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Nonhuman Primate Model for *Listeria monocytogenes*-Induced Stillbirths

Mary Alice Smith,[1]* Kazue Takeuchi,[2] Robert E. Brackett,[2]† Harold M. McClure,[3] Richard B. Raybourne,[4] Kristina M. Williams,[4] Uma S. Babu,[4] Glenn O. Ware,[5] J. Roger Broderson,[6] and Michael P. Doyle[2]

Department of Environmental Health Science,[1] Experimental Statistics,[5] and Office of the Vice President for Research,[6] University of Georgia, Athens, Georgia 30602; Center for Food Safety, University of Georgia, Griffin, Georgia 30223[2]; Yerkes Regional Primate Research Center, Emory University, Atlanta, Georgia 30322[3]; and U.S. Food and Drug Administration, Laurel, Maryland 20708[4]

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***Listeria monocytogenes*, isolated from outbreaks in either human or nonhuman primate populations, was administered orally at doses ranging from 10^6 to 10^{10} CFU. Four of 10 treated animals delivered stillborn infants. *L. monocytogenes* was isolated from fetal tissue, and the pathology was consistent with *L. monocytogenes* infection as the cause of pregnancy loss. For all pregnancies resulting in stillbirths, *L. monocytogenes* was isolated from maternal feces, indicating that *L. monocytogenes* had survived and had probably colonized the gastrointestinal tract. Antibodies and antigen-specific lymphocyte proliferation against *Listeria* increased in animals that had stillbirths.**

Listeriosis resulting from exposure to food containing the bacterium *L. monocytogenes* causes serious disease, with case fatality rates between 20 and 40% (33). Listeriosis is especially serious in susceptible populations such as immunocompromised persons and pregnant women (11, 14, 16, 20, 24, 26, 29, 32, 35). For healthy nonpregnant adults, listeriosis has a relatively low incidence, presumably due to its low infectivity in immunocompetent individuals.

Pregnancy-related listeriosis primarily affects the fetus or neonate. The maternal reaction to the presence of *Listeria* infection is generally an influenza-like episode with fever, backache, and perhaps diarrhea (7, 11, 13, 24, 29). The effect of fetal *Listeria* infection is dependent on the point in gestation time when infection occurs. First-trimester infection leads to spontaneous abortion, whereas second- and third-trimester infections lead to preterm birth followed by neonatal illness or fetal death with preterm delivery of a stillborn (7, 11, 13).

The rhesus monkey (*Macaca mulatta*), with a reproductive cycle and placenta comparable to those of humans (31), is widely used as an experimental model for human reproduction and development. As with humans, exposure to *L. monocytogenes* in pregnant nonhuman primates may result in abortions, stillbirths, or neonatal deaths (4, 27; J. Paul-Murphy, J. E. Markovits, I. Wesley, and J. A. Roberts, Lab. Anim. Sci. **40:**547 [abstr.], 1990). For humans and nonhuman primates, the pathogenesis and morphological findings associated with stillbirths due to *L. monocytogenes* are essentially the same (1, 4, 28, 37).

Despite several epidemiological studies confirming the relationship between *L. monocytogenes* and specific foods (soft cheeses, undercooked chicken, paté, etc.) (2, 30), an infectious dose has not been established for healthy or susceptible human populations due to the delay between exposure and the onset of symptoms. The severe ramifications of the disease in high-risk human populations such as pregnant women precludes the use of humans in volunteer feeding studies. Recently, a draft risk assessment of *L. monocytogenes* in ready-to-eat foods (36) reviewed human epidemiological and animal study data. The risk assessment concluded that mouse studies provide the only acceptable data for developing dose-response information at this time and acknowledged the difficulty with the use of this model because of its differences from human listeriosis (36). The quality and accuracy of the data used to develop dose-response models have a great effect on the predicted infectious dose (21). Thus, the better the animal model, the more accurate the predictions of the dose-response model and the subsequent risk assessment for humans will be.

