui et al, 2007). The bacteria now have the ability to replicate and continue the intracellular infectious process.

While the intracellular life cycle of *L. monocytogenes* has been studied extensively, how the bacteria gain access to organs and systemic circulation is still questionable. *L. monocytogenes* entry into the intestinal epithelial cells is due to the InlA-E-cadherin interaction (Menguad et al, 1996). The initial entry into intestinal epithelial cells allows for bacterial replication in the lamina propina (Lecuit et al, 2001) and subsequent dissemination to the lymph nodes, liver, and spleen; however, it is still unknown how bacterial dissemination to various organs occurs, including whether the bacteria are in the systemic circulation extracellularly or in macrophages (Figure 2.1). It has been hypothesized that the bacteria in the maternal systemic circulation are

extracellular; however in a pregnant nonhuman primate model, the bacteria were not

cultured from whole blood at four or 14 days post-treatment (Smith et al, 2003).

Antibody titer levels against *L. monocytogenes* were not significantly increased until

premature delivery of a stillborn fetus, which suggests the bacteria are intracellular.

It has been proposed that the InlA-E-cadherin interaction is also responsible for

placental translocation of extracellular bacteria (Lecuit et al, 2004). However, the

mechanism of placental translocation and subsequent fetal infection after oral exposure of

L. monocytogenes are still unknown.

Figure 2.1: Schematic diagram of proposed *L. monocytogenes* movement
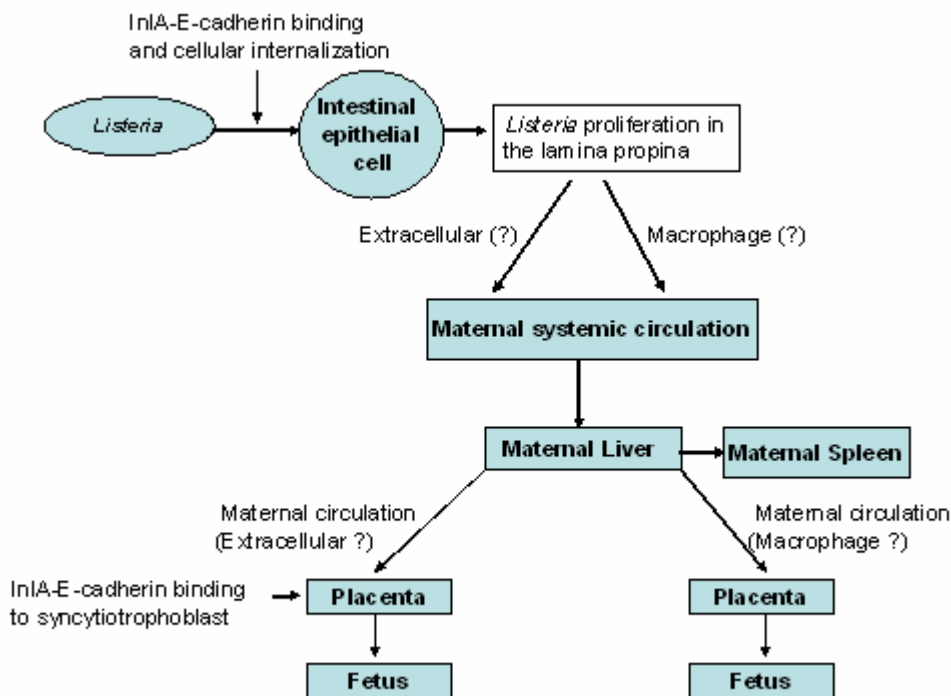
L. monocytogenes has the ability of cell-to-cell spread without extracellular

movement. The ability of the bacteria to replicate intracellularly allows the pathogen to

initially circumvent the host's immune system. Additionally, the intracellular movement

of *L. monocytogenes* does not elicit a large antibody response during infection. During

pregnancy, the immune system is altered with a preference toward B cell mediated

response, including antibody response, after pathogen exposure (Luppi, 2003).

Hormone production alterations during pregnancy could also result in a difference

in immune response; progesterone production is increased during pregnancy, which

induces IL-4 and IL-5 production (Piccinni, 2002). The ability of *L. monocytogenes* to

evade early immune recognition, a lack of B cell mediated response to *L. monocytogenes*,

and an increase in anti-inflammatory cytokine production paired with a suppression of

inflammatory cytokine production could allow for increased invasion and bacterial

growth in pregnant women.

Immune response to *Listeria monocytogenes* in healthy individuals

The innate immune response is the initial immune system response to infection

with *L. monocytogenes* and provides a non-specific inflammatory response. The adaptive

immune response to *L. monocytogenes* occurs after the initiation of the innate immune

response and is T cell mediated rather than antibody producing B cell mediated. Two

specific types of T cells involved with the adaptive immune response to *L.*

monocytogenes are CD4+ and CD8+ T cells. In a murine model of listeriosis, CD4+ T

cells are involved with cytokine production in response to invasion by *L. monocytogenes*

(Mosmann and Coffman, 1989), while CD8+ T cells lyse the infected host cell (Portnoy

et al, 2002). However, no protective immunity is developed after exposure to killed or

heat-attenuated bacteria (Portnoy et al, 2002). Heat killed *L. monocytogenes* is able to

prime *L. monocytogenes* specific CD8+ T cells; however, only treatment with live *L.*

monocytogenes allowed for the differentiation of CD8+ T cells into effector CD8+ T cells

(Lauvau et al, 2001; Muraille et al, 2005). Additionally, it has been shown that the

inability of heat killed *L. monocytogenes* to provide protective immunity is related to the inhibition of optimal CD11c^{hi} dendritic cells conditioning, including the reduction of IFN-γ secretion (Muraille et al, 2005). The inability of heat killed *L. monocytogenes* to provide protective immunity is important when considering the development of vaccines, especially vaccine development for susceptible populations.

Innate immune response

The innate immune response acts in controlling the bacterial burden during the beginning of infection. The initial control of bacteria is important as it allows the body time to mount the necessary adaptive or acquired response. Innate immune response occurs rapidly after initial exposure to *L. monocytogenes*. Mice have been used to extensively study the innate immune response to infection with *L. monocytogenes*.

During the innate immune response to *L. monocytogenes*, neutrophils, macrophages, and natural killer cells react to invasion, both in vitro and after IV inoculation (Bancroft et al, 1991; Tripp et al, 1993; Rogers and Unanue et al, 1993). The depletion of neutrophils causes an increased susceptibility to *L. monocytogenes* infection (Conlan and North, 1994). Macrophage activation after pathogen invasion stimulates the release of IL-12 and TNF-α, which recruits natural killer cells via a Toll-like receptor (Lucas et al, 2007). Macrophages secrete TNF-α and IL-12 which is essential for the subsequent production and secretion of IFN-γ via NK cell activation (Bancroft et al, 1987; Tripp et al, 1993). Natural killer cells stimulate the production and secretion of IFN-γ during early infection with *L. monocytogenes* (Dunn and North, 1991). The activation of natural killer cells is part of a positive feedback loop for early innate immune response (Thäle and Kiderlen, 2005). The production of IFN-γ and TNF-α is

necessary for innate defense against *L. monocytogenes* (Buchmeier and Schreiber, 1985; Havell, 1989). The decreased production of IFN-γ and suppression of TNF-α and cytolytic activity by NK cells during pregnancy (Luppi et al, 2002) could add to the impairment of *L. monocytogenes*-defense during pregnancy and in immunocompromised/suppressed individuals.

Adaptive immune response

The adaptive immune system is comprised of both B cells and T cells. B cell-stimulated antibody production generally provides very little contribution to the protective adaptive immune response to *L. monocytogenes* (Portnoy et al, 2002). However, in a pregnant nonhuman primate study, the *L. monocytogenes* antibody titer levels were significantly increased in dams undergoing delivery of a stillborn fetus (Smith et al, 2003). This suggests that the maternal immune system is capable of providing an antibody response against *L. monocytogenes*, but does not provide protection from adverse fetal outcome. The T cell-mediated adaptive immune response to *L. monocytogenes* is necessary for bacterial clearance. In T cell deficient immunocompromised mice, the number of viable *L. monocytogenes* cells was initially controlled leading to a significant decrease in *L. monocytogenes* early during infection. However, the inability to mount a T cell response resulted in a chronic infection (Tripp et al, 1993; Bancroft et al, 1991).

L. monocytogenes has been used to characterize T cell mediated immune responses. Infection with *L. monocytogenes* in mice initiates a robust CD4+ T cell response (Mittrucker et al, 2000; Kursar et al, 2002). Mice have been used extensively to study the role of CD4+ T cells after *L. monocytogenes* infection. In a previous study, *L.*

monocytogenes-specific CD4+ T cells were found in all organs after mice were inoculated via different routes of exposure; however, the route of exposure did affect the relative distribution of the CD4+ T cells in specific organs (Kursar et al, 2002). However, in both IV and oral exposure, the liver had high levels of CD4+ T cell accumulation (Kursar et al, 2002).

In both in vitro studies and in vivo studies using mice, CD4+ T cells differentiate into Th1 and Th2 cells after antigen recognition (Mosmann and Coffman, 1989). CD4+ T cell differentiation into the Th1 subtype is initiated by the secretion of IL-12 by macrophages during the innate immune response after *L. monocytogenes* infection (Hsieh et al, 1993; Unanue, 1997). CD4+ derived Th1 cells produce and secrete IL-2, IFN-γ, and TNF-α which are involved in inflammatory reactions (Mosmann and Coffman, 1989); while Th2 cells provide an anti-inflammatory cytokine response via IL-4, IL-5, and IL-10 (Mosmann and Coffman, 1989). *L. monocytogenes* infection produces a mainly inflammatory-type response via the secretion of IFN-γ, TNF-α, and IL-2 (Mosmann and Sad, 1996). A previous study showed that following infection with *L. monocytogenes*, CD4+ Th1 cells co-express IFN-γ, TNF-α, and IL-2 (Mielke et al, 1993; Freeman and Ziegler, 2005). However, CD4+ derived Th2 cells which can secrete anti-inflammatory cytokines have been detected after *L. monocytogenes* infection (Marzo et al, 2002). IL-4 and IL-5 producing CD4+ T cells were observed by Marzo et al (2002); however a subsequent study by Freeman and Ziegler (2005) showed no IL-4 producing CD4+ T cells. Additionally, Freeman and Ziegler (2005) found no IL-10 producing CD4+ T cells. The cytokine response via CD4+ T cells is necessary for effective control and clearance of *L. monocytogenes*. A suppression or disruption in the CD4+ T cell-

mediated cytokine response could allow for increased bacterial proliferation and tissue invasion.

A CD8+ T cell response is initiated after infection with *L. monocytogenes*. Elimination of *L. monocytogenes* is accomplished by lysis via cytotoxic T cells (Finelli et al, 1999). CD8+ T cell lysis of infected cells contributes to control of bacterial burden (Portnoy et al, 2002). Bacterial proteins are released into the infected cell cytosol via LLO breakdown after the dissipation of the vacuole membrane (Pamer et al, 1997). The major histocompatability complex (MHC) molecule recognizes the proteins and presents an antigen on the host cell surface (Pamer et al, 1997). The cytotoxic T lymphocytes (CTLs) are released in response to the presentation of the antigen. The CTLs recognize the antigen and a response is initiated (Ladel et al, 1994; Egan and Carding, 2000). It is the recognition of the antigen which stimulates lysis via *Listeria*-specific CTLs (Bouwer et al, 1997). The lysis of *L. monocytogenes*-infected host cells disrupts the intracellular replication and spread of *L. monocytogenes*.

L. monocytogenes is capable of causing severe illness in fetuses and neonates; however, the mechanism of infection is unknown. The decreased activity of cytolytic NK cells, cytotoxic T cells, and the suppression of inflammatory mediators during pregnancy could allow for increased bacterial burden and a decreased clearance capacity. An inability to clear the bacteria would allow for increased replication and invasion and in the initiation of an inflammatory response, which subsequently could be deleterious to fetuses and neonates and immunocompromised/suppressed individuals**.**

Pregnancy related immune system alterations

Due to fetal antigens that are present in the maternal circulation during pregnancy, an alteration in the maternal immune system must occur to prevent rejection of the conceptus by the mother (Bonney and Matzinger, 1997; Bianchi et al, 1996; Invernizzi et al 2002). During pregnancy, if the immune system response to fetal antigens is not suppressed, spontaneous abortion could occur. Initially, the maternal immune system during pregnancy was considered a time of overall immunosuppression. Pregnancy is now considered a time of immune system alteration with areas of suppression and areas of upregulation (Sacks et al, 1999). The maternal immune system undergoes an alteration to suppress inflammatory responses and to increase activation of antibody and anti-inflammatory responses.

Innate immune system during pregnancy

The innate immune system is nonspecific and is often considered the primitive aspect of the immune system. The innate immune system differs from the adaptive immune system not only in function but in cell types. Monocytes, macrophages, granulocytes, and natural killer cells are several cell types involved in the innate immune system during pregnancy (Abba and Lichtman, 2005). The innate immune response cell types are also involved in the immune response against *L. monocytogenes*. The complement immune system is active during pregnancy; however, *L. monocytogenes* is an intracellular pathogen and does not elicit a large antibody response, which would activate the complement immune system.

Monocytes/macrophages and granulocytes play a part in the body's initial inflammatory response to infection or invasion. Macrophage and granulocyte activation

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is thought to increase during pregnancy (Luppi, 2003). Both cells act via phagocytosis of the invading organism (Abba and Lichtman, 2005). Granulocytes are activated during pregnancy; however, excessive levels of granulocyte activation have been associated with preeclampsia (Redman et al, 1999). During pregnancy, monocytes/macrophages and granulocytes are present in the maternal circulation. An increase in their numbers and in phagocytosis is seen and some cytokine secretion is activated during normal pregnancy (Sacks et al, 1999).

Natural killer (NK) cells are a cytotoxic group of lymphocytes; NK cells act on the pathogen directly, via cell destruction, and indirectly, via cytokine production (Abba and Lichtman, 2005). The suppression of the cytotoxic effects of NK cells during pregnancy could result in increased pathogen invasion. While the normal cytotoxic function of NK cells in the maternal circulation is suppressed, NK cells are present in the uterus. Approximately 70% of the total leukocytes in the uterine lining are NK cells; uterine NK cells are present during the time of trophoblast invasion and are present in the placenta but disappear starting at mid-gestation (Sacks et al, 1999; Moffett-King, 2002). Initially after microbial exposure, NK cells are a major source of IFN-γ (French and Yokoyama, 2003). It has been hypothesized that the ability of NK cells to recognize human leukocyte antigens (HLA) class 1 in the placenta blocks the normally cytotoxic effect of the cells and allows for conceptus tolerance (Moreau et al, 1998). NK cells express an inhibitory receptor for HLA class 1, CD94/NKG2A, which could inhibit lysis of maternal and fetal tissues (King et al, 2000; Haedicke et al, 2000). Additionally, trophoblasts are resistant to lysis via NK cells (Santoni et al, 2007).

Adaptive immune system during pregnancy

In pregnant women, the adaptive immune system functions to target specific antigens; the system requires more time to become activated but the response is more specific than the initial innate immune response. The adaptive immune system has two main cell types, B cells and T cells. B cells are responsible for the antibody response of the adaptive immune system while T cells are involved with cytokine production and cytotoxicity.

