APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1992, p. 3991–4000
0099-2240/92/123991-10$02.00/0

Vol. 58, No. 12

Determination of Virulence of Different Strains of *Listeria monocytogenes* and *Listeria innocua* by Oral Inoculation of Pregnant Mice

A. M. LAMMERDING,[1]† K. A. GLASS,[1] A. GENDRON-FITZPATRICK,[2] AND M. P. DOYLE[1]‡*

Food Research Institute, 1925 Willow Drive,[1] and Research Animals Resources Center,[2] University of Wisconsin, Madison, Wisconsin 53706

Received 29 June 1992/Accepted 1 October 1992

A pregnant mouse model was developed to follow the course of infection after peroral inoculation with six different strains of *Listeria monocytogenes* and one strain of *Listeria innocua*. Tissues were sampled and analyzed by microbiologic and histologic methods for 5 days postinoculation. In gnotobiotic pregnant BALB/c mice, *L. monocytogenes* Scott A (SA), serotype 4b, colonized the gastrointestinal tract, translocated to the livers and spleens of mice by day 1 postinoculation, and multiplied in these tissues until day 4. Infection of the placental tissues occurred by days 3 and 4 and was followed by infection of the fetuses. Little damage of colonic and cecal tissues was evident by histologic examination. Livers and spleens showed a cellular immune response; a similar immune response was not detected in the placentas or fetuses. A rough variant of *L. monocytogenes* SA which was as virulent as the parent strain in mice when injected intraperitoneally was less virulent perorally and did not consistently infect the fetuses. *L. monocytogenes* ATCC 19113, serotype 3a, did not colonize the gastrointestinal tract, nor was it isolated from any internal tissue. *L. monocytogenes* strains of serotypes 1/2a and 1/2b behaved like the SA strain in this mouse model. *L. innocua* colonized the gastrointestinal tract and translocated to the livers and spleens but did not survive in these organs and rapidly disappeared without infecting placental and fetal tissues. In comparison with gnotobiotic mice, conventional pregnant mice inoculated with *L. monocytogenes* strains showed less consistent infection. These results suggest that the gnotobiotic pregnant mouse is a useful model for detecting differences in virulence relating to colonization, invasiveness, and uteroplacental infection which cannot be detected by intraperitoneal inoculation of mice.

Until the early 1980s, human cases of listeriosis were considered, for the most part, "accidental with no relevant source found" (48). Now, it is recognized that ingestion of foods contaminated with *Listeria monocytogenes* is a major route of infection in human listeriosis. Outbreaks as well as isolated cases of the disease have been associated epidemiologically with foods (17, 40, 46). Asymptomatic intestinal carriage of *L. monocytogenes* is hypothesized to lead to listeriosis as a secondary infection in cases of enteritis in susceptible individuals (44, 47). The incidence of human listeriosis, however, is relatively infrequent, despite the fact that *L. monocytogenes* is a common contaminant in many foods (17, 24). A recent survey found that the annual incidence of listeriosis was 7.4 cases per million population in the United States (45).

Host susceptibility is a major factor in the epidemiology of listeriosis, as most outbreaks and sporadic cases involve persons with impaired cell-mediated immunity due to disease processes, medications, pregnancy, or aging (35, 45, 46). The infective dose of the organism is not known. Illness has been reported in apparently immunocompetent individuals after ingestion of 10^6 to 10^9 cells (17), and it is probable that smaller populations will cause illness in immunocompromised persons. However, food surveys have identified retail foods that were not associated with any illness and contained 10^2 to 10^7 cells per gram (17). Of the 13 known serotypes of *L. monocytogenes*, many of which are found in foods, only three (serotypes 1/2a, 1/2b, and 4b) are associated with the majority of human illness (46). Strains of a single serotype, 4b, are predominantly responsible for confirmed outbreaks and sporadic cases of food-borne listeriosis (17, 35, 46).

Characterization of *L. monocytogenes* by tests more discriminating than serotyping, such as multilocus enzyme electrophoresis, reveals that outbreak strains are phenotypically identical or closely related (6, 39). Although host susceptibility and undoubtedly the populations of organisms ingested are significant factors in the incidence of food-borne listeriosis, the epidemiologic data also suggest that not all strains of *L. monocytogenes* are equally pathogenic for humans. Studies seeking to identify possible virulence factors on the basis of in vitro characteristics have not provided an explanation for the predominance of certain serotypes causing most cases of human listeriosis.

