Comparison of *Listeria monocytogenes* Virulence in a Mouse Model

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**ABSTRACT**

Listeriosis results from exposure to the foodborne pathogen *Listeria monocytogenes*. Although many different strains of *L. monocytogenes* are isolated from food, no definitive tests currently predict which isolates are most virulent. The objectives of this study were to address two major data gaps for risk assessors, variability among *L. monocytogenes* strains in pathogenicity and virulence. Strains used in our monkey clinical trial or additional food isolates were evaluated for their virulence and infectivity in mice. All strains were equally pathogenic to immunocompromised mice, causing deaths to 50% of the population 3 days after exposure to doses ranging from 2 to 3 log CFU. Doses resulting in 50% deaths on the fifth day after administration were 1 to 2 log lower than those on the third day, indicating that the full course of pathogenicity exceeds the 3-day endpoint in immunocompromised mice. Three strains were chosen for further testing for their virulence and infectivity in liver and spleen in normal (immunocompetent) mice. Virulence was not significantly different (\(P > 0.05\)) among the three strains, all resulting in deaths to 50% of mice at 5 to 7 log CFU by 5 days after administration. All strains were equally infective in liver or spleen, with higher numbers of *L. monocytogenes* directly correlated with higher doses of administration. In addition, there was no preference of organs by any strains. The lack of strain differences may reflect the limitation of the mouse model and suggests the importance of using various models to evaluate the pathogenicity and virulence of *L. monocytogenes* strains.

The Centers for Disease Control and Prevention, based on active surveillance from 2000, estimates that approximately 2,500 people in the United States become ill annually from foodborne *Listeria monocytogenes*, and approximately 500 die (6). The consumption of contaminated ready-to-eat foods is considered to be the principal route of *L. monocytogenes* infection. Soft cheese (3, 7, 19), pasteurized milk (4), and deli meats (5) have been associated with outbreaks of listeriosis. Four serotypes (4b, 1/2a, 1/2b, and 1/2c) among 13 known serotypes have been isolated from a wide range of foods (12). Three of these serotypes (4b, 1/2a and 1/2b) are associated with the majority of human listeriosis (12).

Risk factors for infection are underlying illnesses, impaired immune system, extreme age, and pregnancy (12, 20). In healthy individuals, infections are generally rare, sporadic, and mild, with symptoms characterized as flu-like (12, 19, 20). However, the outcome of listeriosis is serious in susceptible individuals, potentially leading to septicemia and meningitis (12, 19, 20). In pregnant women, listeriosis primarily affects the fetus or neonate and can result in spontaneous abortion or stillbirth. Studies have linked listeriosis to 1 to 6% of spontaneous abortions in western European countries (10).

Because human trials for *L. monocytogenes* are unethical because of the potential fatal outcome for susceptible individuals, animal surrogate studies must be developed to describe dose-response relationships for various adverse endpoints and for extrapolation to human dose-response models. Nonhuman primates have been used for dose-response studies (11, 28), but the cost and limited numbers of primates per dose group make the use of nonhuman primates for *L. monocytogenes* strain comparisons prohibitive. The objective of our study was to screen a panel of *L. monocytogenes* strains for pathogenicity and invasion of the liver and spleen in mice. The panel included strains used in a primate study (28) and additional strains isolated from food linked to human illness (5, 13, 27).

The mouse is the most commonly used species for the study of *L. monocytogenes*, and there are numerous publications on the pathogenicity, virulence, infectivity, genetic factors, and other characteristics of different *L. monocytogenes* strains. However, many studies use three or fewer strains and cannot be directly compared to other studies because of differences in strains used, animal models used for testing, or interlaboratory methods. Results obtained in this study provide information on virulence that can aid in direct comparisons of *L. monocytogenes* strains that are known human or primate pathogens and three food isolates. Additionally, we knew little about the virulence of the nonhuman primate clinical isolate, strain 12443, which we used in our pregnant nonhuman primate model (28), and this...
TABLE 1. Isolation source and serotype of Listeria monocytogenes strains