B cells are the main producers of antibodies. After antigen recognition, B cells secrete antibodies specific for the antigen. B cell frequency is unaltered during pregnancy (Luppi et al, 2002; Brabin and Perrin, 1985). Instead of the normal inflammatory response after pathogen invasion, it has been suggested that during pregnancy an antibody response is activated (Luppi, 2003).

T cell immune activity is altered during pregnancy. The difference in T cell population during pregnancy could affect maternal ability to eliminate pathogens. Several types of T cells are produced by the immune system, including CD4+ and CD8+ (Abba and Lichtman, 2005). Some studies have shown a generalized increase in the number of CD8+ T cells when compared to CD4+ T cells during pregnancy (Luppi, 2003; Piccinni, 2006). However, CD8+ T cell cytotoxic function via CTLs is decreased during pregnancy which could result in increased susceptibility to pathogens. A decreased lysis of infected host cells due to a decrease in CTL activity could result in increased bacterial replication and spread. Additionally, a decrease in CD4+ T cell activity during pregnancy could result in an impaired immune response to an increased bacterial burden.

Two main subsets of CD4+ T helper cells exist, Th1 (inflammatory) and Th2 (anti-inflammatory), each with different roles in immune response (Mosmann and Sad, 1996). Each T helper subset secretes specific cytokines, some of which are necessary for pregnancy maintenance and some are detrimental to pregnancy. An alteration in the normal Th1 and Th2 cytokine production must occur for the maintenance of a successful pregnancy (Raghupathy, 2001). A shift in the production of Th1 cytokines to increased production of Th2 cytokines was detected in pregnant women in the first and second trimester when compared to nonpregnant women (Kruse et al, 2000). The highest ratio corresponded with the second trimester; however, Th1 cytokines were present during the third trimester at decreased levels (Kruse et al, 2000).

Th1 cells secrete several cytokines that are altered during pregnancy, including IFN-γ, TNF-α, and IL-2 (Raghupathy, 2001). IFN-γ is necessary for the maintenance of the placenta during pregnancy (Gustafson et al, 2006). Th1 type cytokines are elicited during the immune response and function to activate macrophages and react to infection, mainly with intracellular pathogens; Th2 type cytokines are mainly used in association with antibody response, usually after infection from extracellular pathogens (Lucey et al, 1996). IL-4, IL-5, IL-6, and IL-10 are four Th2 type cytokines that are associated with antibody production during pregnancy (Lucey et al, 1996; Wegmann et al, 1993).

In human and animal studies, specific cytokines are measured to determine the pregnancy-associated cytokine shift. In mice, IL-2 secretion is decreased while IL-4 and IL-6 production is increased (Dudley et al, 1993). In humans, a significant decrease in IL-2 production occurs. A decrease in IFN-γ secretion also occurs along with a significant increase in the production of IL-10 (Kruse et al, 2000; Hanna et al, 2000).

After pathogen exposure, an induced alteration in the normal pregnancy cytokine secretion could be deleterious to the fetus.

During pregnancy, specific cell types from both innate and adaptive immune responses are altered in quantity and activity to avoid fetal rejection. A decrease in the cytolytic function of NK cells could impair the control of bacterial burden early during infection. Additionally, the suppression of inflammatory cytokines and cytotoxic T cell activity could result in the inability to effectively clear the bacteria from infected organs.

Apoptosis

Apoptosis, also called programmed cell death, is an active process during development that is induced by physiological signals; however, apoptosis can be initiated in response to cell injury. Two separate signaling pathways can be activated, the receptor mediated (extrinsic) pathway or the mitochondrial (intrinsic) pathway.

The receptor mediated pathway is activated by extrinsic signals binding to receptors and initiating the death induced signaling (Mirkes et al, 2002) (Figure 2.2). This pathway can be activated by specific ligand-receptor binding, including Fas ligand (FasL), tumor necrosis factor -related apoptosis inducing ligand (TRAIL), Apo 3 ligand (APO-3L), and APO-2L. The ligand-receptor binding results in the activation of caspase 8. Extrinsic signals could also be initiated by infection resulting in unregulated apoptosis; *L. monocytogenes* induces the expression of FasL on T cells (Zenewicz et al, 2004) possibly leading to unregulated apoptosis. Programmed cell death via the receptor mediated signaling pathway is necessary during development; the activation of caspase 8 is essential for normal heart development (Varfolomeev et al, 1998).

The mitochondrial pathway is activated after the initiation of Bax on the surface of the mitochondria; however, the intrinsic signal which results in Bax initiation is still unknown. The initiation of Bax results in the release of cytochrome c from the mitochondria (Adams and Cory, 1998). Cytochrome c activates apoptosis protease-activating factor 1, which allows for the formation of an apoptosome and the subsequent activation of caspase 9 (Cecconi et al, 1998) (Figure 2.3). During development, programmed cell death via the mitochondrial signaling pathway is necessary for normal CNS development (Kuida et al, 1998).

DNA insult is also capable of initiating both the receptor mediated pathway and the mitochondrial pathway via the activation of p53 protein (Uberti et al, 1999). The p53 protein can increase the expression the FAS protein and Bax resulting in the activation of either the receptor mediated or mitochondrial pathways (Egle et al, 1997). However, radiation during pregnancy has been shown to induce digital defects via a p53-depedent apoptosis (Wang, 2001).

While both signaling pathways operate independently of each other, both end with the activation of caspase 3; caspase 8 and caspase 9 each activate caspase 3 (Gross et al, 1999). After caspase 3 initiation, endonucleases cause the chromatin to become condensed which leads to DNA fragmentation (Figure 2.4). The degradation of cellular proteins, via caspase 3 initiation, results in cell shrinkage and the formation of apoptotic bodies, which are membrane-encapsulated (Figure 2.4). The apoptotic bodies are removed via phagocytosis by macrophages or surrounding cells.

Figure 2.2: Receptor-mediated pathway (Adapted from Mirkes et al, 2002).
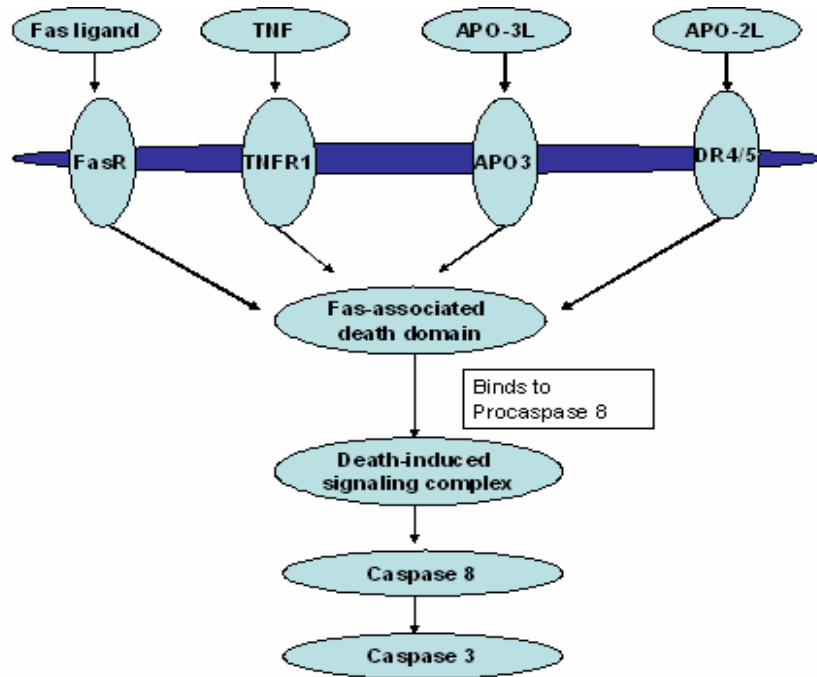

Figure 2.3: Mitochondrial pathway (Adapted from Mirkes et al, 2002).
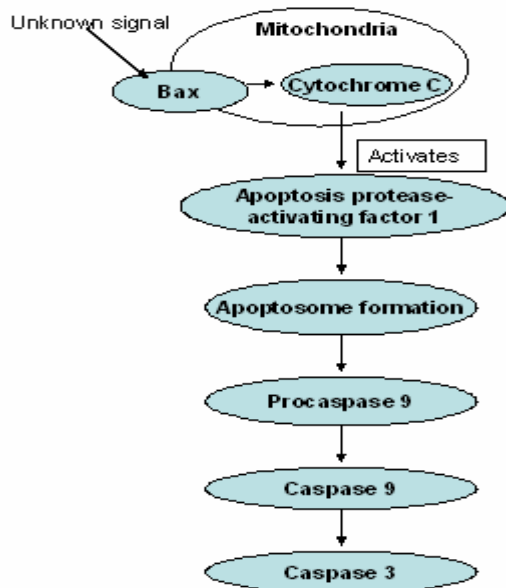
Figure 2.4: Caspase 3 initiation via caspase 8 or caspase 9 and subsequent cellular events (Adapted from Mirkes et al, 2002).
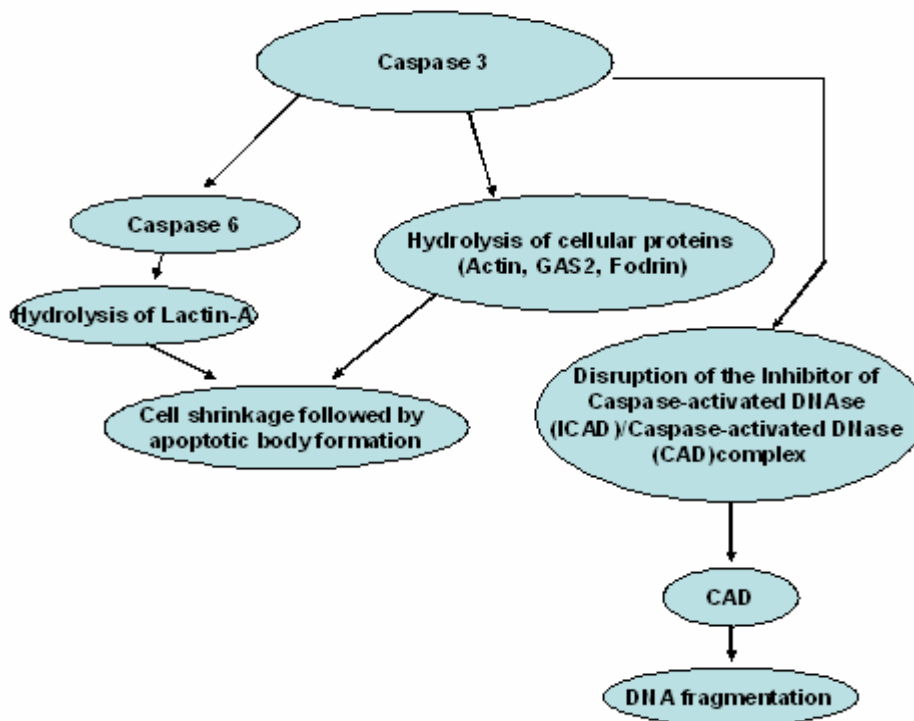
   While programmed cell death is a naturally occurring process during

development, unregulated programmed cell death, as seen with teratogen exposure, has

been associated with abnormal development (Mirkes et al, 2002). The teratogen

thalidomide induces limb anomalies via both the FasL receptor pathway and the

mitochondrial pathway (Knobloch et al, 2008). Additionally, pathogen initiated

apoptosis during pregnancy, possibly via the stimulation of FasL as seen with *L.*

monocytogenes (Zenewicz et al, 2004), could lead to unregulated apoptosis in the fetus or

the placenta. Unregulated apoptosis in the placenta could result in placental degradation,

possibly leading to a disruption in functions at the maternal-fetal interface.

Placenta

The placenta serves as an important factor in fetal growth and development by providing nutrients to the fetus, removing waste products, and providing a protective barrier against some harmful products. After fertilization, fetal cells begin to form connections with maternal blood vessels to divert blood flow from the uterus to the placenta (Red-Horse et al, 2004). The placenta barrier consists of four main layers which separate maternal and fetal circulation, the syncytiotrophoblasts, cytotrophoblasts, connective tissue of the chorionic villous, and the fetal capillaries (Red-Horse et al, 2004). The placenta can be an area of infection after pathogen exposure. *L. monocytogenes* is capable of invading syncytiotrophoblasts and replicating in the placenta villi (Lecuit et al, 2004)

The majority of maternal and fetal exchange occurs in the connective tissue of the chorionic villous. Syncytiotrophoblasts line the intervillous space between the maternal and fetal compartments. The maternal blood and feto-placental blood are separated by a layer of syncytiotrophoblasts (Kingdom et al, 2000). Cytotrophoblast cells are the only trophoblast cell population that is mitotically active, allowing for cell renewal during gestation (Redline, 1997). The cytotrophoblast layer is connected to the syncytiotrophoblast layer. The cytotrophoblast cell layer can disappear in some areas during gestation, resulting in a reduction of the placenta to three layers (Kingdom et al, 2000). Because of the importance of the placenta for normal fetal development, it is an area of interest for pregnancy-related infections.

Placental apoptosis

Apoptosis is a natural process in the placenta (Huppertz et al, 1998). Apoptosis, via the apoptotic cascade, occurs in the cytotrophoblast layer to allow for syncytial fusion (Huppertz et al, 2006). The syncytial fusion of cytotrophoblasts occurs continuously throughout pregnancy and allows for the growth and maintenance of the syncytiotrophoblast layer (Huppertz et al, 2006). The release of the apoptotic material, termed syncytial knots, into the maternal circulation does not initiate an inflammatory response due to the apoptotic bodies being surrounded by an intact, sealed plasma membrane (Huppertz et al, 1998). While apoptosis is a naturally occurring event in the placenta during gestation, abnormalities in the occurrence of apoptosis and in the shedding of apoptotic materials could present a danger to the fetus. Preeclampsia and intrauterine growth restriction have been linked to abnormal apoptosis in the placenta (Smith et al, 1997; Allaire et al, 2000).

Placental apoptosis during infection

Maternal infection during pregnancy can lead to disruption in the normal regulation of apoptosis in the placenta. Human cytomegalovirus has been implicated as a causative agent in fetal death and mental retardation (Chan and Guilbert, 2005). Human cytomegalovirus has been shown to induce apoptosis of villous trophoblasts and initiate the accumulation of monocytes resulting in placental villitis (Chan et al, 2002; Chan and Guilbert, 2005). Infection with *Chlamydia trachomatis*, an intracellular pathogen, is known to cause early pregnancy loss and premature delivery. The bacteria have been shown to cause apoptosis during infection (Miyairi and Byrne, 2006). Additionally,

trophoblast apoptosis induced via Toll-like receptor 4 stimulation may lead to placental degradation during maternal *Chlamydia* infection (Equils et al, 2006).

Placental immune status during normal pregnancy

Cytokine production in the placenta is essential for regulation and maintenance of the maternal-fetal interface. During gestation, the types and functions of cells in the different layers of the placenta vary. Placental macrophages, syncytiotrophoblasts, and cytotrophoblasts are capable of cytokine production in the placenta (Mouzon and Guerre-Millo, 2006).

