Hemolysin Is Required for Extraintestinal Dissemination of *Listeria monocytogenes* in Intragastrically Inoculated Mice

JON T. ROLL† AND CHARLES J. CZUPRYNSKI*

Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, Madison, Wisconsin 53706

Received 9 March 1990/Accepted 6 June 1990

In this study we demonstrated that a hemolytic strain of *Listeria monocytogenes*, but not a nonhemolytic mutant derived from it, translocated in substantial numbers to the mesenteric lymph nodes, spleen, and liver after intragastric inoculation of mice. Growth at 4°C prior to inoculation did not increase the virulence of the nonhemolytic mutant. These results indicate that hemolytic activity is required for the virulence of *L. monocytogenes* via the gastrointestinal tract, as has been shown previously for parenteral challenge.

*Listeria monocytogenes* is a gram-positive, facultative, intracellular pathogen of animals and humans that can cause severe disease in pregnant women and in immunocompromised individuals. Two recent severe outbreaks of human listeriosis were traced to ingestion of listeria-contaminated dairy products (1, 14), in which *L. monocytogenes* can grow to large numbers at ambient temperatures (15). The ability of *L. monocytogenes* to secrete a hemolysin appears to be the principal determinant of its ability to multiply within macrophages in vitro and to be virulent for parenterally injected mice (2, 5, 6, 9, 12, 16). This hemolysin is sulfhydryl dependent and is antigenically and structurally similar to streptolysin O (6). The purpose of our study was to determine whether hemolytic and nonhemolytic strains of *L. monocytogenes* differ in their abilities to establish infection when they are inoculated intragastrically (i.g.), since the gastrointestinal tract is the natural route of infection.

Five- to six-week-old male (C57BL/6 × DBA/2)F<sub>1</sub> mice (BDF1) were obtained from The Jackson Laboratory (Bar Harbor, Maine). These mice were housed under plastic microisolator cages (Lab Products, Frederick, Md.) at the Charmany Animal Care Facility of the School of Veterinary Medicine (an American Society for the Accreditation of Laboratory Animal Care-approved facility) and given Purina Lab Chow (Ralston-Purina, St. Louis, Mo.) and water ad libitum. In all instances, mice were 8 to 12 weeks of age when used in experiments. Animals were cared for and used according to the Public Health Service Guide for the Care and Use of Laboratory Animals.

All strains of *L. monocytogenes* were maintained in tryptose phosphate broth (Difco Laboratories Detroit, Mich.) with 20% glycerol at −70°C. Before each experiment, an aliquot was thawed, inoculated into 5 ml of tryptose phosphate broth, and incubated overnight at 37°C. The next morning the contents of this culture were inoculated into a flask containing 30 ml of fresh tryptose phosphate broth and incubated at 37°C until visibly turbid. To obtain a culture grown at 4°C, the overnight culture was similarly inoculated into fresh tryptose phosphate broth and incubated at 4°C for approximately 1 week. The bacteria were harvested by centrifugation; enumerated by turbidometric readings, using a previously determined standard curve; and resuspended in pyrogen-free saline at the appropriate concentration. Mice were anesthetized with an intraperitoneal injection of sodium pentobarbital and then inoculated i.g. with 2 × 10<sup>9</sup> *L. monocytogenes* cells in 0.4 ml of pyrogen-free saline by using an infant feeding tube (3.5 French) as described previously (4). This challenge dose was chosen because with hemolytic *L. monocytogenes* EGD, it results in a solid sublethal infection in all animals inoculated (4, 10; unpublished observations). We have previously determined that intraperitoneal injection of sodium pentobarbital does not influence the severity of listeriosis in mice (4). The bacterial burdens in various organs of the mice were determined as described previously (4). Mice were sacrificed by cervical dislocation, and their spleens, mesenteric lymph nodes, and portions of the livers and ceca were removed to separate sterile-glass tissue grinders that contained cold pyrogen-free saline. The spleens, livers, and mesenteric lymph nodes were thoroughly homogenized, serially diluted, plated on Trypticase soy agar with 5% sheep blood (Baxter, Chicago, Ill.), and incubated at 37°C for 24 h. The ceca and their contents were similarly homogenized, diluted in pyrogen-free saline, and then plated on a differential selective agar (17) that was incubated at 37°C for 47 to 72 h. Results are expressed as the mean ± standard error of the mean CFU of *L. monocytogenes* per organ.

Three strains of *L. monocytogenes*, i.e., the parental hemolytic wild type (1 wk 39), a nonhemolytic mutant (DP-L215), and a hemolytic revertant of this mutant (DP-L291), were generously provided by D. Portnoy (Philadelphia, Pa.) and used in this study (12, 16). It is not known whether strain DP-L215 is a true null mutant or produces a truncated, inactive hemolysin (D. Portnoy, personal communication). In our first experiment, we compared the abilities of these three strains to establish systemic infection of the spleen and liver after i.g. inoculation (Fig. 1). When the mice were sacrificed at 72 h postinoculation, we recovered similar and substantial numbers of the wild-type and revertant strains from the spleens and livers, whereas in contrast, the nonhemolytic mutant was not recovered from the spleen or liver of any mouse (limit of detection, 100 viable listeriae per tissue homogenate).