<table>
<thead>
<tr>
<th>Clinical isolate</th>
<th>Serotype</th>
<th>Source of isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scott A</td>
<td>4b</td>
<td>Human clinical isolate</td>
</tr>
<tr>
<td>G3982</td>
<td>4b</td>
<td>Human clinical isolate associated with outbreak linked to Mexican-style cheese</td>
</tr>
<tr>
<td>G3990</td>
<td>4b</td>
<td>Human clinical isolate associated with outbreak linked to hot dog</td>
</tr>
<tr>
<td>H7550</td>
<td>4b</td>
<td>Human clinical isolate associated with outbreak linked to hot dog</td>
</tr>
<tr>
<td>H9666</td>
<td>1/2c</td>
<td>Human clinical isolate from blood</td>
</tr>
<tr>
<td>12443</td>
<td>1/2a</td>
<td>Monkey clinical isolate</td>
</tr>
<tr>
<td>12375</td>
<td>4b</td>
<td>Monkey clinical isolate</td>
</tr>
<tr>
<td>101M</td>
<td>4b</td>
<td>Beef pork sausage</td>
</tr>
<tr>
<td>F6854</td>
<td>4b</td>
<td>Turkey frankfurters</td>
</tr>
<tr>
<td>H7776</td>
<td>4b</td>
<td>Frankfurters</td>
</tr>
</tbody>
</table>

This study helps provide information on this strain compared with known human pathogens.

MATERIALS AND METHODS

Mice. Female ICR mice, 18 to 20 g, were obtained from Harlan Sprague-Dawley (Indianapolis, Ind.). Mice were housed in groups of five in cages covered by wire lids and filters with free access to sterile food and water. The mice were held under these conditions for 24 h before the study began and throughout the study period. All animals used in this study were handled in accordance with National Institutes of Health guidelines, and their use was approved by the University of Georgia Institutional Animal Use and Care Committee.

Bacterial strains and preparation of inocula. The L. monocytogenes strains used in this study included several from our nonhuman primate study (human clinical isolates Scott A, G3982, G3990, and H7550) and monkey clinical isolates 12443 and 12375 (28) in addition to three foodborne isolates (101M, beef-pork sausage (13); F6854, turkey frankfurter (27); and H7776, frankfurter (5)) provided by the U.S. Department of Agriculture, Agricultural Research Service (USDA-ARS), Wyndmoor, Pa. (Table 1). Cultures were stored on latex beads at —80°C. Cultures were grown during three successive overnight transfers at 37°C in tryptic soy broth (Difco, Becton Dickinson, Sparks, Md.). Cells were washed twice with 0.01 M potassium phosphate-buffered saline (PBS; pH 7.2) by centrifugation (3,500 × g) for 10 min at room temperature, sedimented in a final wash, and then resuspended in PBS. Cell numbers in suspensions were determined by plate plating serial dilutions (1:10) on tryptic soy agar (Difco, Becton Dickinson) in duplicate. Colonies were enumerated after 24 h of incubation at 37°C. Inocula were prepared by diluting cell suspensions serially (1:10) in PBS.

Assessment of mouse pathogenicity. The pathogenicity of L. monocytogenes isolates was determined in immunocompromised female ICR mice by intraperitoneal (i.p.) injection of test strains. Mice were immunocompromised by i.p. injection of 200 mg/kg of body weight of carrageenan (Sigma type II, Sigma Chemical Co., St. Louis, Mo.) 24 h before inoculation with Listeria (16, 30). Five mice were injected with each strain in 0.1 ml PBS at approximately 4 log CFU. L. monocytogenes H9666 and Listeria innocua were used as positive and negative controls, respectively. Strain H9666 was previously tested in our laboratory and found to infect both immunocompromised and normal mice (30). Control mice were administered 0.1 ml of sterile PBS i.p. Mice were observed for death three times daily for 5 days. Any signs of illnesses, such as rough fur and lethargy (lack of movement), were also recorded. Isolates that caused at least one death within 5 days were designated as pathogenic (16). Strains that did not cause death within 5 days were designated as nonpathogenic.

Evaluation of dose response. The effect of different doses on mice was determined for L. monocytogenes strains that were pathogenic to immunocompromised mice. Mice were immunocompromised as previously described. Five concentrations of each L. monocytogenes strain were prepared as described above and inoculated in mice (five mice per concentration). Concentrations administered were based on the outcome of the pathogenicity screening test. Control mice were given 0.1 ml of sterile 0.01 M potassium PBS (pH 7.2). L. monocytogenes H9666 and L. innocua were inoculated in mice to serve as positive (virulent) and negative (nonvirulent) controls, respectively. Mice were observed for death three times a day for 5 days. The method of Reed and Muench (26) was used to estimate doses that resulted in the death of 50% of mice. When 50% of deaths occurred outside the range of the dose tested, the 50% lethal dose (LD50) was determined by extrapolation.