Different cell types play a role in the production and secretion of inflammatory cytokines in the placenta during gestation. While the inflammatory cytokines are necessary during gestation, abnormal activation of inflammatory cytokines prior to term has been associated with pre-term labor (Mitchell et al, 1991). During the first trimester, endometrial granulated lymphocytes are a large part of the lymphocyte population of the decidua, the maternally-derived portion of the placenta, with the lymphocytes displaying natural killer cell type activities including cytokine secretion (Bulmer et al, 1988; Nagler et al, 1990). Macrophages are responsible for some cytokine secretion (Vince et al, 1992); however, the majority of inflammatory cytokine secretion is done by trophoblasts (Steinborn et al, 1998). IL-2 has been identified in the placenta throughout gestation (Boehm et al, 1989); however, IL-2 RNA expression was weak during the first trimester (Bennett et al, 1998). In the placenta, IFN-γ, TNF-α, and IL-1 have been identified in the first, second, and third trimesters (Paulesu et al, 1991; Pijnenborg et al, 1998; Librach et al, 1994; Paulesu et al, 1991). IL-1β is the main form of IL-1 found in the placenta (Taniguchi et al, 1991). While TNF-α and IL-1β are secreted by the placenta throughout

gestation, the concentration appears to decrease as pregnancy continues (Pijnenborg et al, 1998; Paulesu et al, 1991). Any disruption in normal inflammatory cytokine secretion could produce deleterious effects for the placenta and the fetus.

Anti-inflammatory cytokine production in the placenta occurs throughout gestation (Piccinni et al, 1998; Saito, 2000). IL-4 has been shown to be present in the placenta throughout pregnancy (Haynes et al, 1993; De Moraes-Pinto et al, 1997). Additionally, it has been suggested that IL-5 is present early during pregnancy (Saito et al, 1993). In previous studies, IL-10 has shown regulatory properties in the placenta throughout gestation (Bennett et al, 1999). In a previous study, cytotrophoblasts were obtained from first, second, and third trimester human placentas (Roth et al, 1996). Bioactive IL-10 was produced from the cytotrophoblasts throughout gestation; however, cells from different pregnancies did produce variable amounts of IL-10 (Roth et al, 1996). It has been hypothesized that the placental production of anti-inflammatory cytokines, including IL-5 and IL-10, suppresses the production of placental inflammatory cytokines (Wegmann et al, 1993). The disruption of the normal production and secretion of anti-inflammatory cytokines could allow for an increase in the production of deleterious inflammatory cytokines.

Interaction of apoptosis and cytokines

Pathogen invasion during pregnancy can cause deleterious effects to the placenta by inducing apoptosis and inflammatory responses (Mor and Abrams, 2003). *L. monocytogenes* infection during pregnancy has been shown to cause an increase in apoptosis in the placenta (Irvin et al, 2008). One possible mechanism is via the increase in Fas ligand, which is an initiator of the receptor-mediated signaling pathway for

apoptosis. Specific virulence factors secreted by *L. monocytogenes*, LLO and PI/PL-

PLC, have been shown to induce cell surface expression of Fas ligand (Zanewicz et al,

2004). An increase in apoptosis in the placenta could result in inefficient clearing of

apoptotic bodies, resulting in secretion of inflammatory cytokines, IFN-γ and TNF-α. An

increase in secretion of IFN-γ and TNF-α might induce unregulated apoptosis (Figure

2.5; Mor and Abrams, 2003). Changes in the placenta cytokine milieu and apoptosis

during infection could lead to placental degradation and fetal distress.

Figure 2.5: Pathogen effects on apoptosis and cytokine secretion (Adapted from Mor and

Abrams, 2003)
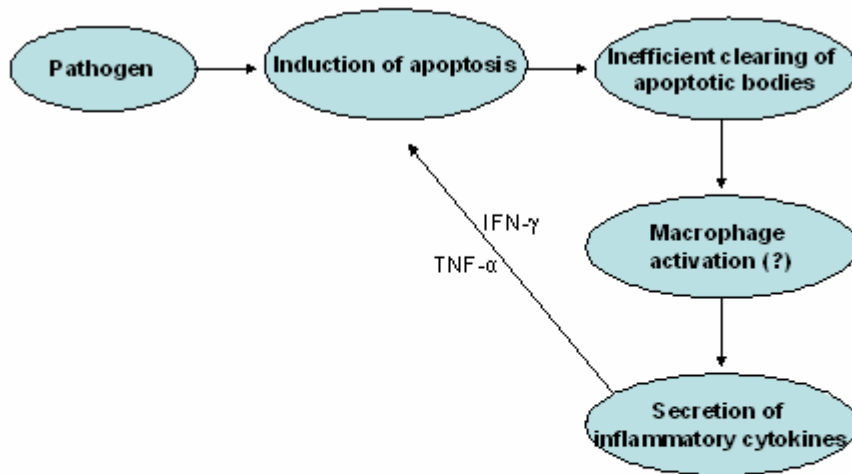
Placental cytokine production during infection

Infections during pregnancy are known to cause deleterious effects to the fetus, including miscarriage, stillbirth, and neonatal illness. However, the mechanisms of fetal effects are largely unknown. Some research has shown that during pregnancy, alterations in normal cytokine secretion in the placenta could lead to abortion. In a pregnant mouse model of *Brucella abortus* infection, it was shown that increased production of IFN-γ contributed to abortion (Kim et al, 2005). Similarly, it has been hypothesized that IFN-γ in the placenta helps control infection with *Chlamydophila abortus* during human pregnancy but could be detrimental to fetal survival (Entrican, 2002). Maternal secretion of inflammatory cytokines has been correlated with unexplained recurrent abortions (Makhseed et al, 2001).

L. monocytogenes is able to invade and infect the feto-maternal barrier (Lecuit et al, 2004; Spencer, 1987). However, little is known about placental cytokine production during human listeriosis. In animal models of pregnancy-associated *L. monocytogenes* infection, the placenta reacts to the bacterial invasion via cytokine secretion. In an IV inoculated mouse model, the secretion of Th1 type cytokines, TNF-α and IFN-γ, was sufficient to control infection within the placenta (Barber et al, 2005). TNF-α and IFN-γ are produced and function to control the bacteria in the placenta; however, the mechanism is still unknown (Barber et al, 2005). In contrast, one study using IV inoculated mice showed increased levels of TNF-α and IFN-γ in the placenta; however, necrotic lesions associated with the bacteria were present (Abram et al, 2002). The cytokine production was not sufficient to control infection, and successful pregnancy outcome was decreased (Abram et al, 2002). While the placenta mounted a response to

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infection with *L. monocytogenes* via cytokine secretion, pregnancy-associated stillbirths occurred with the bacteria being cultured from the fetuses. Unfortunately, the animal models investigating placental cytokine secretion after *L. monocytogenes* infection were exposed through IV inoculation. Because the primary route of exposure in humans is through ingestion of contaminated foods and *L. monocytogenes* is an intracellular pathogen, an appropriate pregnant animal model is needed to investigate the effects of placental cytokine secretion after oral exposure to *L. monocytogenes*.

Project Rationale

Listeria monocytogenes (*L. monocytogenes*) infection is known to cause spontaneous abortions and stillbirths in pregnant women. Non-human primates have been shown to be an appropriate model for human *L. monocytogenes*-induced stillbirths, however, the expensive nature of primate research is limiting. Guinea pigs are proving to be a good model for *L. monocytogenes*-induced stillbirths, and more invasive studies can be done with guinea pigs (Williams et al, 2007).

The role of the placenta during infection with *L. monocytogenes* is unknown. The maintenance of normal maternal and placental cytokine secretion is very important for a successful pregnancy. A disruption in maternal secretion of inflammatory and anti-inflammatory cytokines during pregnancy has been correlated with spontaneous abortions (Raghupathy et al, 2000). If *L. monocytogenes* infection occurs during pregnancy, an increase or decrease in the cytokine secretion could be detrimental to the fetus. The placental cytokine concentrations could also be affected and could increase the possibility of harm to the fetus. Many factors are needed for a successful pregnancy, and disruption of the finely balanced system can cause or allow harm to the fetus.

L. monocytogenes could also affect the fetus directly. Gastrointestinal E-cadherin receptors allow *L. monocytogenes* to cross the intestinal barrier. For fetal infection to occur, *L. monocytogenes* must transmigrate the placental barrier and infect the fetus. Blood-borne *L. monocytogenes* is capable of infecting the placenta through internalization via E-cadherin receptors expressed on syncytiotrophoblasts (Lecuit et al, 2004).

Hypothesis and Specific Aims

The placenta is necessary for fetal survival. It is an organ that delivers nutrients to the growing fetus and removes wastes. *L. monocytogenes* must transmigrate across the placental border for fetal infection to occur. It is unknown whether placental degradation occurs during this process and if it is dose-dependent. Regulation of maternal serum cytokine levels and placental cytokine expression is necessary for normal pregnancy to occur. A serious infection could disrupt the maternal serum cytokine balance and placental cytokine expression resulting in harm to the fetus.

Hypothesis: Maternal infection with *L. monocytogenes* produces a dose response of deleterious effects in maternal and placental tissue and an alteration in placental cytokine expression.

Specific Aims

1. To determine effects of infection with *L. monocytogenes* on apoptosis and necrosis in the placenta.

2. To characterize the effects of infection with *L. monocytogenes* on maternal circulating serum cytokine levels and determine if a dose-dependent relationship occurs.

3. To determine the differences in cytokine expression in the placenta during infection with *L. monocytogenes*.

4. To determine whether antibodies to human E-cadherin will bind to guinea pig E-cadherin.

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CHAPTER 3

LISTERIA MONOCYTOGENES INFECTION IN PREGNANT GUINEA PIGS IS
ASSOCIATED WITH MATERNAL LIVER NECROSIS, A DECREASE IN
MATERNAL SERUM TNF-α CONCENTRATIONS, AND AN INCREASE IN
PLACENTAL APOPTOSIS [1]

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[1] Irvin, E.A., D. Williams, K.A. Voss, and M.A. Smith. Submitted to *Reproductive Toxicology*, 01/25/2008.

Abstract

Listeriosis, the result of infection with *Listeria monocytogenes,* primarily targets fetuses and pregnant women, elderly, and immunocompromised persons. Fetuses and pregnant women account for one-third of the 2500 cases annually. The objectives were to determine the dose dependent trends of immunological and pathogical effects in pregnant guinea pigs after infection with *L. monocytogenes*. Timed pregnant guinea pigs were treated on gestation day (gd) 35 with oral doses of 10^4 to 10^8 CFUs and sacrificed on gd 56. Hepatic lesions were found in dams treated with $\geq 10^5$ CFUs. Apoptosis was detected in significantly more placentas from dams treated with $\geq 10^6$ CFUs compared to controls. Maternal serum TNF-α concentrations were significantly decreased in all dose groups compared to controls. In conclusion, increases in premature delivery, maternal hepatic effects and placental apoptosis along with a decrease in TNF-α concentrations were associated with *L. monocytogenes* infection in pregnant guinea pigs.

Introduction

Listeriosis is the clinical manifestation of *Listeria monocytogenes* (*L. monocytogenes)* infection. In the United Stated, it is estimated that 500 deaths occur each year due to listeriosis (1). The primary route of exposure in humans is through the consumption of contaminated foods. Ingestion of *L. monocytogenes* by pregnant women carries the risk of feto-placental transmission (2). The mother may be asymptomatic or may experience a slight fever while the fetus suffers more serious consequences, such as stillbirth or septicemia of the neonate (3, 4).

To gain entry into the systemic circulation after oral ingestion, *L. monocytogenes* must cross the intestinal barrier. A specific protein receptor, E-cadherin, is used to mediate gastrointestinal invasion and transmission of *L. monocytogenes* across the intestinal barrier (5). The amino acid sequence in the active site of E-cadherin is identical in humans and guinea pigs (6). After exposure to 10^6 or greater CFU *L. monocytogenes*, pregnant guinea pigs have the same etiology and pregnancy outcome (7) as humans (4).

 In pregnant guinea pigs, maternal liver invasion appears to occur prior to fetal infection after oral treatment with *L. monocytogenes* (7). Because maternal exposure to *L. monocytogenes* and subsequent liver invasion can lead to fetal infection, placental transmigration of *L. monocytogenes* is an essential step in *L. monocytogenes*-induced stillbirths. Humans have a hemochorial placenta and guinea pigs are the rodent species with the most similarity to human placentas (8). Because of the importance of the maternal-fetal interface and placental transmission of *L. monocytogenes*, the guinea pig's similarity to the human placenta is very important. In addition, placental infection and degradation may be an important aspect of infection-induced stillbirths. Previously,

Toxoplasma gondii, an intracellular parasite, has been shown to cause apoptosis in placental trophoblasts after infection (9). The induction of apoptosis within the placenta following infection could result in increased pathogen transmission to the fetus.

After pathogen exposure, the immune system's ability to recognize and target the pathogen is necessary to prevent systemic infection. The immune system is altered during pregnancy, likely to prevent allograft rejection. It has been suggested that after exposure to an infectious pathogen during pregnancy, a B cell antibody response is elicited instead of an inflammatory response, which occurs in non-pregnant individuals (10). Two subsets of cytokines elicit different responses after production. T helper type-1 (Th1) cytokines induce an inflammatory response which can be harmful to the developing fetus. Specific Th1 cell products have been characterized as inflammatory mediators; including interleukin (IL)-2, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α. In contrast, T helper type-2 (Th2) cytokine activation results in an anti-inflammatory response. Th2 cytokine anti-inflammatory response is elicited via B cell antibody production (11). IL-4, IL-5, IL-6, and IL-10 are Th2 cytokines that are present throughout pregnancy (12, 13).

The purpose of this study was to investigate the maternal and fetal responses after oral exposure to *L. monocytogenes* using timed pregnant guinea pigs. Previously, pregnant guinea pigs and primates have been used to develop a dose-response model for *L. monocytogenes*-induced stillbirths. In both animal models, the estimated LD50 for fetal mortality was approximately 10^7 CFUs *L. monocytogenes* (7, 14), which is similar to an estimated LD50 of 1.9 x 10^6 CFUs from a human outbreak of listeriosis (15). However, no studies using pregnant guinea pigs have been done to determine the effects

of infection on the maternal immune system and the possible consequences to the fetus. Our objectives were to (1) determine the effects of infection on the maternal liver; (2) characterize the effects of infection on the maternal immune system; and (3) determine the effects of infection on apoptosis in the placenta.

Materials and Methods

Animals

Forty timed pregnant guinea pigs were purchased from Elm Hill breeding laboratories (Chelmsford, MA) on gestation day (gd) 28 and were housed at the University of Georgia (UGA) animal facility. For details on animal husbandry and treatment, see Williams et al (7). Briefly, animals were housed individually with air filters fitted on each cage, and control animals were housed in a separate room from treated animals. The animals were provided sterilized water and chow and were maintained on a 12 hour light/12 hour dark cycle. Guinea pigs were allowed a one-week acclimation period before treatment. After treatment, the animals were observed daily for any overt changes in food consumption, fecal output, and behavior, and the animals were weighed once a week. Pregnancy was allowed to continue normally until sacrifice on gd 56 or until premature delivery occurred. All animal work was approved by University of Georgia's IACUC committee, and was done in compliance with NIH guidelines and the Animal Welfare Act.

Preparation of Inoculum

Details of *L. monocytogenes* preparations were described in our earlier study (7). Briefly, *L. monocytogenes* cells (strain 12443) were activated by three successive transfers to 10 ml of tryptic soy broth (Difco Laboratories; Detroit, MI) and incubated at

35°C for 24 hours. Cultures were then harvested by centrifugation (9,000 x g at 4°C for 30 min), washed twice and resuspended in sterile phosphate buffer saline (PBS, pH 7.2). The number of *L. monocytogenes* cells in the inoculated sample was determined by serially diluting the cell suspension in PBS (0.01M) and plating onto *Listeria* Selective Agar (Oxoid; Ogdenburg, NY). The cell populations obtained were used to confirm the colony forming units per ml (CFU/ml) of *L. monocytogenes* administered to the guinea pigs.