These results led us to investigate more closely the effects

---

*Corresponding author.
† Present address: Department of Bacteriology, E. B. Fred Hall, University of Wisconsin-Madison, Madison, WI 53706.
of hemolytic activity on the course of *L. monocytogenes* infection in i.g. inoculated mice. In this experiment, one group of mice was inoculated with the hemolytic parental strain grown at 37°C, a second group was inoculated with the nonhemolytic mutant grown at 37°C, and a third group was inoculated with the nonhemolytic mutant grown at 4°C. This last group was included because of evidence that growth at 4°C can increase the virulence of *L. monocytogenes* under some circumstances (4). All three inoculants exhibited a similar poor ability to survive within the cecum, being largely eliminated within 4 to 6 days (Fig. 2A). However, we recovered only a few viable nonhemolytic *L. monocytogenes* from the mesenteric lymph nodes at any time (Fig. 2B), which suggests either that the mutant did not translocate across the intestinal epithelium or that it did not survive after having done so. As a result, the nonhemolytic mutant was unable to establish a systemic infection in either the spleen (Fig. 2C) or the liver (Fig. 2D). In contrast, the parental hemolytic strain was recovered in substantial numbers from the mesenteric lymph nodes, spleens, and livers at 2 through 6 days after inoculation. Previous results with hemolytic *L. monocytogenes* EGD suggest that the listeriae would have been cleared from the spleen and liver in 10 to 14 days after challenge (10; unpublished observations), but we did not examine that possibility in this study.

We went on to ask whether the self-limiting infection that resulted from i.g. inoculation of nonhemolytic *L. monocytogenes* would be sufficient to generate protective immunity against a subsequent i.g. re-challenge with virulent hemolytic *L. monocytogenes*. In a single preliminary experiment, we observed that mice inoculated i.g. with 2 x 10^8 nonhemolytic *L. monocytogenes* cells 2 weeks before rechallenge with the hemolytic parental strain yielded significantly fewer (P < 0.05) viable listeriae from the mesenteric lymph nodes at 3 days after challenge than did nonimmunized control mice (mean ± standard error of the mean, 1.96 ± 0.33 and 3.48 ± 0.31 log_{10} CFU, respectively). There was no statistically significant difference between immunized and nonimmunized mice in the numbers of listeriae recovered from the ceca, spleens, and livers (data not shown).

Previous reports have documented the importance of the hemolysin of *L. monocytogenes* to its ability to survive intracellularly in vitro (5, 7, 9, 12, 16) and to its virulence after parenteral inoculation in vivo (2, 12). The results of the present study extend these observations to experimental infection of the gastrointestinal tract, which is likely to be the predominant route of natural infection (1, 14). Nonhemolytic and hemolytic *L. monocytogenes* did not differ in their abilities to survive in the gastrointestinal tract; both were eliminated in 4 to 6 days after i.g. inoculation. Nonhemolytic *L. monocytogenes* were rarely recovered, and then in low numbers, from the mesenteric lymph nodes, spleens, and livers after i.g. inoculation. In contrast, i.g. inoculation of the hemolytic parental strain resulted in substantial infection of these organs, as has been described previously for hemolytic wild-type *L. monocytogenes* in guinea pigs (13), mice (4, 10, 11, 17), and rats (3).

The results of this study do not allow us to explain how hemolytic *L. monocytogenes* traverses the gut epithelium. Previous in vivo (13) and in vitro (5) studies have described the ability of *L. monocytogenes* to invade and multiply within intestinal epithelial cells. Other workers have proposed that the major route of invasion of *L. monocytogenes* after i.g. inoculation was through the Peyers' patches and other gut-associated lymphoid tissues (10). The inability of nonhemolytic *L. monocytogenes* to infect the mesenteric lymph nodes, spleen, and liver may reflect its inability to attach to and invade gut epithelial and lymphoid cells. Existing evidence suggests, however, that the hemolysin is principally involved in intracellular survival and multiplication rather than in the initial invasion of cells (9, 12) and that some other determinant may regulate cell invasion (8). If so, then a more likely interpretation of our data may be that nonhemolytic *L. monocytogenes* failed to survive and multiply after gaining access to the intestinal mucosa in our experiments.

Acquisition of antilisteria resistance generally requires clearance of a sublethal infection of sufficient intensity and duration to activate a protective cellular immune response. Previous results have indicated that clearance of an intestinal infection with virulent *L. monocytogenes* protected mice against subsequent rechallenge with the same organism (3, 10; unpublished observations). We therefore were intrigued with determining whether the limited infection caused by nonhemolytic *L. monocytogenes* would be sufficient to engender resistance to rechallenge with virulent hemolytic listeriae. Our preliminary results indicate that there may have been stimulation of some local immunity in the mesenteric lymph nodes but no significant reduction in the bacte-
were nonhemolytic strains of L. monocytogenes. By nodes (MLN) (B), spleen (C), and liver (D) of each mouse were determined as described in the text. *, P < 0.05 for hemolytic versus nonhemolytic strains of L. monocytogenes.

FIG. 2. Course of infection of hemolytic and nonhemolytic L. monocytogenes in i.g. inoculated mice. Mice received 2 × 10⁶ hemolytic (○) or nonhemolytic (●) L. monocytogenes cells grown at 37°C or 2 × 10⁶ nonhemolytic L. monocytogenes grown at 4°C (△). Groups of four mice were killed at the indicated times, and the mean ± standard error of the mean log₁₀ L. monocytogenes in the cecum (A), mesenteric lymph nodes (MLN) (B), spleen (C), and liver (D) of each mouse were determined as described in the text. *, P < 0.05 for hemolytic versus nonhemolytic strains of L. monocytogenes.

rial burden in the spleen and liver. Although these results are modest, it might be possible to develop strategies (repeated administration, use of adjuvants, etc.) to use avirulent nonhemolytic L. monocytogenes to protect against gastrointestinal listeriosis.

This work was supported by Public Health Service grant AI 21343 and by funds from the University of Wisconsin-Madison graduate school.

We thank Daniel Portnoy for generously providing the L. monocytogenes strains that are described in this paper. We thank the School of Veterinary Medicine word processing personnel for preparing the manuscript.

LITERATURE CITED