Virulence of *Listeria* spp. is most frequently assessed by lethality in adult mice inoculated intraperitoneally (i.p.) with the organism (2, 5, 11, 22, 34). Because this assay sometimes fails to differentiate between virulent and avirulent *Listeria* spp., a modified method in which mice are immunocompromised by pretreatment with carrageenan, a macrophage-suppressing agent, has been used (13, 50). The resulting immunosuppression decreases the i.p. 50% lethal dose (LD_{50}) of *L. monocytogenes* but not of the nonpathogenic species *Listeria ivanovii*, *Listeria seeligeri*, and *Listeria innocua*. Both these techniques assess lethality as the criterion for virulence and fail to measure gastrointestinal inva-

* Corresponding author.
† Present address: Food Safety/New Hazards Laboratory, Food Production and Inspection Branch, Agriculture Canada, Guelph, Ontario N1G 3W4, Canada.
‡ Present address: Food Safety and Quality Enhancement Laboratory, University of Georgia, Georgia Station, Griffin, GA 30223.

siveness, a criterion important in naturally occurring infection. The ability of the organism to cause placental and fetal infection is also an important aspect of the pathogenesis of human disease, since pregnant women and neonates represent a significant proportion of the cases in food-borne outbreaks as well as in sporadic listeriosis (17, 36, 45, 46). Previous studies in pregnant mice have used intravenous routes of infection (32, 42).

The objectives of this study were to characterize the pathogenesis of peroral listeric infection and then determine whether peroral inoculation of pregnant mice could be used as a better model to differentiate between strains of *L. monocytogenes* by using invasiveness and infection of placental and fetal tissues as the criteria for virulence. Characterizing isolates on the basis of differences in these criteria may provide insights for further in vitro studies to distinguish among strains of *L. monocytogenes*. The ability to differentiate between virulent and possibly avirulent or less virulent strains is needed to understand the significance and epidemiology of *L. monocytogenes* in the food supply.

MATERIALS AND METHODS

Bacterial strains and preparation of inocula. Serotypes of the test strains were determined by the U.S. Centers for Disease Control, Atlanta, Ga., and/or by the subfactor typing scheme of the U.S. Food and Drug Administration (FDA), Minneapolis, Minn. (3). The six *L. monocytogenes* strains were Scott A (SA; serotype 4b), a human clinical isolate from a food-borne outbreak in Massachusetts (19); V7 (serotype 1/2a), a raw milk isolate; 316 (serotype 1/2a), a meat isolate; Scott A Rough (SAR), a spontaneous variant of SA which produced filamentous colonies on solid agar media; ATCC 19113 (serotype 3a); and 108 (serotype 1/2b [Centers for Disease Control] and serotype 1a factor 1 [FDA]), which was isolated from meat. Strains SA and V7 were obtained from J. D. Lovett, FDA, Cincinnati, Ohio; ATCC 19113 was purchased from the American Type Culture Collection; and strains 316, SAR, and 108 were isolated at the Food Research Institute, Madison, Wis. The SAR strain was isolated after successive transfers of the parent strain on nutrient agar (Difco Laboratories, Detroit, Mich.) and tested to ensure its stability and phenotypic and serologic identity with the parent strain. Strain ATCC 19113 was submitted to the FDA for confirmation of serotype and was typed as 3a factor 4 but also reacted as serotype 1a factor 2. *L. innocua* 404 (serotype 4b factor 6 [FDA]) was isolated from a meat product at the Food Research Institute.

Cultures were grown overnight at 37°C in 100 ml of tryptose broth (Difco). Cells were harvested by centrifugation (3,500 × *g* for 30 min at 4°C) and suspended in 0.01 M potassium phosphate-buffered saline (PBS), pH 7.2, to an optical density at 500 nm of 0.5 (approximately 5×10^9 CFU/ml). Cell suspensions were serially diluted 1:10 in sterile PBS and surface inoculated onto duplicate plates of tryptose agar (Difco) and enumerated after 48 h of incubation at 37°C. Lethality of the test strains was assessed by i.p. inoculation of 10^9 CFU of listeriae into nonpregnant ICR mice (Harlan Sprague Dawley, Indianapolis, Ind.) according to FDA protocol (18). The i.p. LD_{50}s (16) were determined for SA, SAR, and ATCC 19113 in normal and immunocompromised ICR mice. Mice were immunocompromised by i.p. injection of 200 mg of carrageenan (Sigma Type II, Sigma Chemical Co., St. Louis, Mo.) per kg of body weight 24 h before inoculation with listeriae (50).