Treatments

Animals were treated with a *L. monocytogenes* strain that was isolated from a spontaneously occurring rhesus monkey stillbirth (strain 12443). The details of the treatment and inocula preparation are described in Williams et al (7). Briefly, *L. monocytogenes* was administered to the guinea pigs in sterilized whipping cream sweetened with 0.5g Splenda® to make it more palatable. Two consecutive days prior to treatment with *L. monocytogenes,* the guinea pigs were trained to ingest the vehicle by feeding 5 ml of the treatment vehicle using a transfer pipette. Controls animals were given sterilized whipping cream with 0.5g Splenda®.   The animals were fed a dose of 0, 10^4, 10^5, 10^6, 10^7, or 10^8 CFUs *L. monocytogenes* strain 12443 in 5 ml sweetened whipping cream on gd 35.

Sample and Tissue Collection

Animals were sacrificed by carbon dioxide asphyxiation at gd 56. If premature labor occurred prior to date of sacrifice, the dam was euthanized at time of delivery and routine samples were collected. Upon necropsy, visible signs of infection in the dam, such as hepatic lesions, and fetal viability were noted. Each placenta was sectioned to

obtain a representative sample, and sections were flash frozen for immunological and pathological analysis or fixed in formalin for histological examination. Upon sacrifice, maternal and fetal blood was collected by cardiac puncture. Whole blood was centrifuged at 2500 x g for 25 minutes at 4°C. Serum was collected, aliquoted (100 μL), and stored at -20°C.

Fetal viability

Fetal viability at the time of sacrifice was based on several criteria: physical characteristics, including eyelid skin formation and opening of the eyelid; skin color and formation; hair growth; pinna formation; and the appearance of placental blood and cord blood. The appearance of cyanosis was also noted.

Histology

Maternal liver samples were fixed in 10% neutral buffered formalin for histological examination. Samples were paraffin embedded, and sections of 4-5 μm placed on slides. Slides were stained with hematoxylin and eosin. Liver samples were scored on two criteria, extent of necrosis and appearance of mononuclear cells. Slides were scored without knowledge of identity and treatment (Table 3.2).

Humoral immune response

To investigate maternal antibody response following infection with *L. monocytogenes*, antibody titer levels against listeriolysin O (LLO) were measured. The antibody response was measured as described in Smith et al (16), with a few modifications, including the use of an antibody against LLO. For plate preparation, 100 μL of diluted listeriolysin O antigen (FDA, Bethesda, MD) was added to each well of Immulon 1 Flat Bottom ELISA plates (Thermo LabSystems, Franklin, MA). The plates

were incubated overnight at 4°C; fluid was aspirated and washed three times with PBS (0.01M). Antibody titers were determined by incubating the plates with two-fold serial dilutions of serum (100μL/well). Serum dilutions were made in PBS containing 5% heat-inactivated fetal bovine serum and 0.05% Tween 20 (Biorad, Hercules, CA). The plates were incubated at room temperature for 2 hours and then washed with PBS with 0.1% Tween 20. Bound antibody was detected by incubating the plates for 2 hours at room temperature with 100μL of a 1/8000 dilution of peroxidase-conjugated donkey anti-guinea pig immunoglobulin G (Jackson Immuno Research, West Grove, PA). Titers were normalized to a baseline value derived from the absorbance obtained with a 1:400 dilution of normal (control) guinea pig serum at a wavelength of 450 nm.

Measurement of serum cytokine levels

To determine the maternal cytokine response, serum cytokine levels were measured using a mouse enzyme-linked immunosorbent assay (ELISA) (R & D Systems Minneapolis, MN). Previously, mouse ELISAs have been used to analyze guinea pig serum for cytokine determination due to the lack of guinea pig specific reagents (17, 18). Tumor necrosis factor (TNF)-α, interleukin (IL)-4, and IL-10 concentrations in maternal serum were quantified using commercially available mouse kits following the manufacturer's protocol using a cytokine specific polyclonal antibody. The optical density was measured using a μQuant microplate spectrophotometer (Bio-Tek Instruments, Winooski, VT) at 450 nm using a wavelength correction of 540 nm. A standard curve was generated using a four parameter logistic curve fit with KC4 version 3.0 (Bio-Tek Instruments, Winooski, VT).

A commercially available mouse cytokine/chemokine Linco*plex* kit (Linco Research, St. Charles, MO) was also used to measure cytokines. Cytokines analyzed were granulocyte macrophage colony-stimulating factor (GM-CSF), IFN-γ, IL-12p40, IL-12p70, IL-13, IL-17, IL-1a, IL-1b, IL-2, IL-3, IL-5, IL-6, IL-9, keratinocyte-mediated chemokine (KC), monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1b (MIP-1b), regulated upon activation normal T-cell expressed and secreted protein (RANTES), and vascular endothelial growth factor (VEGF). Briefly, a 1:1:1 solution of assay buffer (PBS with 0.08% sodium azide and 1% BSA), standards or samples, and antibody-immobilized beads were added to the microplate. A biotinylated antibodies solution was added; a strepavidin-phycoerythrin conjugate was used to bind to the antibodies. The microparticles were resuspended in sheath fluid (Luminex Corporation, Austin, TX) and the concentrations were determined using the Luminex$^{\circledR}$ 100 analyzer (Luminex Corporation, Austin, TX). Analysis of the cytokines using the Linco*plex* kit was completed in the laboratory of Dr. Steven Offenbacher, University of North Carolina at Chapel Hill, NC.

Determination of placental apoptosis

Apoptosis in the placenta was determined by PCR. DNA was extracted using a DNeasy mini tissue kit according to the manufacturer's protocol (Qiagen Inc, Valencia, CA). Briefly, a 25 ± 1 mg section of placenta was placed in a sterile, DNA-free centrifuge tube and was subjected to mechanical grinding. Lysis buffer and proteinase K were added and mixed by vortexing. The sample was incubated at 55°C for a minimum of three hours in a shaking water bath. A DNA binding buffer was added to the sample and mixed thoroughly. The sample was incubated at 70°C for ten minutes. DNA was

collected through a series of three washes and a final elution and centrifugation. The optical density was measured using a spectrophotometer (BioPhotometer, Eppendorf, Hamburg, Germany) to determine the amount of genomic DNA present.

Apoptosis was analyzed using a commercially available apoptosis-specific PCR kit, DNA Ladder Assay (Maxim Biotech Inc, Rockville, MD). The ligation mix was added to 1 μg genomic DNA and cooled from 55°C to 10°C over one hour. The reaction was incubated at 16°C overnight. Ligated DNA was added to the PCR mixture, a Hot start PCR method was used and Taq Polymerase was added. The recommended temperature profile for PCR reaction (72°C for 10 minutes, 94°C for 1 minute, a 2-step cycle of 94°C for 1 minute and 70°C for 2 minutes was run for 40 cycles, then 72°C for 10 minutes, and 20°C for a minimum of 30 minutes) was completed using a Mastercycler EP (Eppendorf, Hamburg, Germany).

The completed reaction solution was loaded on a 1.75% agarose gel. The gel was run at 100 mV for approximately 1 hour. The gel was viewed under an ultraviolet hood after staining with ethidium bromide, and pictures were taken using a Nikon camera with a 590 lens.

Statistical analysis

Data were analyzed using SAS version 8.2 (Cary, N.C.). Statistical significance between control groups and treated groups for maternal antibody response, cytokine concentrations, and placental apoptosis was calculated using an analysis of variance test (ANOVA, $p \leq 0.05$). Differences between groups were determined by using a Student-Newman-Keuls (SNK) test ($p \leq 0.05$). Comparisons between the number and day of premature delivery were analyzed using a Chi-square test ($p \leq 0.05$).

Results

Of the forty guinea pigs treated with *L. monocytogenes* (doses ranging from 10^4 to 10^8 CFUs), ten animals had adverse pregnancy outcomes. Microbiology analyses of these guinea pigs are reported in Williams et al (7). When premature labor occurred, all fetuses delivered were stillborn. No premature deliveries occurred in guinea pigs treated with $\leq 10^5$ CFUs *L. monocytogenes* (Table 3.1), however two dams treated with 10^5 CFUs *L. monocytogenes* each had one nonviable fetus with viable littermates at the time of sacrifice (gd56), but *L. monocytogenes* was not recovered from the fetuses (7). One dam treated with 10^6 CFUs *L. monocytogenes* had one nonviable fetus at the time of sacrifice with five viable littermates (data not shown).

Premature deliveries resulting in stillbirths occurred at doses of $\geq 10^6$ CFUs *L. monocytogenes* (Table 3.1). The number of days until a guinea pig prematurely delivered a stillbirth was dose-dependent. One dam treated with 10^6 CFUs *L. monocytogenes* delivered stillbirths on gd 55 (average full term for guinea pigs is 65 days). However, three dams treated with 10^7 CFUs had stillbirths at an average of gd 48 (range gd 44-54). At the highest dose tested, 10^8 CFUs, three dams had stillbirths occurring at an average of gd 45, which was significantly earlier when compared to the animals whose pregnancy continued until the time of sacrifice (gd 56) ($p < 0.05$).

At necropsy, maternal livers were observed for gross hepatic lesions. Dams treated with 10^4 CFUs *L. monocytogenes* had no visible hepatic lesions. Focal hepatic lesions (Figure 3.1) were visible at higher doses, and the number of lesions appeared to increase as the dose increased. Microscopic examination of the liver revealed two main findings. There were multiple, randomly distributed round foci of hepatocellular

necrosis. The foci were surrounded by mixed acute, chronic inflammation and fibrosis (Figure 3.2). An increase in the extent of necrosis and chronic inflammation was noted as the dose increased (Table 3.2). Secondly, there appeared to be a dose-related increase in the numbers of mononuclear cells scattered throughout the sections (Table 3.2). Identifying their origin is beyond the scope of this study, but their appearance (round to slightly oval shape, eosinophilic cytoplasm, darkly-stained variably shaped nuclei) indicate that they are likely histiocytes or macrophages of monocyte origin.

Maternal serum samples were collected at the time of sacrifice to determine immunological changes including changes in antibody levels. Maternal *Listeria* antisera titer levels were significantly increased ($p < 0.05$) from the control levels in dams treated with 10^8 CFUs *L. monocytogenes*. However, *Listeria* antisera titer levels in dams exposed to $\leq 10^7$ CFUs *L. monocytogenes* were not significantly altered (Figure 3.3).

In addition to maternal *Listeria*-induced antibody changes, maternal cytokine production was analyzed. An ELISA assay was used to analyze maternal serum for selected cytokines: tumor necrosis factor (TNF)-α, interleukin (IL)-4, and IL-10. TNF-α was significantly decreased ($p < 0.05$) in all dose groups, however all other cytokine levels were not significantly altered in dams treated with *L. monocytogenes* (Table 3.3). In addition, a wide range of pro-inflammatory and anti-inflammatory cytokines were analyzed using the Linco*plex* kit. No significant differences were seen in the maternal serum cytokines analyzed using the Linco*plex* kit (Table 3.3). The lack of significant changes in cytokine concentrations after maternal treatment with *L. monocytogenes* is likely due to large variation between individual animals, or the length of time between infection and sample collection.

Placentas from all dose groups were analyzed to determine whether maternal treatment with *L. monocytogenes* affected placental apoptosis. Some placental apoptosis is normal for late gestation; however the number of placentas with a detectable amount of apoptosis showed a significant increase in dams treated with $\geq 10^6$ CFUs (Figure 3.4). Interestingly, in dams treated with 10^8 CFUs *L. monocytogenes*, all placentas were positive for apoptosis. In addition, when amplified DNA was viewed under UV lighting comparing placentas from treated dams with nonviable fetuses to those with viable fetuses, there was greater intensity staining of the bands in placentas from treated dams with nonviable fetuses (Figure 3.5).

Discussion

Exposure to *L. monocytogenes* can result in spontaneous abortions and stillbirths in humans and other mammals. To develop drug therapies and diagnostic tools for early intervention for human listeriosis during pregnancy, animal models are needed that mimic human disease using common routes of exposure. Previously, primates and mice have been used to study the effects of infection with *L. monocytogenes* on pregnancy. In humans, the bacteria use E-cadherin, an internalinA receptor, to transmigrate the gastrointestinal tract which allows for systemic invasion. Non-human primates appear to be a good model for human listeriosis during pregnancy although the E-cadherin amino acid sequence has not been determined (16). However, nonhuman primates are expensive and invasive studies are not easily done. Mice are less susceptible to *L. monocytogenes*-induced stillbirth after oral exposure, due to a difference in E-cadherin, more specifically a substitution of a glutamic acid for proline at site 16. Interestingly, using anesthesia during inoculation of mice resulted in increased susceptibility to

infection with *L. monocytogenes* than non-anesthetized mice (19). In a previous study, we developed a pregnant animal model for oral exposure to *L. monocytogenes* using timed pregnant guinea pigs (7). In our current study, we investigated the effects of maternal exposure to *L. monocytogenes* on maternal liver, the maternal immune system, and placenta using timed pregnant guinea pigs.

In our study, stillbirth occurred at doses of $\geq 10^6$ CFUs *L. monocytogenes*. At the highest dose tested (10^8 CFUs), dams went into premature labor significantly earlier, an average of gd 45 (range gd 42 – gd 50) compared to sacrifice at gd 56 (post-treatment day 21). This is in agreement with a previous primate study that allowed animals to go to full term after treatment with *L. monocytogenes*. The primates with adverse pregnancy outcomes on average delivered earlier than the animals with normal pregnancy outcomes (14, 16).

After human exposure, the mother will often be asymptomatic until the time of fetal distress or may experience slight nausea or flu-like symptoms (3, 4). In our study, the dams were asymptomatic until the time of premature delivery and this is similar with that seen in our previous study in primates (14, 16).

Grossly visible lesions, confirmed by microscopic examination to be necrotic foci, were seen on the maternal liver at doses of $\geq 10^5$ CFUs *L. monocytogenes*. Listeria-induced liver damage has been shown in IV challenged mouse studies and after human exposure (20, 21). Severe necrotizing hepatitis with multiple hepatic lesions resulted from IV infection of *L. monocytogenes* in pregnant mice (20). In cases of human exposure to *L. monocytogenes*, solitary liver abscesses have been noted (21). Guinea pig dams with multiple necrotic foci were not moribound and did not have an increase in

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serum alanine aminotransferase (ALT) levels (7). Interestingly in human listeriosis, solitary and multiple liver abscesses have been present in patients with an underlying condition, such as diabetes mellitus (22). In these cases of listeriosis, serum ALT levels and aspartate aminotransferase (AST) levels were not helpful in diagnosis (22). However, one study suggests that alkaline phosphatase levels might be a better indicator of *Listeria*-induced liver damage (21).

We measured antibody production against the listeriolysin O (LLO) protein in the circulating maternal serum. At the highest dose of 10^8 CFUs *L. monocytogenes*, maternal antibody titer levels were significantly higher (4-fold), when compared to the control group. Previously, rhesus monkeys showed an eight to 32-fold increase in antibody titer levels from dams with a stillbirth when compared to levels from mothers with a normal birth (16). The lack of response seen in lower dose groups probably results from the limited contribution of humoral immunity to listeric infection. However at the time of stillbirth or sacrifice, high antibody titer levels were seen both in primates and guinea pigs indicating that the maternal immune system is capable of mounting an immune response (16). .