Infectivity determination. L. monocytogenes inocula of pathogenic strains were prepared as described above. Based on results of the pathogenicity experiment, one concentration that resulted in consistent death of the mice was selected for the evaluation of infection of liver and spleen. Three L. monocytogenes strains were inoculated i.p. in normal (immunocompetent) mice. A known virulent strain of L. monocytogenes (H9666) and a non-virulent strain (L. innocua) were used as positive and negative controls, respectively. Control mice were injected with PBS. Based on preliminary experiments and previous work in our laboratory (30), necropsy dates were selected. Mice were euthanized by carbon dioxide asphyxiation, and necropsy was performed. Livers and spleens were aseptically removed from mice, weighed, and macerated in PBS. The tissue suspensions were serially diluted in PBS and surface plated on tryptic soy agar in duplicate to determine the bacterial counts in each organ. Plates were incubated at 37°C for 24 h prior to enumeration. Colonies were examined for typical Listeria appearance by hemolysis test. Select colonies were confirmed as L. monocytogenes by standard tests (12, 16). In addition, pulsed-field gel electrophoresis (PFGE) patterns of isolates from tissue were compared with the strains used for inoculation to determine the stability of the original strains during passage through the mouse. PFGE (15) was performed using Ascl as a restriction enzyme and L. monocytogenes strain H2446, which produces 14 bands with Ascl, as a standard.

Statistical analysis. Data were analyzed statistically by analysis of variance and Duncan’s multiple range test using Statistical Analysis Software (version 6.12, SAS, Cary, N.C.) at 5% level of significance.

RESULTS

Assessment of mouse pathogenicity and lethality dose determination. All strains except for L. innocua (non-pathogenic control) were pathogenic to immunocompromised mice. PBS injected i.p. did not kill any mice, either immunocompromised or normal. Inoculation with L. monocytogenes caused mouse coats to appear rough 2 to 3 days after inoculation. If the illness progressed, mice developed...
TABLE 2. Dose of Listeria monocytogenes strains required to result in deaths to 50% of immunocompromised mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Day 3</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. innocua</td>
<td>No death</td>
<td>No death</td>
</tr>
<tr>
<td>H9666</td>
<td>4.4 ± 0.5</td>
<td>2.5 ± 0.5</td>
</tr>
<tr>
<td>G3982</td>
<td>3.6 ± 1.4</td>
<td>2.4 ± 0.4</td>
</tr>
<tr>
<td>12443</td>
<td>5.7 ± 1.2</td>
<td>3.3 ± 0.9</td>
</tr>
<tr>
<td>Scott A</td>
<td>3.8 ± 1.4</td>
<td>2.5 ± 0.6</td>
</tr>
<tr>
<td>H7550</td>
<td>4.4 ± 1.5</td>
<td>2.9 ± 1.3</td>
</tr>
<tr>
<td>12375</td>
<td>4.0 ± 1.6</td>
<td>3.0 ± 1.4</td>
</tr>
<tr>
<td>G3990</td>
<td>4.8 ± 0.9</td>
<td>3.2 ± 1.0</td>
</tr>
<tr>
<td>F6854</td>
<td>4.2 ± 1.3</td>
<td>2.5 ± 0.8</td>
</tr>
<tr>
<td>H7776</td>
<td>5.3 ± 1.0</td>
<td>3.2 ± 1.7</td>
</tr>
<tr>
<td>101M</td>
<td>4.7 ± 0.1</td>
<td>2.7 ± 0.1</td>
</tr>
</tbody>
</table>

There were no significant differences among strains. LD_{50} values were significantly higher on day 3 compared to day 5 for all L. monocytogenes strains tested.

The invasion and persistence of each strain in the liver and spleen was evaluated in normal mice for two L. monocytogenes test strains, 12443 and G3982, and pathogenic and nonpathogenic controls, L. monocytogenes H9666 and L. innocua (Figs. 1 and 2). There were no significant differences (P > 0.05) among strains in their persistence in liver or spleen. L. monocytogenes strains (CFU) were equally recovered from both organs of all treated mice. An increase in recovery was observed at higher doses for all strains. For mice treated with L. innocua, 60% had livers and spleens positive for L. innocua. There were no incidences where L. innocua was recovered from only one of the two organs without recovery from the other.

**DISCUSSION**

All L. monocytogenes strains tested in our study, except the negative control strain L. innocua, were pathogenic to

![FIGURE 1. Comparison of the infectivity of different Listeria monocytogenes strains in liver of normal mice.](image-url)
immunocompromised mice regardless of the source. The LD50 doses determined in the study were comparable to those observed in other studies using i.p. injection (1, 8, 21, 25). However, the differences in virulence among strains reported by other researchers were not observed in our study (21, 25, 29). Barbour et al. (2) has shown that intravenous injections (i.v.) result in lower LD50 estimates in mice than intragastric exposures. This result is expected because i.v. injection bypasses host defense mechanisms that normally constrain progression of disease from oral exposure routes. For the same reasons, differences may also be expected for LD50 estimates from i.p. injections compared with oral infection (17). The increased dose of L. monocytogenes strains required to cause deaths in 50% of normal (immunocompetent) mice as compared with immunocompromised mice confirms that impaired immunity increases the risk of listeriosis (29). However, in our study, pathogenesis in both normal and immunocompromised mice appears to require more than 3 days for full development based on the significantly lower (P < 0.05) LD50 at five days posttreatment compared with 3 days posttreatment (Tables 2 and 3).