Animals and inoculation procedure. Germfree pregnant BALB/c mice, 8 to 10 weeks old and at 9 to 15 days of gestation, were obtained from the Gnotobiote Center, University of Wisconsin, Madison. During the study, the mice were housed in sterile, wire-bottom cages covered by filter hoods, with free access to sterile food and water. The mice were held under these conditions for 24 h before inoculation. Gnotobiotic mice were inoculated perorally with 1.0×10^9 to 5.0×10^9 CFU of listeriae in 0.3 ml of PBS, administered with a sterile 20-gauge stainless steel gavage. This dose was determined in preliminary studies to result in consistent invasive infection by *L. monocytogenes* SA. Control mice were given 0.3 ml of sterile PBS. Conventional BALB/c mice, bred and raised at the Food Research Institute, were similarly inoculated with *L. monocytogenes* strains. All animals used in the course of this work were handled in accordance with National Institutes of Health guidelines.

Sampling. Mice perorally inoculated with *L. monocytogenes* strains were killed by carbon dioxide asphyxiation on days 1, 3, 4, and 5 postinoculation. Mice inoculated with *L. innocua* 404 and control mice for all studies were sampled on days 1, 3, and 5 postinoculation. Livers, spleens, and intestinal tissues (ceca/colons) were removed aseptically and separated into approximately equal portions for microbiologic and histologic analyses. Fetal-placental units were removed by incising along the long axis of the uterine horn. Approximately one-half of the fetal-placental unit from each mouse was used for microbiology, and one-half was used for histology. For bacterial culture, the units were dissected to separate the placental tissues from the fetus. The placental tissues were composed of the chorionic plate, the placental labyrinth, the spongiotrophoblast, the decidua, and the maternal uterine wall. Fetal tissues included the fetus itself, the remnants of the yolk sac placentation, the amnion, and umbilical vasculature surrounding the fetus. Fetal tissues for bacterial enumeration from a single mouse were pooled, as were the placental tissues. Maternal blood for microbiologic culture was drawn by cardiac puncture.

A further study was conducted to examine the early invasive stages with *L. monocytogenes*. Four gnotobiotic pregnant mice were inoculated perorally with SA and killed at 0, 12, 16, and 20 h postinoculation. The small intestines of these mice were included in the samples for analysis.

Enumeration of *L. monocytogenes*. Tissue samples were weighed and macerated with 9 volumes of sterile 0.1% peptone. Samples of cecum/colon tissues and their contents from each animal were tested as one sample. The tissue suspensions were serially diluted 1:10 in sterile PBS, and 0.1 ml of each dilution was surface inoculated onto duplicate plates of lithium chloride-phenylethanol-moxalactam agar (31) (Difco; moxalactam, Sigma). Blood samples were directly surface inoculated without dilution. Typical listeria-like colonies were enumerated after 48 h of incubation at 37°C. Two isolates from each culture-positive tissue were confirmed as *L. monocytogenes* or *L. innocua* by standard tests (9). Serologic confirmation of the same isolates was done by slide agglutination with *Listeria* O Types 1 and 4 and Poly 1,4 antisera (Difco). Antiserum for *L. monocytogenes* serogroup 3 was not commercially available; hence, strain ATCC 19113 could not be confirmed by a positive slide agglutination reaction. However, the isolates recovered from mice inoculated with ATCC 19113 were tested for absence of agglutination with Types 1 and 4 and Poly antisera. Representative isolates were inoculated into ICR mice (10^9 CFU per mouse) to test lethality by i.p. injection.

Histopathology. Reagents for preparation of histologic specimens were obtained from Sigma unless otherwise

TABLE 1. Numbers of pregnant BALB/c mice perorally inoculated with *L. monocytogenes* and *L. innocua* examined on each sampling day

Class of mice	Strain injected	No. of pregnant mice on postinoculation day:				No. of pregnant mice (total)
		1	3	4	5	
Gnotobiotic	*L. monocytogenes* SA	13	14	13	8	48
	L. monocytogenes SAR	10	9	8	7	34
	L. monocytogenes ATCC 19113	5	5	7	5	22
	L. monocytogenes V7	5	3	2	2	12
	L. monocytogenes 316	3	3	3	1	10
	L. innocua 404	12	11	ND[a]	13	36
Conventional	*L. monocytogenes* SA	5	5	4	5	19
	L. monocytogenes 108	4	3	4	4	15
	L. monocytogenes ATCC 19113	5	5	5	9	24