Cell mediated immunity is largely involved in the destruction of intracellular pathogens, including *L. monocytogenes* (23). Th1 cytokine production plays a large role in the cell mediated inflammatory immune response; however, Th1 cytokine production, including TNF-α, IL-2, and IFN-γ, is reduced during pregnancy leading to a shift in the Th1/Th2 cytokine ratio (24, 25, 26). In our study maternal levels of circulating serum cytokines were generally below the detection limit or not significantly altered upon

infection with *L. monocytogenes*, with the exception of TNF-α concentrations which were significantly decreased after maternal treatment with *L. monocytogenes* (Table 3).

For the cytokines analyzed in our study, no guinea pig specific ELISA assays were available, and mouse specific ELISA assays were used. Mouse specific ELISA assays have previously been used to analyze guinea pig serum cytokine levels (17, 18). A guinea pig model of chorioamnionitis induced via cervical inoculation with *E. coli* analyzed maternal serum cytokine concentrations, and similar to our results many samples tested below the minimal detectable limit (17). A guinea pig model of lipopolysaccharide-induced fetal brain injury showed significant increases of maternal TNF-α concentrations using a mouse specific ELISA assay (18). Our study was designed to analyze only one time point, 21 days post-treatment. We are currently examining cytokine levels at earlier post-infection time points to determine temporal changes in cytokine concentrations and analyzing cytokines within the placenta.

Guinea pigs are the rodent species with a placenta most similar to humans, a hemochorial placenta (8). Bakardjiev et al (27) found that the placenta was targeted for proliferation more than other organs after intravenous exposure to the bacteria (27). In our study, the number of placentas positive for apoptosis increases as the dose increases with the three highest doses having a significant increase in the number of placentas positive for apoptosis when compared to the control animals. The increase in degradation could be due to significant infection within the placenta, which could lead to adverse fetal effects. In our previous study (7), the number of placentas from which L. *monocytogenes* is isolated is directly correlated with increasing dose.

In conclusion, our study provides additional information on the pregnant guinea pig as a surrogate model for human listeriosis. With exposures to increasing doses of *L. monocytogenes*, the severity of maternal liver damage increases, the number of placentas with detectable levels of apoptosis increases, and the percent of nonviable fetuses increases. Maternal serum TNF-α concentrations were significantly decreased by infection with *L. monocytogenes*, however all other cytokine levels are not significantly altered at 21 days post-treatment.

Acknowledgements

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Table 3.1: Average day of premature delivery after oral exposure of pregnant guinea pigs to *Listeria monocytogenes*

Dose	Number of dams	Number of premature deliveries	Average post-treatment day of premature deliveries
Control	9	0	-
10^4 CFUs	4	0	-
10^5 CFUs	11	0	-
10^6 CFUs	9	1	20
10^7 CFUs	10	3	14
10^8 CFUs	4	3^a	10^b

[a] statistically significant when compared to control group ($p < 0.05$)
[b] statistically significant when compared to control group ($p < 0.05$) sacrificed at gd 56

Table 3.2: Maternal liver pathology scores for pregnant guinea pigs treated with *L. monocytogenes*

Dose	N	Number of dams with ≥ 1 lesion	Necrosis score[a] (Average ± sd)	Mononuclear cells[b] (Average ± sd)
Control	4	0	0	0.5 ± 0.58
10^4 CFUs	4	0	0	0.75 ± 0.5
10^5 CFUs	4	2	0.75 ± 0.96	1.5 ± 0.58
10^6 CFUs	3	2	1.67 ± 1.53	1.0 ± 1.0
10^7 CFUs	3	2	1.33 ± 0.58	1.67 ± 0.58
10^8 CFUs	2	2	2.0 ± 1.4	2.5 ± 0.7

[a] Necrosis subjectively scored on a 0-3 scale averaged per dose group: 0 = no necrosis present, 1 = few small foci of necrosis with little inflammation, 2 = well developed focal area of necrosis with increased inflammation, 3 = more extensive lesion as described in score 2

[b] Value indicates subjective scoring for the number of mononuclear cells (see text) noted in the sections: 0 = within normal limits; 1 = a minimal to slight increase in the number of cells not involving all fields; 2 = a further increase in number with multiple cells present in most fields; 3 = a noticeably larger numbers of cells present in all fields.

Table 3.3: Maternal serum cytokine concentrations (pg/ml) in pregnant guinea pigs 21 days after treatment with *L. monocytogenes*[a]

Dose	GM-CSF[b] (± sd)	MIP-1β[b] (± sd)	IFN-λ[b] (± sd)	IL-12p70[b] (± sd)	IL-9[b] (± sd)	TNF-α[c] (± sd)	IL-4[c] (± sd)	IL-10[c] (± sd)
Control	24.59 (± 22.4)	41.9 (± 39.5)	1.76 (±0.9)	3.6 (± 0.3)	101.4 (± 16.5)	14.01 (± 16.7)	8.79 (± 7.3)	≤ 2.0[d]
10^4 CFUs	20.3 (± 33.4)	≤ 7.7[d]	≤ 0.3[d]	≤ 3.4[d]	65.5 (± 45.7)	≤ 2.5[d,e]	ND	ND
10^5 CFUs	5.44 (± 6.3)	34.6 (± 14.6)	1.45 (± 1.6)	10.6 (± 9.9)	137.3 (± 74.9)	5.12[d,e] (± 6.9)	2.55 (± 2.4)	19.8 (± 18.1)
10^6 CFUs	9.35 (± 11.8)	≤ 7.7[d]	0.75 (± 0.6)	24.75 (± 38.2)	42.35 (± 54.5)	≤ 2.5[d,e]	7.56 (± 13.1)	45.09 (± 32.9)
10^7 CFUs	24.43 (± 27.8)	40.28 (± 22.5)	4.22 (± 3.0)	8.3 (± 8.2)	71.48 (± 28.1)	3.41[d,e] (± 1.8)	6.89 (±5.6)	37.63 (± 52.3)
10^8 CFUs	≤ 1.0[d]	≤ 7.7[d]	1.13 (± 1.2)	3.6 (± 0.3)	24.2 (± 28.8)	≤ 2.5[d,e]	16.97 (± 8.0)	2.67 (± 1.2)

[a] Each sample was analyzed in duplicate and serum from ≥ 3 dams was analyzed per dose group; all samples recorded in pg/ml
[b] Cytokine levels were analyzed by Linco*plex* (see Methods for details)
[c] Cytokine levels were analyzed by ELISA (see Methods for details)
[d] Cytokines were measured at or below the detection limit; the detection limit was used for statistical comparisons when all samples were less than the detection limit.
[e] Statistically significant ($p < 0.05$) when compared to control
[f] ND=not done

Figure 3.1: Representative pictures from livers of a control dam and of dams treated with *L. monocytogenes*. A: Control animals had no visible lesions present on the liver. B: Liver from a dam treated with 10^5 CFUs *L. monocytogenes* and sacrificed 21 days post treatment; 2 visible lesions on the right lobe. C: Liver from a dam treated with 10^7 CFUs *L. monocytogenes* and sacrificed 21 days post treatment; 5 visible lesions on right lobe. Lesions confirmed by histopathology to be necrotic foci (see Figure 3.2).

Figure 3.2: Maternal livers were examined to determine pathological effects of infection with *L. monocytogenes*. Liver sections were stained with H & E; microphotographs were taken at a 200x magnification. A: Microphotograph of a liver section from a control guinea pig. No areas of chronic inflammation were seen. B: Microphotograph of a liver section from an animal treated with 10^7 CFUs *L. monocytogenes* showing an area of focal necrosis with peripheral chronic inflammation. Necrotic lesions were found scattered throughout the hepatic parenchyma of animals treated with a dose $\geq 10^5$ CFUs *L. monocytogenes*.

Figure 3.3: Antibody titers against listeriolysin-O antigen in sera from guinea pigs 21 days after treatment with *L. monocytogenes*. At the highest dose of 10^8 CFUs *L. monocytogenes*, the titer level was significantly increased ($p \leq 0.05$, $n \geq 3$).
* Statistically significant

Figure 3.4: Placental tissue samples analyzed for apoptosis by PCR. All samples were adjusted to 1μg DNA and subjected to 40 thermacycles. A significant increase in the number of placentas positive for apoptosis was seen in treatment groups 10^6, 10^7, and 10^8 CFUs *L. monocytogenes* compared to the control group. * Statistically significant ($p < 0.05$, $n \geq 3$).
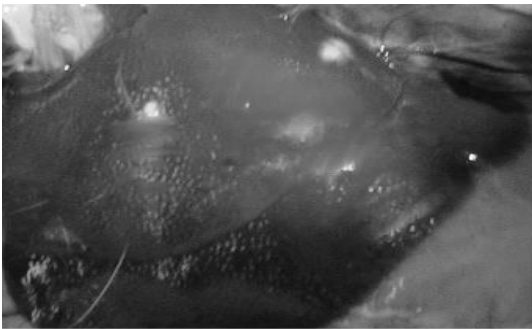
Figure 3.5: Comparison of apoptosis in placentas of viable and nonviable fetuses from dams treated with 10^6 CFUs *L. monocytogenes*. Fetuses A, B, and C were littermates. An increase in banding intensity is seen in lanes 1-3 (nonviable fetuses) compared to lane 4 (viable fetus). A placenta from a control fetus in lane 5 shows no banding.
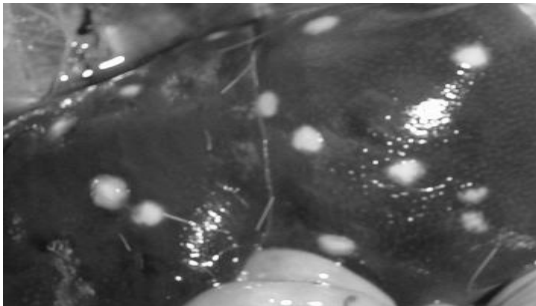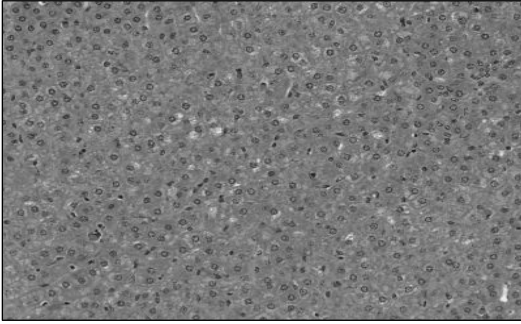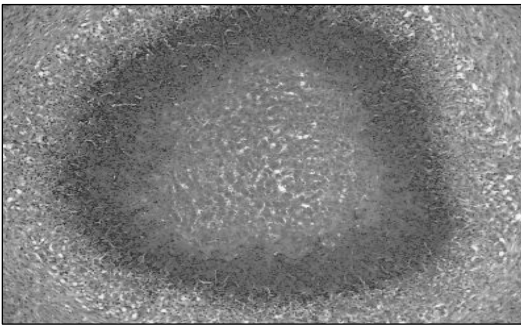
Figure 3.1

A.

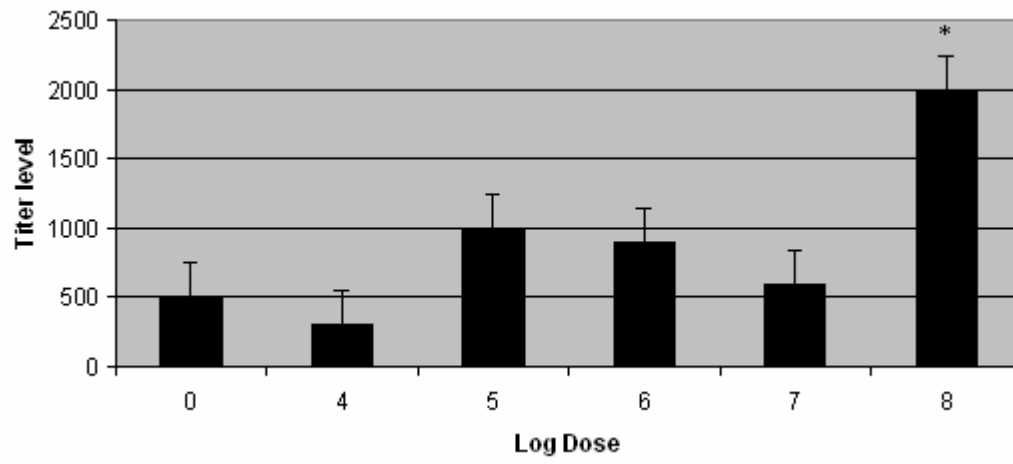
B.

C.

Figure 3.2

A.

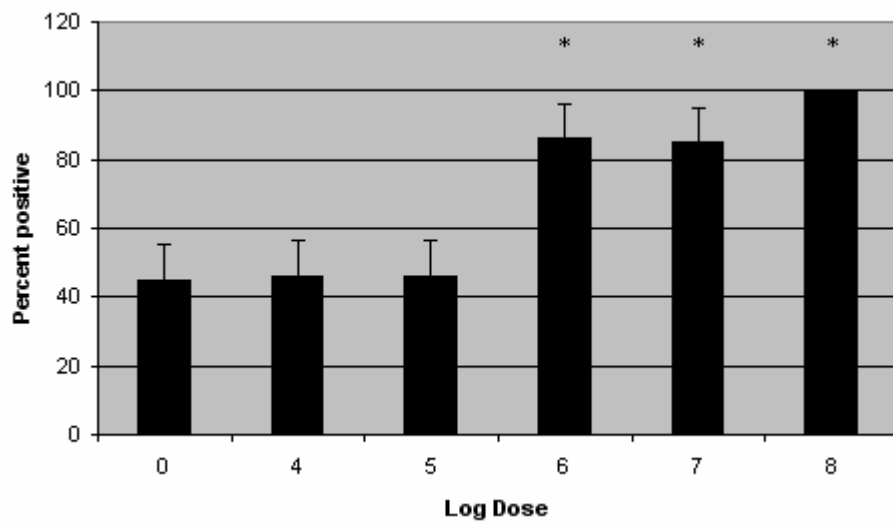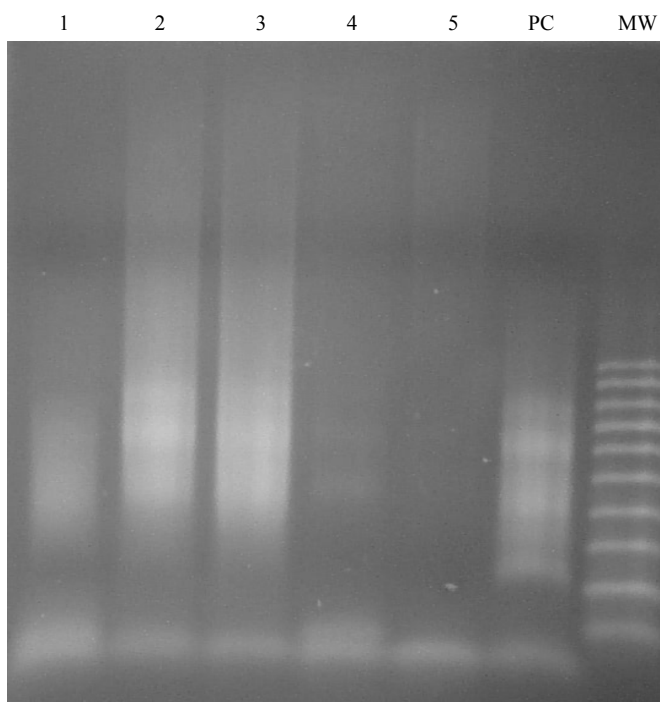B.