Farber et al. (12) fed *L. monocytogenes* to healthy adult (nonpregnant) cynomolgus monkeys (*Macaca fascicularis*) and found fecal shedding of *L. monocytogenes* but no adverse health effects for doses below 10^9 CFU. At a dose of 10^9 CFU, symptoms included septicemia, irritability, loss of appetite, and occasional diarrhea. In addition, reports from several primate research centers implicated listeriosis as a cause of spontaneous abortions, stillbirths, and neonatal deaths in macaques housed in outdoor compounds (27; Paul-Murphy et al., Lab. Anim. Sci. abstract). Interestingly, there are no published reports of, nor has the Yerkes Center ever detected, cases of spontaneous listeriosis occurring in macaques housed indoors, probably because the monkeys receive less exposure to *L. monocytogenes* in a controlled indoor environment.

The purpose of this study was to establish the pregnant rhesus monkey as an experimental model for listeriosis in pregnant humans. The results of our study indicate that pregnant rhesus monkeys are susceptible to infection by *L. monocytogenes*. There were no outward signs of illness among any of the animals, whether the pregnancy resulted in a stillbirth or a normal birth. Although *L. monocytogenes* was not detected in blood samples obtained 4 days postexposure, *L. monocytogenes*

* Corresponding author. Mailing address: 206 Environmental Health Science Building, University of Georgia, Athens, GA 30602-2102. Phone: (706) 542-2454. Fax: (706) 542-7472. E-mail: masmith@uga.edu.
† Present address: U.S. Food and Drug Administration, College Park, MD 20740.

TABLE 1. Strains of *L. monocytogenes* used in treatments

Strain	Source	Serotype
G3982	Human clinical isolate associated with listeriosis outbreak linked to Mexican-style-cheese consumption	4b
H7550	Human clinical isolate associated with listeriosis outbreak linked to hot dog consumption	4b
Scott A	Human clinical isolate	4b
12443	Monkey clinical isolate	1/2a
12375	Monkey clinical isolate	4b

was detected in fecal samples from all animals whose pregnancies resulted in stillbirths.

The vehicle, the strain of *L. monocytogenes*, and the number of *L. monocytogenes* organisms administered to the animal were varied in the experiments to determine the best experimental protocol. The outcomes of the different treatment protocols are described in the following paragraphs.

Animals. Ten pregnant rhesus monkeys (*M. mulatta*) with no previous history of listeriosis were selected from the Yerkes National Primate Research Center's timed-breeding colony. Pregnancies were confirmed at gestation day (gd) 30 and allowed to proceed normally until the day of *Listeria* exposure. At that time, the pregnant animal was sedated with ketamine (10 mg/kg of body weight) and given *L. monocytogenes* by nasogastric intubation. The animals were observed daily for any signs of illness, such as diarrhea or a change in eating behavior or degree of activity. The animal work was done in full compliance with all federal regulations, including the Animal Welfare Act. The Yerkes Center is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

Experimental design and treatments. The experimental design was based on a phase I clinical trial commonly used in human pharmacological studies (15). However, rather than starting with a relatively low dose and increasing the dose with each positive outcome, our treatments began with a relatively high dose. When a stillbirth occurred, the next treatment dose was decreased, thereby conserving a larger number of animals for the lower-dose groups.

The normal gestation period for rhesus monkeys is 165 ± 14 days (mean ± standard deviation), and the last third of the pregnancy begins at approximately gd 110. Because humans are known to be susceptible to *L. monocytogenes* during the last trimester, gd 110 and 127 were chosen as the days for treatment. Two animals treated at gd 127 had normal birth outcomes. Because adverse pregnancy outcome resulted from treatment at gd 110 and not at gd 127, all further treatments were administered at gd 110 (±4).