[a] ND, not done.

noted. Tissues were fixed according to the procedures of Isobe et al. (23) and embedded with JB-4 glycol methacrylate (Polysciences, Inc., Warrington, Pa.) according to the manufacturer's instructions. Specimens were sectioned at a thickness of 4 to 5 μm and stained with hematoxylin and eosin (HE) and by Gram stain. From animals inoculated with strain SA, fixed tissue sections cut sequential to the sections stained by HE and Gram stain were immunostained (StrAviGen Supersensitive System, BioGenex Laboratories, San Ramon, Calif.) to confirm the presence of *L. monocytogenes*. The immunoperoxidase staining procedure was carried out at room temperature with minor modifications to the manufacturer's instructions as follows: the glycol methacrylate sections were pretreated in saturated sodium ethoxide, and endogenous peroxidase activity was blocked with 3% methanolic hydrogen peroxide (10). Sections were digested in a 1:100 dilution of ficin in 0.05 M Tris-buffered saline, pH 7.2. The primary antibody was *Listeria* O Type 4 (Difco), diluted 1:100 in Tris-buffered saline. The chromogen, 3,3'-diaminobenzidine tetrahydrochloride, prepared in substrate buffer but without hydrogen peroxide, was applied to the sections for 30 min. The sections were drained, and the reaction mixture was developed with 3,3'-diaminobenzidine tetrahydrochloride plus hydrogen peroxide in the same substrate solution. Sections were counterstained with Harris's hematoxylin and sequentially dehydrated before mounting. Slides were read by a board-certified veterinary pathologist (A.G.-F.).

The χ^2 test (49) was used to evaluate the statistical significance of differences between proportions of infected tissues among groups of inoculated mice.

RESULTS

Characterization of strains. On tryptose agar, all listeriae except SAR produced colonies of typical morphology. Strain SAR cells were linked in long chains and on solid media produced filamentous colonies. Otherwise, the rough strain was identical to SA in hemolytic activity, biochemical tests, and serotyping. The i.p. LD_{50}s for *L. monocytogenes* SA, SAR, and ATCC 19113 in nonpregnant ICR mice were 7.8×10^5, 2.6×10^5, and $>10^9$ CFU, respectively. In mice immunocompromised by pretreatment with carrageenan, the LD_{50}s decreased to 6.3×10^1 CFU for SA and 1.4×10^2 CFU for SAR, whereas that of ATCC 19113 was $>10^9$ CFU. Actual LD_{50}s for *L. monocytogenes* V7, 316, and 108 were not determined, but each of these strains killed five of five mice within 5 days, usually in 1 to 3 days, when the mice were inoculated i.p. with 10^9 CFU. *L. innocua* 404 occasionally caused the death of one to five ICR mice at i.p. doses of 10^9 CFU per mouse, with deaths occurring between days 2 and 6 postinoculation. This was observed mainly when young mice weighing 16 to 19 g were used for these inoculations (repeated three times) but not when larger mice were used (repeated twice). *L. innocua* 404 was not tested in immunocompromised mice.

Peroral infection studies. (i) Microbiologic findings. *L. monocytogenes* SA, V7, 316, SAR, and ATCC 19113 and *L. innocua* 404 were tested in gnotobiotic pregnant BALB/c mice. Of these, strains SA and ATCC 19113 were also tested in conventional mice, and *L. monocytogenes* 108 was tested only in conventional mice. The type and number of mice inoculated with each strain are summarized in Table 1. The overall progression of infection resulting from peroral inoculation of pregnant mice was determined by calculating the mean $\log_{10}$ CFU of listeriae per gram of tissue from mice sampled each day from all trials. When no recoveries were made (i.e., <50 CFU/g, the threshold of detection), a $\log_{10}$ value of zero was given.

All strains of *L. monocytogenes* except ATCC 19113 colonized the gastrointestinal tract, were invasive, and translocated to and multiplied within the livers and spleens of perorally inoculated gnotobiotic pregnant mice. The spread of infection to the placentas and fetuses occurred mainly at 3 or more days after inoculation. During initial studies, maternal blood from mice inoculated with SA and SAR was sampled and cultured by direct plating. Recoveries were made from the blood of 11 of 32 mice inoculated with SA and 10 of 23 mice inoculated with SAR, regardless of the extent of infection in other tissues at any time during the 5-day study. When listeriae were detected in blood, their populations ranged from 10^2 to 10^4 CFU/ml. Because isolations were inconsistent, blood was not sampled in subsequent inoculation trials.