In a normal course of infection by L. monocytogenes from foods, the organism will enter the host through epithelial cells in the intestinal tract (9, 22). Once inside the host cell, L. monocytogenes can evade cell-mediated immunity, multiply in the cytoplasm, and migrate toward the cell periphery to invade macrophages and enter the lymphatics and lymph nodes. At this point, L. monocytogenes can enter the bloodstream and further disseminate to tissues such as liver and spleen. The extent of invasion was previously related to the virulence of strains (2, 23). In our study, we recovered similar levels (CFU) of all three strains from liver and spleen of normal mice. These results are not surprising because there was no significant difference (P > 0.05) in doses required to cause deaths in 50% of mice. However, higher rates of invasion observed at higher levels of inoculum provide evidence of dose dependency for severity of listeriosis regardless of strain. Such a mechanism suggests that a high dose of pathogens may overwhelm the host defenses, resulting in an increased likelihood that a fraction of the pathogen dose successfully evades the host immune defenses, survives to invade host tissues, and replicates in host tissues to the magnitude necessary to cause severe damage. In contrast, this mechanism of density-dependent severity suggests that low dose challenges may be effectively eliminated by host defenses prior to significant tissue damage.

In our study, as in others, invasion of liver and spleen was also observed with nonpathogenic L. innocua. Mendier et al. (23) observed infection of spleen and liver in 5 and 10% of mice at 0.13 and 0.24 log CFU/g of organ, respectively, after exposure to 10^6 CFU L. innocua by the i.p. route of exposure. von Koenig et al. (31) has also demonstrated that L. innocua can enter the spleen at approximately 4 log CFU per spleen when mice are i.p. infected at 9 log CFU, although L. innocua was incapable of growing in the spleen and steadily decreased in numbers with time. After 3 days postinoculation, the numbers decreased to approximately 2 log CFU per spleen and became undetectable after 6 days (31). Our observed rates and levels of infection of liver and spleen by L. monocytogenes are higher than these observations. Differences in susceptibility to listeriosis among different mouse strains has been demonstrated (14, 21) and may account for the differences in our results from previous studies.

Golnazarian et al. (14) used hydrocortisone acetate or cimetidine to immunocompromise mice and subsequently i.p. injected mice with L. monocytogenes strains in doses ranging from 1.0 to 7.0 log CFU/ml. In our study, we used carrageenan to immunocompromise mice and 24 h later injected L. monocytogenes in doses ranging from 1 to 7 log CFU/ml. Carrageenan was selected as the macrophage-suppressing agent because of its ability to cause an eightfold decrease in the number of macrophages in mouse peripheral blood within 2 days and because of its long-lasting effect, with full recovery requiring 15 days (29). Cortisone acetate has also been shown to suppress cellular immunity and to increase the susceptibility of mice to Listeria infection (14, 24). However, cortisone acetate's effect wanes rapidly after 24 h (24), indicating that it would probably not be effective for the duration of the infection. Golnazarian et al. (14) reported LD50 results from i.p. injected mice ranging from 3 to 6 log CFU/ml. Other reported values for the LD50 of L. monocytogenes in mice range from 3 to 10 log CFU, depending on the strain of Listeria, the strain of mouse, and the route of inoculation (1, 14). In order for valid inferences to be drawn from the diverse reports of LD50 from various mouse models to predict likelihood and severity of adverse effects in humans, a conceptual model is needed that elucidates the controlling events of pathogenesis for scaling within and between host species.

The difficulty of using animal models to study the
mechanisms of pathogenicity and virulence of \textit{L. monocytogenes} to understand human listeriosis has been recognized by several researchers. One example is recent evidence for the role of intercellin for \textit{L. monocytogenes} invasion of human epithelial cells. Mouse E-cadherin is not a receptor for intercalin as it is in humans (18). Other factors may also be different between mouse and human listeriosis. In addition, different methods of administration of \textit{L. monocytogenes} may result in different outcomes due to changes in the course of infection. Our study demonstrates the importance of evaluating the pathogenicity and virulence of \textit{L. monocytogenes} strains in multiple animal models to expand knowledge of the mechanisms of listeriosis and enable more rigorous scientific inferences to build defensible dose-response models for human listeriosis.

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**REFERENCES**