***L. monocytogenes* strains and inoculum preparation.** Five strains of *L. monocytogenes* (serotype 1/2a or 4b) associated with human or primate clinical cases were chosen for the challenge study (Table 1). Cultures were maintained frozen in broth at −20°C until use. Prior to use, cultures were activated by three successive transfers in tryptic soy broth (Difco Laboratories, Detroit, Mich.) at 30°C for 24 h. Cultures were harvested by centrifugation, washed three times, and resuspended in sterile serological saline. Mixtures of strains were prepared by combining equal quantities of the cell suspensions. Skim milk, half-and-half, and whipping cream were purchased from a local store and sterilized by autoclaving at 121°C for 15 min before the mixture was diluted. One milliliter of strain mixture or individual culture was mixed with 9 ml of skim milk, half-and-half, or whipping cream to obtain the desired cell populations. The number of *L. monocytogenes* cells in the strain mixture or individual culture was determined by serially diluting the cell suspension in saline and by surface plating the suspension on tryptic soy agar (Difco) in duplicate. The plates were incubated at 30°C for 24 h before colony enumeration. The cell populations obtained were used to confirm the numbers of CFU of *L. monocytogenes* administered to animals. Fetal tissues from all adverse pregnancy outcomes were confirmed positive for *L. monocytogenes* according to the USDA method described by Cook (5), which includes both qualitative and quantitative detection methods. Briefly, the qualitative method includes two enrichments in nonselective and selective media followed by selective plating on modified Oxford agar plates (Difco), and the quantitative method uses direct plating on modified Oxford agar plates. In both methods, presumptively positive isolates were confirmed by morphological and biochemical methods prior to pulsed-field gel electrophoresis (PFGE) analysis.

Vehicle. Listeriosis outbreaks have been linked to a variety of foods, including dairy products (for a review, see reference 36). Several outbreaks of salmonellosis suggest that low infective doses are associated with high-fat-content vehicles, such as chocolate (6, 8, 18), cheese (9, 19), and paprika-powdered potato chips (23). In developing our treatment strategy, three different vehicles were used: skim milk (0.25% milk fat), half-and-half (11% milk fat), and whipping cream (30% milk fat). Three of four stillbirths occurred in animals receiving *L. monocytogenes* in whipping cream (Table 2). Due to limited numbers of available animals, a decision was made to use whipping cream as the vehicle for all remaining animals.

Pregnancy outcomes. Of the treated pregnant monkeys, four pregnancies resulted in stillborn infants, one pregnancy resulted in a premature, low-birth-weight infant, and five pregnancies resulted in normal infants (Table 2). No overt signs of illness or changes in routine behaviors were noted for any of the pregnant animals. For animals with adverse pregnancy outcomes, there were no indications of an abnormal pregnancy until the animal showed signs of labor and the stillbirth was imminent.

The length of gestation was generally shorter for animals with adverse pregnancy outcomes (average, 150 gd) than for those with normal outcomes (average, 165 gd), with one exception. Three animals delivered stillborn infants at approximately 144 gd, but one animal went full term before delivering a stillborn. For pregnancies resulting in live births, the average length of gestation was 165 days, but one animal gave birth to a live, premature infant at gd 147 (Table 2).

Tissue collection and analysis. Blood samples were collected before treatment, at 4 days posttreatment, at the time of delivery, and at 1 month postdelivery. Tissue samples were collected from all stillborn infants for culture and histologic examination. When available, samples were also collected from the placentas.

Dose. Actual cell numbers were confirmed after treatment by plating the cells on the day of treatment, incubating the plates for 24 h, and determining the counts (Table 2). The

TABLE 2. Summary of outcomes and conditions for administration of *L. monocytogenes* to pregnant monkeys

Animal no.	Day of treatment (gd)	Vehicle	Dose (CFU)	Strain of *L. monocytogenes* [serotype(s)]	Birth outcome	Day at delivery (gd)
RPp4	110	Whipping cream	1.2×10^8	5-strain mixture (1/2a and 4b)	Stillborn	145
RMt2	112	Half-and-half	1.1×10^8	5-strain mixture (1/2a and 4b)	Stillborn	140
RWh3	112	Skim milk	7.1×10^7	5-strain mixture (1/2a and 4b)	Premature (LBW)[a]	147
RVd3	112	Whipping cream	3.7×10^{10}	Scott A (4b)	Stillborn	167
RKq4	106	Whipping cream	5.7×10^8	Scott A (4b)	Normal	166
RKz4	108	Whipping cream	2.8×10^7	H7550 (4b)	Normal	166
RIr2	111	Whipping cream	9.1×10^6	G3982 (4b)	Normal	169
RGj3	127	Half-and-half	7.9×10^6	G3982 (4b)	Normal	172
RJy4	106	Whipping cream	7.2×10^6	12443 (1/2a)	Stillborn	147
RJi2	127	Half-and-half	1.1×10^6	12443 (1/2a)	Normal	170