In gnotobiotic pregnant mice, the reference strain SA was recovered in populations of 10^8 to 10^{10} CFU/g from the cecum/colon of all mice on day 1 (Fig. 1). Although the population decreased with time, SA colonized the cecum/colon throughout the 5-day study period. SA was isolated from the livers of 10 of 13 mice and the spleens of 9 of 13 mice at day 1 and from these organs in all mice sampled on subsequent days. Populations of SA were slightly greater in livers than in the spleens. Mice with hepatic counts greater than 10^5 CFU/g generally had infected placentas and fetuses, whereas mice with hepatic counts less than 10^5 CFU/g had

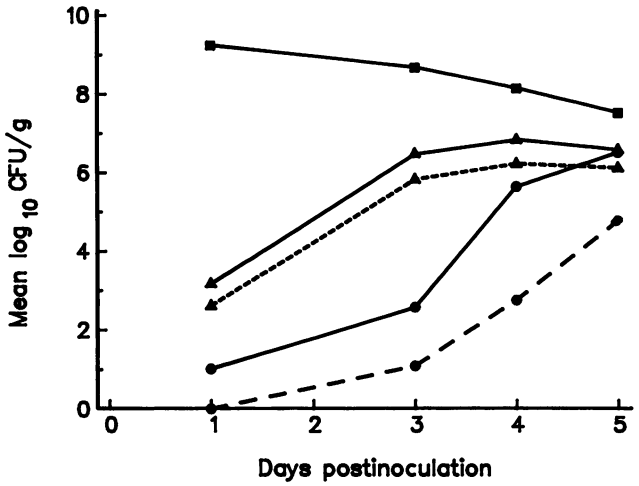

FIG. 1. Recovery of *L. monocytogenes* SA from tissues of gnotobiotic pregnant mice after peroral inoculation. Each value represents the mean count from all mice sampled each day. Symbols: ■——■, cecum/colon; ▲——▲, liver; ▲----▲, spleen; ●——●, placentas; ●----●, fetuses.

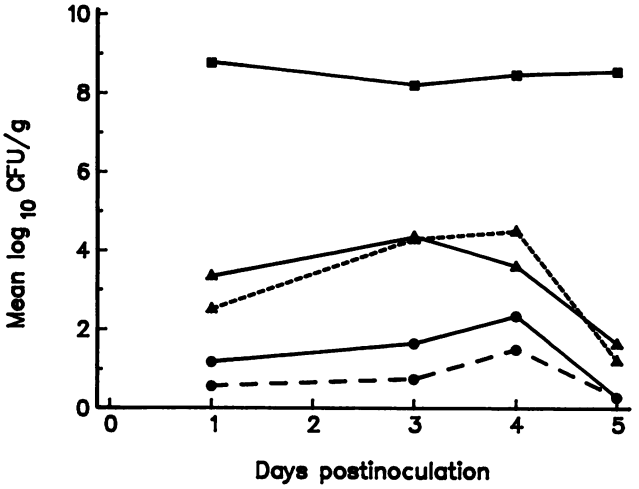

FIG. 2. Recovery of *L. monocytogenes* SAR from tissues of gnotobiotic pregnant mice after peroral inoculation. Each value represents the mean count from all mice sampled each day. Symbols: ■——■, cecum/colon; ▲——▲, liver; ▲----▲, spleen; ●——●, placentas; ●----●, fetuses.

no detectable or minimal infection of these tissues. At day 1, SA was isolated in small populations from the placental tissues of 5 of 13 mice and from none of the fetuses. By day 3, increasing populations of SA were recovered from the placental tissues of 8 of 14 mice that were culture positive and from three fetal cultures. Recoveries of SA from fetal tissues were made only from animals with culture-positive placentas. The populations of SA continued to increase in the placental and fetal tissues on days 4 and 5, and recoveries were made from 20 placental cultures and 15 fetal cultures of 21 mice sampled on days 4 and 5.

L. monocytogenes V7 and 316 were tested in small groups of gnotobiotic mice and yielded patterns of infection that were similar to that observed for SA. The data for these strains are not shown.