Figure 3.3

Figure 3.4

Figure 3.5

Lane 1: Placenta from nonviable fetus A
Lane 2: Placenta from nonviable fetus B
Lane 3: Placenta from nonviable fetus C
Lane 4: Placenta from viable fetus D
Lane 5: Placenta from control fetus E
Lane 6: Positive control
Lane 7: DNA ladder

CHAPTER 4

IMMUNOLOGICAL AND PATHOLOGICAL CHANGES IN THE PLACENTA
DURING INFECTION WITH *LISTERIA MONOCYTOGENES* IN PREGNANT
GUINEA PIGS [2]

Abstract

Exposure to *Listeria monocytogenes* during pregnancy can result in spontaneous abortion and stillbirths; however, the mechanisms are unknown. Our objective was to determine the effects of infection on inflammatory and anti-inflammatory cytokine expression and apoptosis in the placenta after infection with *L. monocytogenes*. Pregnant guinea pigs were treated on gestation day (gd) 35 with 10^8 colony forming units *L. monocytogenes* and sacrificed on gd 37, 41, 44, 55. At gd 41, IFN-γ and IL-2 expression was significantly decreased in placentas from treated dams (0.0012-fold and 0.131-fold respectively). At gd 55, TNF-α expression was significantly decreased (0.19-fold), while IFN-γ expression was significantly increased (32-fold), and apoptosis was detected in 100% of placentas from treated dams. In conclusion, cytokine expression is altered and apoptosis is increased in the placenta after treatment with *L. monocytogenes*, and these changes may contribute to fetal death.

Introduction

Infection with *Listeria monocytogenes* (*L. monocytogenes*) during pregnancy can result in abortions or stillbirths. While pregnant women are normally asymptomatic or experience mild flu-like symptoms (1), the fetus or neonate is twenty times more likely to develop listeriosis than the general population (2). Previously, we have shown that oral exposure of pregnant nonhuman primates (3, 4) and pregnant guinea pigs (5) to *L. monocytogenes* induces delivery of stillborn fetuses in a dose-dependent manner. However, the mechanisms of fetal infection and death are unknown.

Immune regulation during gestation is necessary for maintenance of pregnancy, and placental cytokine secretion is tightly controlled during gestation (6). Inflammatory (7, 8, 9) and anti-inflammatory (6, 10) cytokines are expressed in the placenta throughout pregnancy. It has been hypothesized that anti-inflammatory cytokines are dominant throughout gestation while inflammatory cytokines are suppressed (11, 12). However, maternal infection during pregnancy may cause a disruption in cytokine secretion. In a mouse model of *Brucella*-induced spontaneous abortion, IFN-γ was significantly increased, and the increase contributed to abortion (13). In pregnant mice, *L. monocytogenes* infection results in secretion of inflammatory cytokines in the placenta after intravenous (IV) exposure (14).

Apoptosis in the placenta is a natural, tightly regulated process that occurs throughout gestation (15). However, infection during pregnancy has been associated with an increase in placental apoptosis (16, 17). Like *L. monocytogenes*, *Chlamydia trachomatis* is an intracellular pathogen known to cause early pregnancy loss and premature delivery. *Chlamydia trachomatis* have been correlated with an increase in

apoptosis in the placenta (18, 19). An increase in unregulated placental apoptosis could lead to placental inflammation and degradation resulting in fetal distress.

In our previous study after orally exposing pregnant guinea pigs to *L. monocytogenes*, there was a dose-dependent increase in the number of placentas with a detectable amount of apoptosis at 21 days post-treatment (20). Additionally, *L. monocytogenes* invasion of the placenta increased as the dose increased (5). Our objectives were to determine 1) whether maternal treatment with *L. monocytogenes* induces changes in selected inflammatory and anti-inflammatory cytokine expression in the placenta, and 2) whether the number of placentas with a detectable amount of apoptosis increases as infection proceeds.

Materials and Methods

Animals

Harley timed-pregnant guinea pigs were obtained on gestational day (gd) 28 from Elm Hill Laboratories (Chelmsford, MA). Animals were allowed a one-week acclimation period and were treated with 10^8 colony forming units (CFUs) *L. monocytogenes* on gd 35. Animal husbandry has been described in detail in Williams et al (5). Briefly, guinea pigs were fed sterilized guinea pig chow (Purina, PMI Nutrition International, St. Louis, Mo) and given sterilized water *ad libitum*. The animals were housed in separate cages fitted with filters.

Inoculum preparation

Details of the inoculum preparation were previously described in Williams et al (5). Briefly, *L. monocytogenes* cells (strain 12443) were activated by three successive transfers to 10 ml of tryptic soy broth (Difco Laboratories; Detroit, Michigan) and

incubated at 35°C for 24 hours. Cultures were then harvested by centrifugation (9,000 x

g at 4°C for 30 min), washed twice and resuspended in sterile phosphate buffered saline

(PBS). The number of *L. monocytogenes* cells in the inoculated sample was determined

by serially diluting the cell suspension in PBS and plating onto *Listeria* Selective Agar

(Oxoid; Ogdenburg, NY). The cell populations obtained were used to confirm the CFUs

per ml (CFU/ml) of *L. monocytogenes* administered to the guinea pigs.

Tissue collection

Animals were sacrificed on gd 37, 41, 44, or 55 (post-treatment day (PTD) 2, 6, 9,

or 20)) by CO_2 asphyxiation. Placentas were collected from each maternal-fetal unit.

Placentas from stillborn fetuses were not able to be analyzed due to tissue degradation. A

representative section was dissected through all cell layers of the placenta and was stored

for analysis of cytokine expression, apoptosis, or histology.

Extraction of RNA

At the time of sacrifice, placenta sections were stored in RNA*later*® to prevent

RNA degradation. RNA was extracted with an RNeasy® mini kit (Qiagen Inc, Valencia,

CA) according to the manufacturer's instructions. Briefly, a 30 ± 1 mg sample of

placenta was placed into a guanidine-thiocyanate–containing buffer allowing for RNase

inactivation and purification of intact RNA. The tissue sample was homogenized using a

sonic dismembrator (Fisher Scientific, Pittsburgh, PA). Ethanol was added to the

solution and total RNA was allowed to bind to a silica-based membrane in a spin column.

The sample was washed three times, and RNase-free water was added to elute the RNA.

The concentration of extracted RNA was quantified by measuring the absorbance at 260

nm (Biophotometer, Eppendorf, Hamberg, Germany).

Cytokine mRNA expression

All primers and probes were obtained from Applied Biosystems (Foster City, CA). Specific primers and probes were used for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), tumor necrosis factor (TNF)-α, interleukin (IL)-2, IL-5 (21), interferon (IFN)-γ, and IL-10 (22). Real-time RT-PCR was performed using the Eppendorf Mastercycler EP sequence detection system (Eppendorf, Hamberg, Germany) with a one step RT-PCR kit (Verso™ One step QRT-PCR kit, ABGene, Rochester, NY) following the manufacturer's protocol. Briefly, concentrations in the final solution were: 1 μg RNA, 400 nM of each primer, 240 nM of probe, 1x Verso™ enzyme mix, and 1x QPCR mix. The thermal cycling program was: 50°C for 15 minutes and 95°C for 15 minutes, followed with 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds. In each cytokine analysis, the threshold cycle (Ct) values from the gene of interest (IFN-γ, IL-2, TNF-α, or IL-5) were normalized to the Ct values for the housekeeping gene, GAPDH. The $2^{-\Delta\Delta Ct}$ method of comparative analysis described by Livak and Schmittgen (23) was used to express the fold-difference in cytokine expression in placentas from treated animals compared to expression in placentas from gestation day matched control animals.

Determination of apoptosis in the placenta

Placenta sections were frozen in liquid nitrogen immediately following excision, and stored at -80°C to prevent DNA degradation. DNA was extracted using a DNeasy mini tissue kit according to the manufacturer's protocol (Qiagen Inc, Valencia, CA). Briefly, a 25 mg placenta section was homogenized and incubated overnight in proteinase K for tissue lysis. The lysate was mixed with a binding buffer, and the solution was

added to a silica-based membrane in a spin column. The sample was washed three times, and a low-salt buffer was added to release purified DNA from the membrane. The optical density at 260 nm was measured using a spectrophotometer (BioPhotometer, Eppendorf, Hamburg, Germany) to determine the amount of genomic DNA present. A genomic DNA concentration of $1\mu g$ was used for PCR. Ligation and PCR were done using an apoptosis-specific PCR kit, DNA ladder assay, following the manufacturer's protocols and temperature profile (Maxim Biotech Inc, Rockville, MD).

The completed reaction solution was mixed with loading buffer (4:1) and loaded on a 1.75% agarose gel. The gel was run at 100 mV for approximately 1 hour. The gel was allowed to soak in an ethidium bromide bath for approximately 5 minutes and was rinsed with deionized water. The gel was viewed under an ultraviolet hood and pictures were taken using a Nikon camera fitted with a 590 lens.

Histological examination

A representative sample from each placenta was fixed in 10% neutral buffered formalin and embedded in paraffin. The samples were sectioned at 4-5 μm and stained with hematoxylin and eosin. Histological assessment was completed without knowledge of animals' identity or treatment.

Statistical analysis

Data were analyzed using SAS version 8.2 (Cary, N.C.). Statistical significance was calculated using an analysis of variance test (ANOVA, $p \leq 0.05$). Differences between groups were determined using a Student-Newman-Keuls (SNK) test ($p \leq 0.05$) for analysis of apoptosis in the placenta. To determine significant alterations in mRNA cytokine expression, the Ct values from placentas from treated animals were compared to

the Ct values for placentas from gestation day matched control animals using a SNK test (p ≤ 0.05).

Results

Placentas from all post-treatment days were analyzed to determine if treatment and duration of infection with *L. monocytogenes* affected cytokine expression within the placenta. The microbiological analyses of guinea pigs sacrificed at gd 41, 44, and 55 are reported in Jensen et al (24). Additionally, placentas from stillborn fetuses were not used for analysis due to RNA and DNA degradation. Cytokine expression in placentas from control animals was examined to determine if expression changed as gestation proceeded. IFN-γ expression was significantly decreased at gd 55 compared to expression at gd 37, 41, and 44; however, IL-2, TNF-α, and IL-5 expression did not change as gestation proceeded.

The three selected inflammatory cytokines showed different patterns of expression in response to *L. monocytogenes* infection. While none of the three were significantly different from controls at gd 37 (PTD 2), IFN-γ was significantly decreased (0.0012-fold) by gd 41 (Figure 4.1A). IFN-γ expression was not different from control levels at gd 44, but expression was significantly increased at gd 55, 32-fold (Figure 4.1A). IL-2 was significantly decreased at gd 41 and 44 (0.131-fold and 0.231-fold, respectively), but expression was the same as control levels by gd 55 (Figure 4.1A). TNF-α expression was significantly decreased in placentas from treated dams at gd 55, 0.19-fold (Figure 4.1A).

Two anti-inflammatory cytokines were selected for analysis. IL-5 expression was not altered after maternal treatment with *L. monocytogenes* at any gestation day

examined. IL-5 expression in placentas from treated dams was 0.47-fold lower than expression in control placentas at gd 55; however, the difference was not statistically significant (Figure 4.1B). IL-10 expression was not detected in either control or infected placentas at any gestation day examined.

Histological analyses were performed on placentas from treated and control dams at gd 41, 44, and 55 (PTD 6, 9, and 20). Microscopic examination demonstrated no histological alteration in placentas from treated dams regardless of gestational day. No evidence of hemorrhage, necrosis, or increase in acute or chronic inflammation was seen within the placental tissues. All placentas maintained appropriate vascular and histological architecture (data not shown).

Placentas from treated and control dams sacrificed at gd 41, 44 and 55 (PTD 6, 9, and 20) were analyzed for apoptosis to determine if a temporal change occurred after treatment with *L. monocytogenes*. Approximately 50% of the placentas from control dams exhibited detectable levels of apoptosis on gd 41, 44, and 55 (Figure 4.2). The percentage of placentas positive for apoptosis from the *L. monocytogenes* treated dams was significantly increased at gd 55 (100%, $p < 0.05$, Figure 4.2).

<p align="center">Discussion</p>

Although *L. monocytogenes* exposure during pregnancy can cause spontaneous abortion, stillbirths, and neonatal illness, the mechanisms of disease and adverse pregnancy outcome are unknown. Previously, we have used pregnant guinea pigs as a model for *L. monocytogenes*-induced stillbirths (5, 20, 24). This study shows that placental cytokine secretion and placental apoptosis are affected after maternal exposure

to *L. monocytogenes* in pregnant guinea pigs. Significant changes in cytokine expression are seen earlier, gd 41, than changes in apoptosis, gd 55.

The most common route of exposure for *L. monocytogenes* in humans is through oral consumption of contaminated food with passage of *L. monocytogenes* through the gastrointestinal tract and internalization into intestinal epithelial cells by binding to the intestinal E-cadherin receptor. Guinea pigs are susceptible to *L. monocytogenes*-induced stillbirths after oral inoculation (5). Previously, mice have been used to study the immune response in maternal serum and in the placenta after IV or intraperitoneal (IP) infection with *L. monocytogenes*. However, *L. monocytogenes* is an intracellular pathogen and oral exposure allows the bacteria to initially evade the immune system in humans (25, 26) while IV exposure delivers the bacteria directly into the bloodstream. Mice are generally not susceptible to severe illness or adverse pregnancy outcomes after oral exposure because *L. monocytogenes* does not bind to the mouse intestinal E-cadherin receptor (27, 28) because of one amino acid substitution in the active site. In humans and guinea pigs, the amino acid sequence of the E-cadherin active site is identical (27). Thus, guinea pigs provide a model for human infection by the same oral route of infection allowing *L. monocytogenes* to initially evade the immune system.

Abram et al (14) found an increase in inflammatory cytokine expression and microabscesses in the placenta after IV inoculation with 5×10^3 CFUs *L. monocytogenes* by three days post-treatment in pregnant mice. Elevated TNF-α mRNA expression was identified in placentas from dams treated with *L. monocytogenes* compared to placentas from control dams, and IFN-γ mRNA was only detected in placentas from treated dams at gd 14, three days post-treatment (14). No inflammation was seen in placentas from

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treated dams; however, clusters of bacteria were present in the placenta and associated with hemorrhagic necroses (14). In pregnant guinea pigs treated orally, no necroses were noted after maternal treatment with *L. monocytogenes*, and a significant increase in IFN-γ was not seen until later in pregnancy, gd 55 (Figure 4.1). Additionally, TNF-α expression was significantly decreased at gd 55. A decrease in TNF-α expression in the placenta at gd 55 might be correlated with a decrease in maternal serum cytokine TNF-α levels; a decrease in maternal serum TNF-α levels at gd 56 was seen in pregnant guinea pigs treated with 10^4 to 10^8 CFUs *L. monocytogenes* (20). The ability of *L. monocytogenes* to replicate and spread intracellularly after oral exposure in pregnant guinea pigs could explain the delay in inflammatory cytokine expression and the lack of bacteria-associated necroses in the placenta.