[a] LBW, low birth weight.

doses resulting in stillbirths ranged from 7.2×10^6 CFU for strain 12443 to 3.7×10^{10} CFU for the Scott A strain. Four strains (G3982, 12443, Scott A, and H7550) were tested by administering each strain individually to a pregnant monkey. Only animals treated with Scott A at 3.7×10^{10} CFU and with strain 12443 at 7.2×10^6 CFU had stillbirths (Table 2). Strain H7550 was tested individually because it had been recently found to be responsible for a human listeriosis outbreak associated with the consumption of hot dogs (3). Although the results were not conclusive, the strain associated with hot dog consumption was not isolated from tissue samples of stillborn infants when administered in a mixture of strains nor did it produce a stillbirth in the single case when administered alone.

Routine hematology. There were no unexpected differences in hematology between animals that had normal births and animals that had stillbirths. Although animals with stillbirths had white blood cell counts that were significantly higher ($P < 0.05$) than those of animals with normal births at 1 month postdelivery (data not shown), the values were within the normal range for rhesus monkeys (22). All other parameters examined (number of platelets, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and numbers of neutrophils, lymphocytes, monocytes, and eosinophils) were within the normal reported ranges for rhesus monkeys, with the exception that animals with natural deliveries had red blood cell counts that were below the normal range, likely due to blood loss during the birthing process (22).

Fecal shedding. Fecal samples were collected at specific intervals after treatment to determine the shedding of *L. monocytogenes*. Samples were obtained daily for 1 week, every other day for the second week, and once per week for two additional weeks. After collection, fecal samples were either immediately cultured or refrigerated for not more than 48 h until enumeration. Pregnant animals that subsequently had a stillbirth generally shed *L. monocytogenes* in higher numbers and for a longer period of time than did animals having normal births (Table 3).

Although there were not enough animals for statistical analysis of fecal shedding according to the vehicle used, animals receiving *L. monocytogenes* in whipping cream shed up to 18 times more *L. monocytogenes* per gram of feces than those receiving half-and-half. This suggests that *L. monocytogenes* may be better able to colonize the gastrointestinal tract when delivered to animals in a higher-fat vehicle. Sprong et al. (34) determined that in rats a diet high in fat inhibits colonization of *L. monocytogenes* in the gut; however, in their study, *L. monocytogenes* was administered in a saline solution. This approach is different from our study, where *L. monocytogenes* was administered in a high-fat vehicle to animals on a normal monkey diet. A study is needed to directly test whether the fat content of the vehicle is important in the ability of *L. monocytogenes* to survive and colonize the gastrointestinal tract.

Humoral immune response. Plasma was collected by centrifugation of heparinized venous blood and frozen at −20°C until tested. *L. monocytogenes* strain 12443 from a stationary-phase culture was washed three times in phosphate-buffered saline (PBS) and resuspended to an estimated concentration of 10^8 CFU/ml, based on the optical density at 550 nm. Fifty microliters of bacterial cell suspension was added to each well of Immulon 2 MicroElisa plates (Thermo LabSystems, Franklin, Mass.), and liquid was removed with a vacuum evaporator-concentrator (Heto Corporation, Allerod, Denmark). Nonspecific binding to MicroElisa plates was blocked for 2 h at room temperature by adding 100 μl of PBS containing 5% heat-inactivated fetal bovine serum to each well. The plates were washed two times with PBS–0.05% Tween 20 and stored at −80°C until use. Antibody titers were determined by incubating the plates with twofold serial dilutions of plasma (100 μl/well). Plasma dilutions were made in PBS containing 5% heat-inactivated fetal bovine serum and 0.05% Tween 20. The plates were incubated at 4°C overnight or for 2 h at room