The results for SAR (Fig. 2) in gnotobiotic mice showed a pattern of infection different from that of SA. Like SA, SAR multiplied in the cecum/colon to large populations and translocated to the livers and spleens of all mice within the first day postinoculation. SAR multiplied to some extent in these tissues, but by day 4 the populations of SAR decreased rapidly in the livers and spleens. Also, SAR failed to infect the placental and fetal tissues to the same extent as the smooth parent strain. As with SA, infection of the placental tissues by SAR preceded infection of the fetuses, but overall, significantly fewer mice ($P = 0.008$) inoculated with SAR yielded infected placental and fetal tissues than the mice inoculated with SA (Fig. 3). On a daily basis (values not shown), the proportions of the tissues infected were similar for both SA and SAR until day 4; on days 4 and 5, the number of recoveries made from SA-inoculated mice was significantly higher ($P = 0.013$ and $P = 0.00006$, respectively). The isolates recovered from all mice inoculated with SAR produced rough colonies on solid media, indicating that no reversion to the smooth morphology had occurred in vivo.

Strain ATCC 19113 was not isolated from the internal organs or from the placental or fetal tissues of any of the inoculated gnotobiotic mice (Fig. 4). This strain did not multiply in the cecum/colon like SA, V7, 316, and SAR, and the populations of ATCC 19113 rapidly decreased with time.

Like *L. monocytogenes* SA, 316, V7, and SAR, *L. innocua* 404 colonized the cecum/colon of pregnant gnotobiotic mice throughout the 5-day study period (Fig. 5). Small populations were recovered from the livers and spleens of fewer than 50% of the mice at days 1 and 3 postinoculation. Although invasive, this species did not proliferate in maternal organs and was not recovered from any liver samples taken on day 5. Sporadically, small populations ($\leq 10^4$ CFU/g) were recovered from placental or fetal tissues of mice inoculated with *L. innocua* 404, but there was no

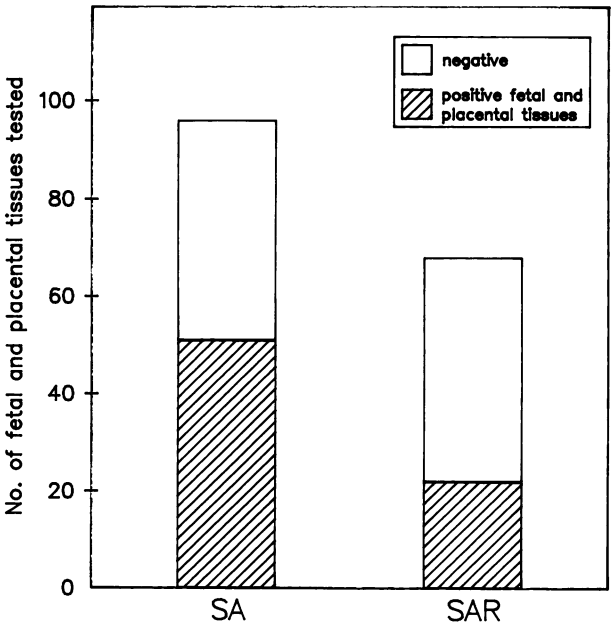

FIG. 3. Frequency of isolations of *L. monocytogenes* SA and SAR from placentas or fetuses from groups of gnotobiotic mice over 5 postinoculation days. Proportions of isolations were significantly higher in the group perorally inoculated with SA ($P = 0.002$).

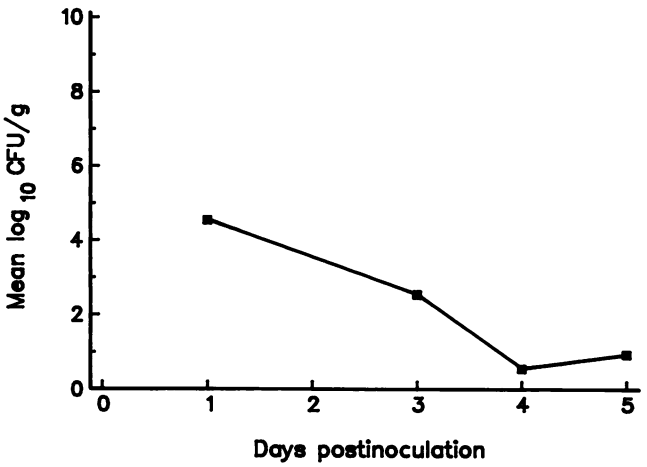

FIG. 4. Recovery of *L. monocytogenes* ATCC 19113 from tissues of gnotobiotic pregnant mice after peroral inoculation. This strain was isolated only from the cecum/colon (■) of inoculated mice; no listeriae were recovered from the liver, spleen, placentas, or fetuses. Each value represents the mean count from all mice sampled each day.