Results of investigations of normal cytokine expression in the placenta of guinea pigs have not been previously reported. IFN-γ, IL-2, and TNF-α have been identified in the placenta throughout human gestation (7, 8, 9). In our study, IFN-γ expression in control animals was significantly decreased at gd 55 compared to the earlier gestation days, 37, 41 and 44. Similar to humans, IL-2 and TNF-α expression in the placenta was not altered as gestation proceeded.

The only inflammatory cytokine that showed a significant increase in expression was IFN-γ, and that occurred at gd 55 (Figure 4.1). This suggests that while *L. monocytogenes* can infect the placenta as early gd 37, PTD 2 (data not shown), an inflammatory response is not elicited until later in infection. The initial suppression of inflammatory cytokines could be due to anti-inflammatory cytokine expression. However in our study, placental IL-5, an anti-inflammatory cytokine, was not altered

after treatment with *L. monocytogenes*, suggesting that an increase in IL-5 is not inducing suppression of inflammatory cytokines. The suppression of inflammatory cytokine expression at gd 41 and 44 (PTD 6 and 9) and the maintenance of IL-5 expression would result in a cytokine ratio that would be favorable for pregnancy maintenance. However, IFN-γ increases at gd 55 could result in detrimental effects to the pregnancy. In humans, maternal serum IFN-γ levels were significantly higher in preterm delivery patients when compared to maternal serum IFN-γ levels in normal second trimester women and women with normal delivery (29).

At gd 56, *L. monocytogenes* induced placental apoptosis in a dose-dependent manner (20). Similarly, placental apoptosis in the present study appeared to be slightly elevated at gd 41 and 44 (PTD 6 and 9) and was significantly increased at gd 55 (PTD 20) after maternal treatment with 10^8 CFUs *L. monocytogenes* (Figure 4.2). While apoptosis during pregnancy is a naturally occurring process, infection related apoptosis could cause detrimental effects to the placenta and fetus. The release of placental apoptotic materials during gestation occurs without a maternal inflammatory reaction due to the containment of apoptotic bodies in a sealed plasma membrane (17). Unregulated apoptosis and inefficient clearing of apoptotic bodies in the placenta during *L. monocytogenes* infection could result in placenta degradation and fetal distress.

Placenta histology did not reveal any microabscesses after maternal treatment with *L. monocytogenes* in pregnant guinea pigs. In human *Listeria*-induced stillbirths, placenta microabscesses have been observed (30, 31). However, in the present study, no placentas from stillborn guinea pig fetuses were available for placental histology and this may have affected the results.

In conclusion, *L. monocytogenes* infection in pregnant guinea pigs is associated with changes in inflammatory cytokine expression and apoptosis in the placenta. IFN-γ expression in the placenta is significantly increased at the time where a significant increase in apoptosis occurs. This suggests there is a shift in the expression of IFN-γ in the placenta as infection proceeds. During infection there is no significant change in expression of anti-inflammatory cytokines, IL-5 or IL-10, but an increase in IFN-γ would result in a shift from the cytokine ratio seen in normal pregnancy to a ratio seen with premature delivery. As additional guinea pig genes are sequenced and made available, further work will help understand the role of cytokine expression in *L. monocytogenes*-induced stillbirths.

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Figure 4.1: Selected inflammatory and anti-inflammatory cytokine expression in the guinea pig placenta after maternal treatment with 10^8 CFUs *L. monocytogenes* at gd 37, 41, 44, and 55. Selected cytokine expression in the placenta for each time point analyzed is reported as a fold-change relative to expression levels in placentas of control animals. Inflammatory cytokine expression, IFN-γ, IL-2, and TNF-α (A). IL-5 cytokine expression (B). For all dams ≥ 2 placentas per litter were analyzed. For controls groups: n ≥ 2 dams. For treated groups: n ≥ 4 dams. Error bars = ± SD.
* Statistically significant (p ≤ 0.05)

Figure 4.2: Apoptosis in the placentas of control guinea pigs and guinea pigs treated with 10^8 CFUs *L. monocytogenes* sacrificed at gd 41, 44, and 55. All available placentas were analyzed for control dams and treated dams. The mean of the number of placentas with a detectable amount of apoptosis for each dam was calculated and a mean for each control group and treated group per gestational day was calculated. For control groups: n ≥ 2 dams. For treated groups: n ≥ 3 dams. Error bars = ± SE.
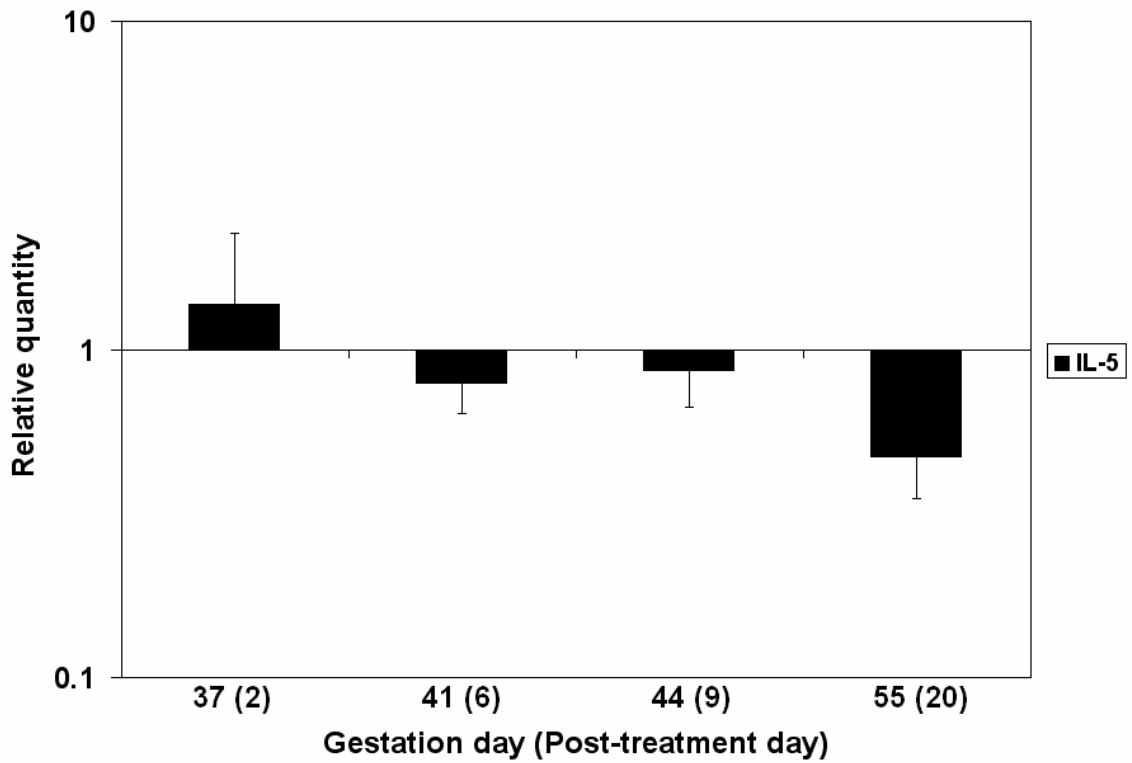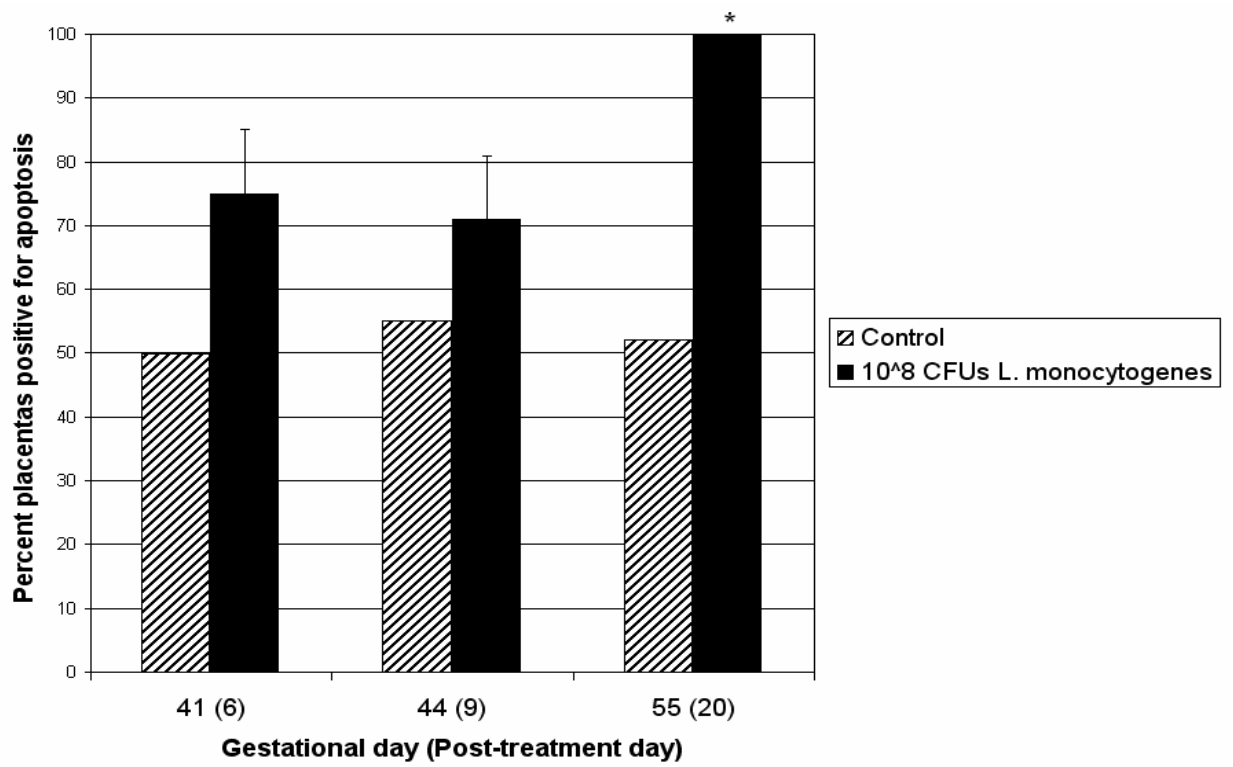* Statistically significant (p < 0.05)

Figure 4.1 (A).
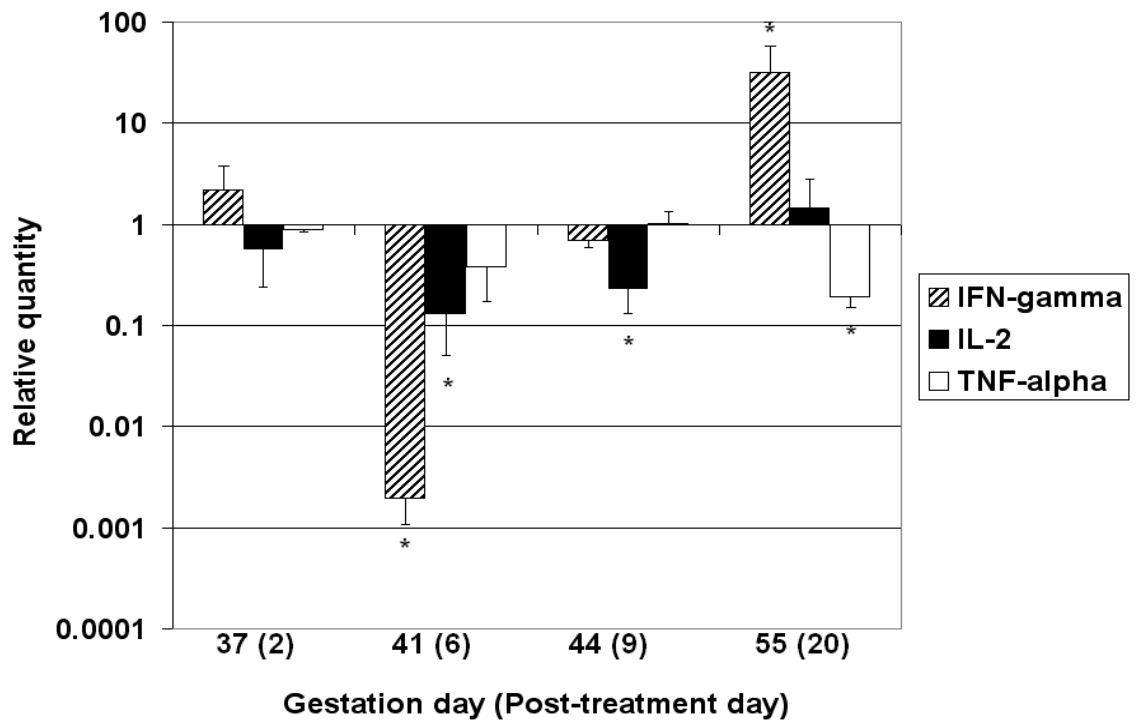
(B).

Figure 4.2

CHAPTER 5

COMPARISON OF IMMUNOREACTIVITY OF A HUMAN ANTI E-CADHERIN
ANTIBODY IN HUMAN, RHESUS MONKEY, AND GUINEA PIG INTESTINAL
AND PLACENTAL TISSUE [3]

Abstract

L. monocytogenes, a foodborne pathogen, causes spontaneous abortion and stillbirth in humans. The bacteria have the ability to cross the intestinal and placental barriers in humans and animals using a species-specific receptor, E-cadherin. Rhesus monkeys and guinea pigs have been used to characterize the effects of *L. monocytogenes* during pregnancy. While humans and guinea pigs have similar E-cadherin, the E-cadherin sequence of the rhesus monkey is unknown. Our objective was to determine the immunoreactivity of a human anti E-cadherin antibody using rhesus monkey and guinea pig intestinal and placental tissue. Rhesus monkey intestinal tissue and term placental tissue was obtained. Placental tissue and intestinal tissue from guinea pigs sacrificed on gestation day 56 was obtained. The human anti E-cadherin antibody was immunoreactive with human, rhesus monkey, and guinea pig intestinal tissue. Human, rhesus monkey, and guinea pig placental tissue was not immunoreactive. In conclusion, the immunoreactivity of the anti E-cadherin antibody to intestinal E-cadherin suggests that both rhesus monkey and guinea pig E-cadherin is functionally similar to human E-cadherin.

Introduction

Listeria monocytogenes (*L. monocytogenes*) is a food-borne pathogen with the ability to cross the intestinal and placental barrier (7). The bacteria contain a surface protein, Internalin A (InlA), which interacts with a receptor, E-cadherin, in human tissues (8). The InlA-E-cadherin interaction allows the bacteria to cross the intestinal barrier and cause systemic infection (8). *L. monocytogenes* infection during pregnancy can result in spontaneous abortion or stillbirth (2, 6).

E-cadherin is expressed in many cell types, including hepatocytes, dendritic cells, epithelial cells, and trophoblasts (11, 1, 5, 14). E-cadherin contains five extracellular domains (12), and InlA binds to the first of the five extracellular cadherin domains (EC1) (8). The C-terminus of the EC1 domain contains an InlA binding site of 35 amino acids (4). The InlA-E-cadherin receptor interaction is dependent on a proline residue at amino acid site 16 in the EC1 domain binding site of E-cadherin (3). *L. monocytogenes* is unable to bind to mouse E-cadherin due to a substitution of glutamic acid at amino acid site 16 (3). The InlA-E-cadherin interaction is essential for bacterial invasion and infection after oral exposure to *L. monocytogenes* (3).