TABLE 3. Fecal shedding of *L. monocytogenes* after treatment of pregnant animals

Birth outcome (no. of animals)	No. of samples positive/total samples collected[a]	Avg % of shedding days (range)	Avg CFU of bacteria/g of feces (range)
Normal (6)	9/60	15 (0–30)	2.9×10^3 (ND[b]–1.6×10^4)
Stillborn (4)	21/40	52 (30–80)	2.6×10^4 (4.2×10^2–7.5×10^4)

[a] Includes all detectable cultures, including those positive by enrichment.
[b] ND, none detected.

TABLE 4. Titers of antibody against *L. monocytogenes* in sera from monkeys before and after infection

Birth outcome and infectious dose (CFU)	Antibody titer at:			
	Preinfection[a]	Day 4 postinfection	Delivery	Recovery
Normal deliveries				
7.1×10^7	800	800	800	800
7.9×10^6	1,600	1,600	1,600	1,600
1.1×10^6	800	800	800	800
9.1×10^6	400	400	800	ND[b]
2.8×10^7	800	800	800	800
5.7×10^8	200	200	800	400
Avg ± SD	800 ± 500	800 ± 500	900 ± 300	900 ± 400
Stillbirths				
1.1×10^8	1,600	1,600	12,800[c]	6,400
1.2×10^8	1,600	1,600	>25,600[c]	25,600
7.2×10^6	800	800	>25,600[c]	>25,600
3.7×10^{10}	1,600	1,600	>25,600[c]	25,600
Avg ± SD	1,400 ± 400	1,400 ± 400	>22,400 ± 6,400	>20,800 ± 9,600

[a] Preinfection sample drawn immediately prior to infection.
[b] ND, not determined for mother; titer for newborn, 400.
[c] Sample drawn at time of stillbirth.

temperature and then washed with PBS containing 0.1% Tween 20. Bound antibody was detected by incubating the plates for 2 h at room temperature with 100 μl of a 1/2,000 dilution of peroxidase-conjugated goat anti-rhesus immunoglobulin G (heavy plus light chains) (Southern Biotechnology, Birmingham, Ala.), as previously reported (38). Titers were normalized to a baseline value derived from the absorbance obtained with a 1:100 dilution of pooled normal rhesus monkey serum (Sigma, St. Louis, Mo.) at 450 nm.

Preinfection antibody titers ranged from 800 to 1,600, with no change in titers at 4 days postinfection regardless of pregnancy outcome (Table 4). At the time of delivery or 30 days postdelivery, all animals with stillbirths had antibody titers that were between 8- and 32-fold higher than the titers of preinfection samples collected immediately prior to infection or at 4 days after infection. Animals with normal births had antibody titers no more than fourfold greater than those measured preinfection (Table 4).

In passive-transfer studies using the mouse listeriosis model, Mackaness (25) demonstrated that there was no protective role for antibodies. More recent reports indicate that neutralizing monoclonal antibodies produced against purified listeriolysin O can protect mice against a potentially lethal infection (10). The lack of protection associated with serum transfers in Mackaness' original studies (25) may be related to the relatively weak anti-*Listeria* antibody response in mice. In contrast, in the rhesus model, a vigorous anti-*Listeria* antibody response was associated with stillbirth. An invasive *Listeria* infection with spreading to the placenta and fetus is clearly a prerequisite for stillbirth. It is likely that the increased antibody response is a result of the invasion of *L. monocytogenes* beyond the gastrointestinal tract, resulting in more extensive exposure to the immune system. The correlation of the antibody response with a negative outcome (stillbirth) does not suggest a protective role with respect to the fetus. A potential role for antibody in protection of the mother cannot be excluded given the lack of clinical disease.