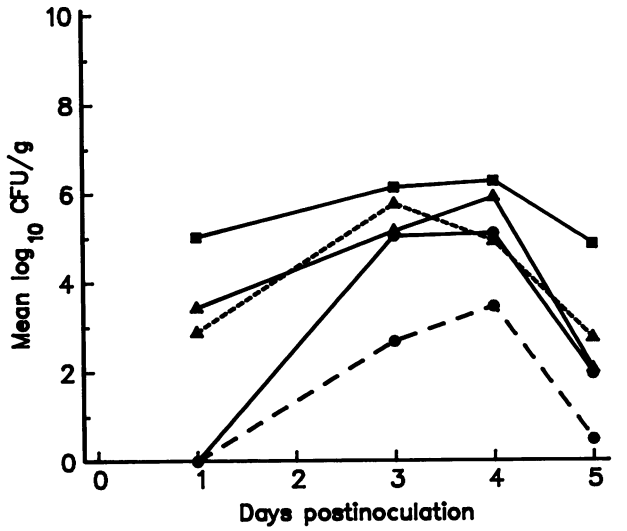

FIG. 6. Recovery of *L. monocytogenes* SA from tissues of conventional pregnant mice after peroral inoculation. Each value represents the mean count from all mice sampled each day. Symbols: ■——■, cecum/colon; ▲——▲, liver; ▲----▲, spleen; ●——●, placentas; ●----●, fetuses.

evidence of overt infection in these tissues as seen with strains of *L. monocytogenes*.

L. monocytogenes SA, ATCC 19113, and 108 were additionally tested by peroral inoculation of conventional mice. SA colonized the cecum/colon of these mice throughout the 5-day study period but in smaller populations than in gnotobiotic mice (Fig. 6). By day 1 postinoculation, SA translocated to the livers and spleens and was recovered in populations similar to those found in gnotobiotic mice. SA proliferated in these organs until day 3; however, the populations decreased dramatically in these tissues from mice sampled on day 5. Of five conventional mice sampled on day 3, four had culture-positive placentas and three of these had infected fetuses. By day 5, two of five mice had culture-positive placentas, and fetal tissues were infected in only one of the mice with a culture-positive placenta. In conventional mice that were inoculated with *L. monocytogenes* 108, the pattern of infection was similar to that produced by SA in conventional mice (data not shown).

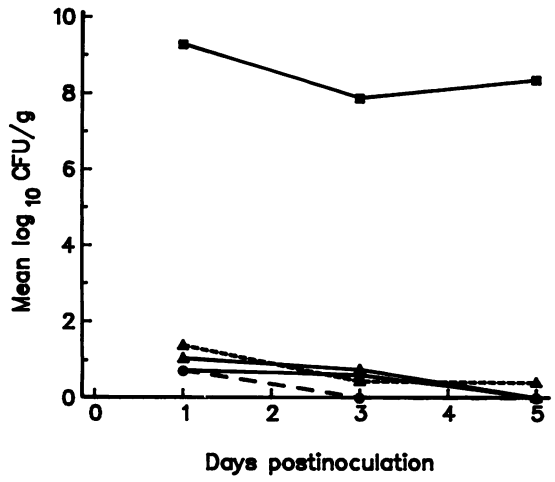

FIG. 5. Recovery of *L. innocua* 404 from tissues of gnotobiotic pregnant mice after peroral inoculation. Each value represents the mean count from all mice sampled each day. Symbols: ■——■, cecum/colon; ▲——▲, liver; ▲----▲, spleen; ●——●, placentas; ●----●, fetuses.

Of 24 conventional mice perorally inoculated with ATCC 19113, the organism was isolated only from the cecum/colon of one mouse on day 1 postinoculation and was not isolated thereafter from any samples (data not shown).

Gnotobiotic pregnant mice perorally inoculated with SA and sacrificed at various times within the first 24 h of infection had large populations ($>10^6$ CFU/g) of SA in the small intestine at 0 and 12 h and in the cecum/colon at 0, 12, 16, and 20 h. By 20 h, counts in the small intestine had decreased to 10^2 CFU/g. Recovery of SA from the livers and spleens occurred at 16 and 20 h (10^2 to 10^3 CFU/g), and no recoveries were made from the placental and fetal tissues.