E-cadherin has been shown to be expressed in human placental syncytiotrophoblasts (5). The syncytiotrophoblast layer has direct contact with maternal blood, and blood-borne *L. monocytogenes* could take advantage of the E-cadherin expression on syncytiotrophoblasts (5). The invasion of syncytiotrophoblasts by *L. monocytogenes* could allow for bacterial replication in the placenta and bacterial transmission to the fetus.

In a previous study using guinea pig cells of epithelial origin, guinea pig E-cadherin was found to be similar to human E-cadherin (3). Our objectives were to determine 1) whether a human anti E-cadherin antibody is immunoreactive with guinea pig and rhesus monkey E-cadherin in intestinal and placental tissue.

Materials and Methods

Tissue slide preparation

Human intestinal tissue slides and A431 (epidermis carcinoma) cell slides were obtained from ProSci Inc. (Poway, CA). The human intestinal tissue slides were used as a positive control and the A431 cell slides were used to check for possible antibody degradation. Formalin-fixed human term placentas were obtained from Athens Regional Medical Center. Four to five μm thick sections were cut onto positively-charged glass slides from paraffin-embedded blocks.

Rhesus monkey duodenum and placenta tissue slides were obtained from Yerkes Primate Center (Atlanta, GA). Guinea pig intestinal tissue and placenta tissue were obtained from timed pregnant guinea pigs (Elm Hill Breeding Labs, Chelmsford, MA) sacrificed at gd 56. Mouse intestinal tissues were obtained from CD-1 adult mice (Charles River Laboratories, Inc., Wilmington, MA). All tissue samples were fixed in 10% neutral buffered formalin and paraffin-embedded. Four to five μm thick sections were cut onto positively-charged glass slides from paraffin-embedded blocks.

Tissue immunohistochemistry

E-cadherin expression was immunohistochemically evaluated using a specific monoclonal primary antibody against human E-cadherin (HECD-1, Zymed Laboratories, San Francisco, CA). Antibody dilutions were made according to manufacturer's protocol

(1:100 for DAB chromagen staining) using an antibody diluent solution containing PBS (pH 7.4), bovine serum albumin, and 0.1% NaN_3 (Zymed Laboratories, San Francisco, CA).

Sections were deparaffinized and rinsed. The sections were subject to heat-induced epitope retrieval; slides were placed in 500 ml citrate buffer (pH 6.0) and heated in a microwave cooker for 15 min at 100°C. Sections were cooled to room temperature and rinsed with PBS. All incubations were done at room temperature. A 3% hydrogen peroxide solution was added to eliminate endogenous peroxide activity. After incubating, sections were washed with PBS. A serum blocking solution was added and the slides were incubated. The primary antibody (HECD-1) was added and slides were incubated in a moist chamber for 1 hour. After PBS washing, a biotinylated secondary antibody was added. After incubation and PBS washing, an avidin-biotin complex with horseradish peroxidase was added. A 1:1:1 solution of copper enhancer, 3,3-diaminobenzidine tetrahydrochloride, and 0.6% hydrogen peroxide (DAB chromagen solution) was added to 1ml deionized water. After the enzyme conjugate solution was rinsed off the slides, the DAB chromagen solution was added, and sections were allowed to incubate for 7 minutes. Slides were rinsed with deionized water and mounted. Sections were viewed with a Nikon 8200 microscope equipped with a 10x objective (Melville, NY). Photographs were taken using a SPOT Flex™ digital camera and SPOT Basic™ software (Sterling Heights, MI).

Results

We analyzed human, rhesus monkey, guinea pig, and mouse intestinal tissue for the presence of E-cadherin as determined by the immunoreactivity of the tissue to a

human anti E-cadherin antibody. Human intestine tissue and A431 cells were immunoreactive to the anti E-cadherin antibody (Figure 5.1 A,B). Additionally, mouse intestinal tissue was used as a negative control, and the tissue was not immunoreactive to the antibody (Figure 5.1C). The rhesus monkey duodenum tissue was immunoreactive with the human anti E-cadherin antibody (Figure 5.2A), as was the guinea pig intestinal tissue (Figure 5.2B).

Because *L. monocytogenes* is known to cross the placental barrier and infect fetuses, we analyzed human, rhesus monkey, and guinea pig placental tissue for the presence of E-cadherin. In all three placental tissues, the human anti E-cadherin antibody was not immunoreactive. However, the placentas examined were from late gestation or term placentas and E-cadherin expression could be expressed at low levels during the end of gestation which would result in faint or no staining.

Discussion

Previously, no data were available on the rhesus monkey E-cadherin receptor. Studies have shown that *L. monocytogenes* utilizes a surface protein, internalin A (InlA), to bind to E-cadherin and invade tissues in humans and guinea pig cells of epithelial origin (3). Additionally, guinea pig E-cadherin has been molecularly characterized and the active site is identical in guinea pigs as in humans (3).

In the present study, rhesus monkey and guinea pig E-cadherin in intestinal tissue was immunoreactive to a human anti E-cadherin antibody. Lecuit et al (3) showed the ability of a human anti E-cadherin antibody to bind to guinea pig cells of epithelial origin (3). While rhesus monkeys have been used as an animal model for *Listeria*-induced stillbirths, the E-cadherin binding site sequence has not been determined (9, 10). We

show that functionally both rhesus monkey and guinea pig E-cadherin will react with a human anti E-cadherin antibody.

E-cadherin was not detected in the placenta tissue from rhesus monkey or guinea pig. Previously in humans, E-cadherin was shown to be expressed on the apical membrane of syncytiotrophoblasts (5). *L. monocytogenes* utilize the syncytiotrophoblast E-cadherin to gain access to the placenta villi (5). While E-cadherin was not detected in term rhesus monkey placentas or guinea pig placentas at gd 56, *L. monocytogenes* was cultured from guinea pig placenta tissue at gd 56 (13) and in placentas after *Listeria*-induced stillbirths in rhesus monkeys (9). The lack of E-cadherin detection in the placenta could be due to low expression levels of E-cadherin at the end of pregnancy in humans, rhesus monkeys, and guinea pigs.

In conclusion, our results show that intestinal rhesus monkey and guinea pig E-cadherin is structurally similar to human E-cadherin and suggests that rhesus monkey and guinea pig E-cadherin are functionally similar to human E-cadherin. Rhesus monkey duodenum tissue and guinea pig intestinal tissue is immunoreactive to a human anti E-cadherin antibody. However, in term rhesus monkey placenta tissue the human anti E-cadherin antibody is not immunoreactive. In guinea pig placenta tissue at gd 56, the tissue is not immunoreactive to a human anti E-cadherin antibody.

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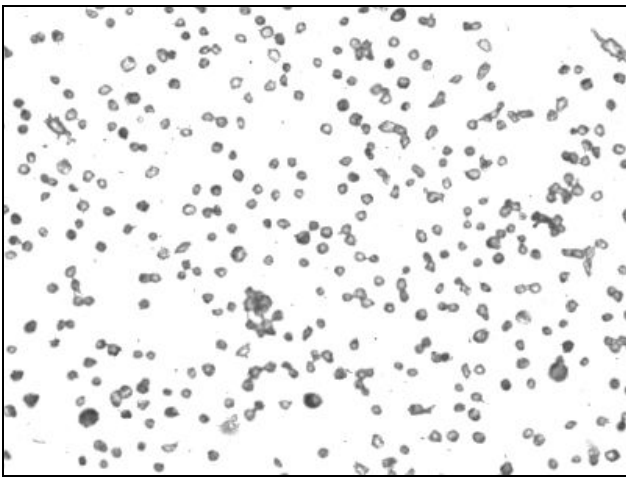
Figure 5.1: Immunohistochemical analysis of a human anti E-cadherin antibody. Human intestinal tissue, 200x (A). Cell line A431, 200x (B). Mouse intestinal tissue, 1000x (C).

Figure 5.2: Immunoreactivity of intestinal tissue to a human anti E-cadherin antibody. Rhesus monkey duodenum tissue, 1000x (A). Guinea pig intestinal tissue, 1000x (B).
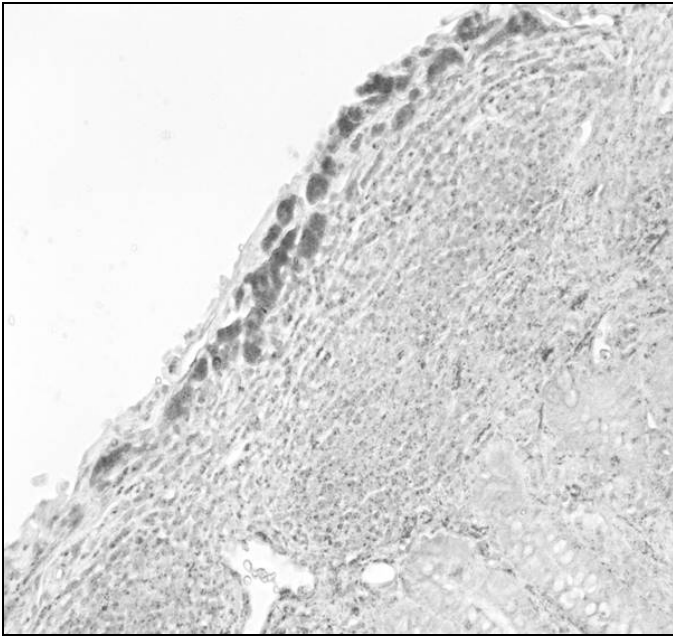
Figure 5.1 (A).
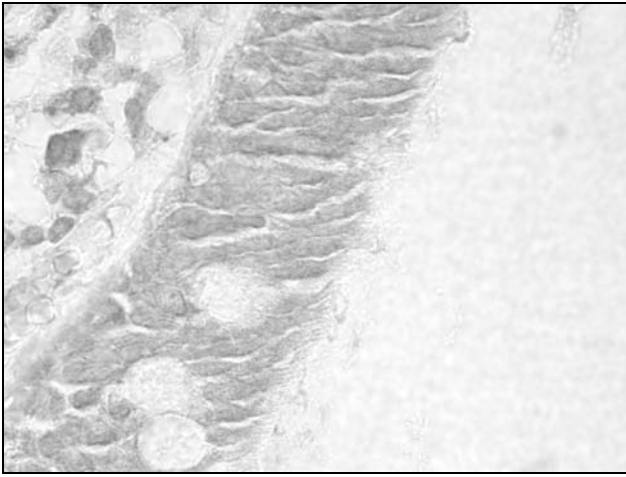
(B).

(C).

Figure 5.2 (A).

(B).

CHAPTER 6

SUMMARY AND CONCLUSIONS

Listeriosis continues to be a problem in the United States with an estimated 500 deaths occurring annually (Mead et al, 1999). One-third of the cases occur in pregnant women with the fetus or neonate 20 times more likely to develop listeriosis than the general population (Mead et al, 1999; Southwick et al, 1996). The mechanisms of fetal infection and death in humans are largely unknown. The objective of our study was to investigate effects of *L. monocytogenes* infection using pregnant guinea pigs. We characterized the effects of infection on the maternal liver, maternal immune response, placental apoptosis and placental cytokine secretion. In addition, we determined the immunoreactivity of a human anti E-cadherin antibody on rhesus monkey and guinea pig intestinal and placental tissue.

Maternal inoculation via oral ingestion of *L. monocytogenes* at gd 35 resulted in deleterious effects to the maternal liver and placental tissue in guinea pigs. As the dose increased, the severity of maternal liver effects increased with microscopic lesions present at $\geq 10^5$ CFUs *L. monocytogenes*. In maternal serum, cytokine secretion was unaltered except for TNF-α concentrations, which were significantly decreased. At doses of $\geq 10^6$ CFUs *L. monocytogenes*, the number of placentas with a detectable amount of apoptosis was significantly increased, and premature delivery of stillborn fetuses occurred. At the highest dose of 10^8 CFUs *L. monocytogenes*, delivery of stillborn fetuses occurred significantly earlier, an average of 10 days post-treatment, compared to the sacrifice day, 21 days post-treatment.

After oral exposure to *L. monocytogenes*, intestinal transmigration via the E-cadherin receptor is essential for systemic infection in humans. While rhesus monkeys and guinea pigs have been used as a model for *Listeria*-induced stillbirths, the amino acid sequence for the active site of E-cadherin has not been published for rhesus monkeys. We determined rhesus monkey and guinea pig intestinal E-cadherin was immunoreactive to a human anti-E-cadherin antibody, however, E-cadherin was not detected in rhesus monkey or guinea pig placentas. The lack of E-cadherin detection in term placentas suggests that *L. monocytogenes* invasion of the placenta is not dependent on the InlA-E-cadherin interaction.

Similar to humans and nonhuman primates, pregnant guinea pigs infected with *L. monocytogenes* deliver premature stillborn fetuses. In humans, however, little is known about the bacterial dissemination and subsequent pathological and immunological changes prior to the occurrence of stillbirth. The effects of *L. monocytogenes* on the maternal liver during human pregnancy are unknown because the infection does not cause maternal death. However, the *Listeria*-induced liver damage in pregnant guinea pigs is similar to abscesses seen in immunocompromised patients with listeriosis (Brönnimann et al, 1998). In humans, the mother is often asymptomatic (Gray and Killinger, 1966); similarly, no maternal illness has been reported in nonhuman primates or guinea pigs that underwent premature delivery of stillborn fetuses (Smith et al, 2003; Williams et al, 2007). The similarities of *Listeria*-induced stillbirths in guinea pigs and humans suggest guinea pigs are an appropriate model to study pregnancy related listeriosis.

L. monocytogenes-induced expression of inflammatory cytokines in the placenta is different following different exposure routes. In a pregnant mouse study, IFN-γ and TNF-α expression in the placenta was significantly increased three days after IV treatment with *L. monocytogenes* (Abram et al, 2002). However, after oral exposure in pregnant guinea pigs, a significant increase in IFN-γ expression in the placenta was not seen until post-treatment day 20. Additionally, TNF-α is decreased by post-treatment day 20 in both expression in the placenta and concentrations in maternal serum. This suggests that after oral exposure, systemic and local suppression of TNF-α occurs. Based on the literature, we expected to see an increase in anti-inflammatory cytokine expression in the placenta, but no alterations were seen in IL-5 or IL-10 at the time points analyzed. However, we did see a significant decrease in placental inflammatory cytokine expression earlier during infection. This might result from the inflammatory and anti-inflammatory pathways acting independently of each other. The initial suppression of placental inflammatory cytokines in pregnant guinea pigs is in contrast to what is seen in the mouse model after IV exposure; however, differences in exposure (IV versus oral) may influence these responses.

Intracellular pathogen invasion has been correlated with an increase in apoptosis in the placenta (Equils et al, 2006); in pregnant guinea pigs, *L. monocytogenes* infection increases apoptosis in the placenta. An increase in apoptosis has been correlated with inefficient clearing of the apoptotic bodies (Mor and Abrams, 2003) and in our study could initiate the IFN-γ inflammatory response; this could lead to changes in the placenta cytokine milieu resulting from an increase in IFN-γ and other inflammatory cytokines. The possible continued increase of apoptotic bodies and inefficient clearing could

continue to initiate an inflammatory response. The inflammatory cytokine secretion and changes in apoptosis could result in fetal distress and eventual death. The characterization of maternal and placental effects following oral ingestion of *L. monocytogenes* during pregnancy allow for a better understanding of the mechanisms behind fetal infection and death, which can lead to development of diagnostic tools for earlier diagnosis and treatment.