Cellular immune response. Peripheral blood mononuclear cells were isolated on Ficoll-Hypaque gradients, washed, and resuspended to a concentration of 5×10^6/ml in RPMI 1640 medium (Sigma) with glutamine plus 10% heat-inactivated fetal bovine serum and antibiotics. Cells were cultured for 72 h with heat-killed *L. monocytogenes* and assessed for [^{3}H]thymidine incorporation, as previously described (38).

Lymphocytes isolated from peripheral blood responded to heat-killed *L. monocytogenes* strain 12443 differently depend-

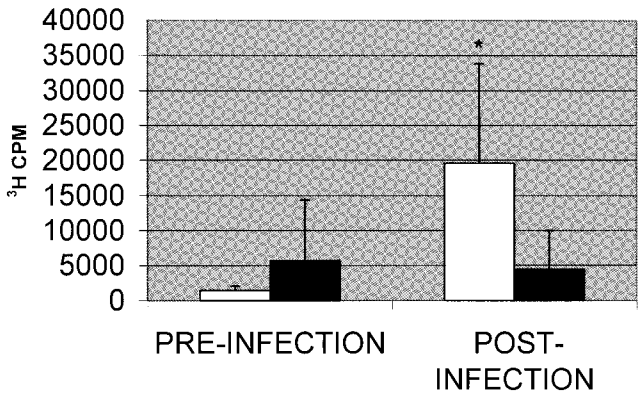

FIG. 1. Antigen-induced proliferation of peripheral blood lymphocytes. Proliferative response to heat-killed *L. monocytogenes* strain 12443 was measured by [^{3}H]thymidine incorporation after 72 h of culture. Bars represent the mean responses ± standard errors of results for animals with normal pregnancy outcomes (filled bars; number of samples, 5) or stillbirths (empty bars; number of samples, 4). Proliferation responses were measured in samples collected immediately prior to infection and approximately 30 days after stillbirth or normal outcome of pregnancy. An asterisk indicates a result that is significantly different from the preinfection level at a P value of <0.05.

ing on the outcome of the infection with respect to stillbirth (Fig. 1). Preinfection, or baseline, average proliferation responses were similar in animals delivering stillborn infants and in those delivering normal infants. Although there was considerable variation in the magnitude of proliferation among individual animals in both the stillbirth and normal-outcome groups, the observed significance level associated with the difference in the means between the two groups postinfection was at a P value of <0.06. Despite the individual variation in postinfection samples, the responses measured in samples from animals with stillbirths were significantly higher than their preinfection levels ($P < 0.04$).

Antigen-induced lymphocyte proliferation also correlates with stillbirth; however, it does not appear to be a more sensitive or specific biomarker of invasive listeriosis than antibody response in this small number of animals. The occurrence of an increased proliferative response in those animals with stillbirths does indicate an activation of cell-mediated immune components. At the exposure levels used in this study, neither antibody response nor proliferation of lymphocytes was capable of indicating any immune system changes in animals with normal birth outcomes.

PFGE. The five strains used in the initial mixture of *L. monocytogenes* were analyzed by PFGE according to the methods of the Centers for Disease Control and Prevention described by Graves and Swaminathan (17), and each strain was distinguishable from the others by using PFGE. When administered as a single strain, *L. monocytogenes* isolates from feces from the mother, placental tissue, and fetal tissue (liver, brain, and blood) were identical to the treatment strain.

Statistical analysis. Statistical differences in hematology and immunology between animals with normal births and those with stillbirths were determined by using the Student's t test (Microsoft Excel 2000). A P value of <0.05 was used to determine significant differences.

In conclusion, the results of our study establish the pregnant rhesus monkey as a model for human listeriosis. When pregnant rhesus monkeys are exposed to *L. monocytogenes* at the beginning of the third trimester, they have an increased risk of delivering a stillborn infant with pathology similar to that of humans, including acute inflammation, placentitis, fetal liver necrosis, and isolation of *Listeria* from the placental and fetal tissues. Animals that have become infected with *L. monocytogenes*, as evidenced by increased antibody response, have stillbirths. This model can be useful for assessing mechanisms of infection and disease, dose response, and intervening therapies for *L. monocytogenes* infection.

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