(ii) Histologic findings. Fixed sections of tissues from control mice and all mice perorally inoculated with SA or SAR and representative sections from mice inoculated with V7, 316, ATCC 19113, 108, or *L. innocua* 404 were examined by HE staining. Sections of the cecum/colon, liver, spleen, and uteroplacental tissues from mice inoculated with *L. monocytogenes* ATCC 19113 or *L. innocua* 404 showed no pathological evidence of infection and appeared normal when compared with sections from control mice.

The pathology in gnotobiotic pregnant mice perorally inoculated with SAR, V7, or 316 was similar to that observed for SA. Examination of the ceca/colons revealed that disruption of the integrity of the epithelium was not extensive at any time during the 5-day study period. Superficial epithelial necrosis was apparent in 2 to 10% of the cecum/colon sections examined and was most evident in tissues sampled on day 3 postinoculation. In general, an inflammatory reaction, which was characterized by edema; infiltration of neutrophils, lymphocytes, and monocytes; and hyperplasia of lymphoid nodules was most often observed in the lamina propria and submucosa. Hemorrhage was occasionally observed. Bacteria were detected within phagocytic cells in the cecal/colonic submucosa by day 1 postinoculation but were

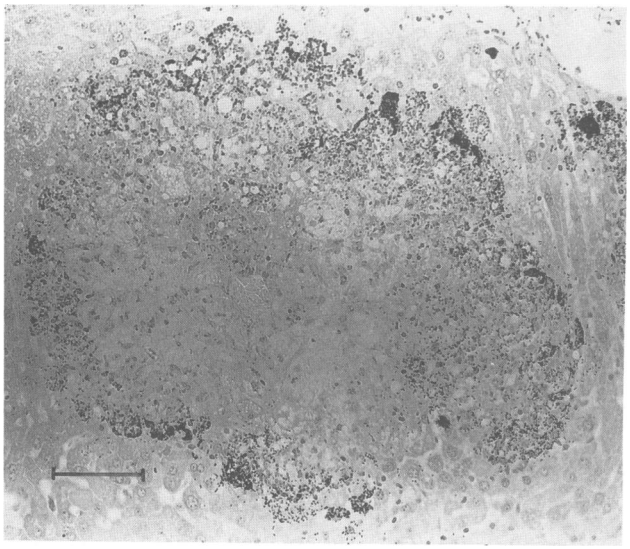

FIG. 7. Gram stain (modified Lilly's stain) of mouse liver, 4 days after inoculation with *L. monocytogenes* SA. Listeriae are localized in the peripheral macrophages and neutrophils of necrotic hepatic lesions. Bar = 150 μm.

not seen within the epithelial mucosa. Comparison of the sections of the small intestine with those of the cecum/colon removed from perorally inoculated gnotobiotic mice sampled at 0, 12, 16, and 20 h postinoculation indicated that SA invaded primarily the small intestinal mucosa. Immunoperoxidase staining revealed listeriae-containing macrophages in the interstitial tissues of the ileum and jejunum at 12 and 24 h. No focal necrosis or disruption of the overlying epithelium was evident by HE staining of the gastrointestinal tissues sampled during this initial stage of infection.

In conventional mice inoculated with SA or 108, little evidence of an inflammatory reaction was detected in the cecum/colon.

In both gnotobiotic and conventional infected mice, liver and spleen sections revealed the development of a cellular inflammatory response. By day 1 postinoculation, characteristic necrotizing and neutrophilic multifocal microabscesses were apparent in the livers. The lesions became more extensive by day 3, with necrotic foci accompanied by neutrophils and macrophages. By days 4 and 5 postinoculation, the lesions developed into pyogranulomas, characterized by an increased infiltration of macrophages and other mononuclear cells. Gram-positive bacteria phagocytized by macrophages were localized primarily at the periphery of the lesions (Fig. 7); the presence of listeriae was confirmed by immunoperoxidase staining (data not shown). By day 3, splenic tissues revealed multifocal necrosis and an increased number of neutrophils and macrophages (Fig. 8). Germinal follicles displayed a "moth-eaten" appearance.

Infected fetal-placental units did not have the inflammatory reaction seen in the liver sections. In mice inoculated with SA, the organism was detected by Gram stain within regions of necrosis in the spongiotrophoblast of the placental tissues 5 days postinoculation (Fig. 9). Severe disruption of this region was evident in one mouse from which 10^9 CFU of SA per gram was isolated from the placental tissues (Fig. 10). Necrosis of fetal tissues, with the absence of a cellular inflammatory reaction, was also observed when 10^7 CFU of SA per gram was isolated from the fetal tissues of a mouse 4 days postinoculation (data not shown).

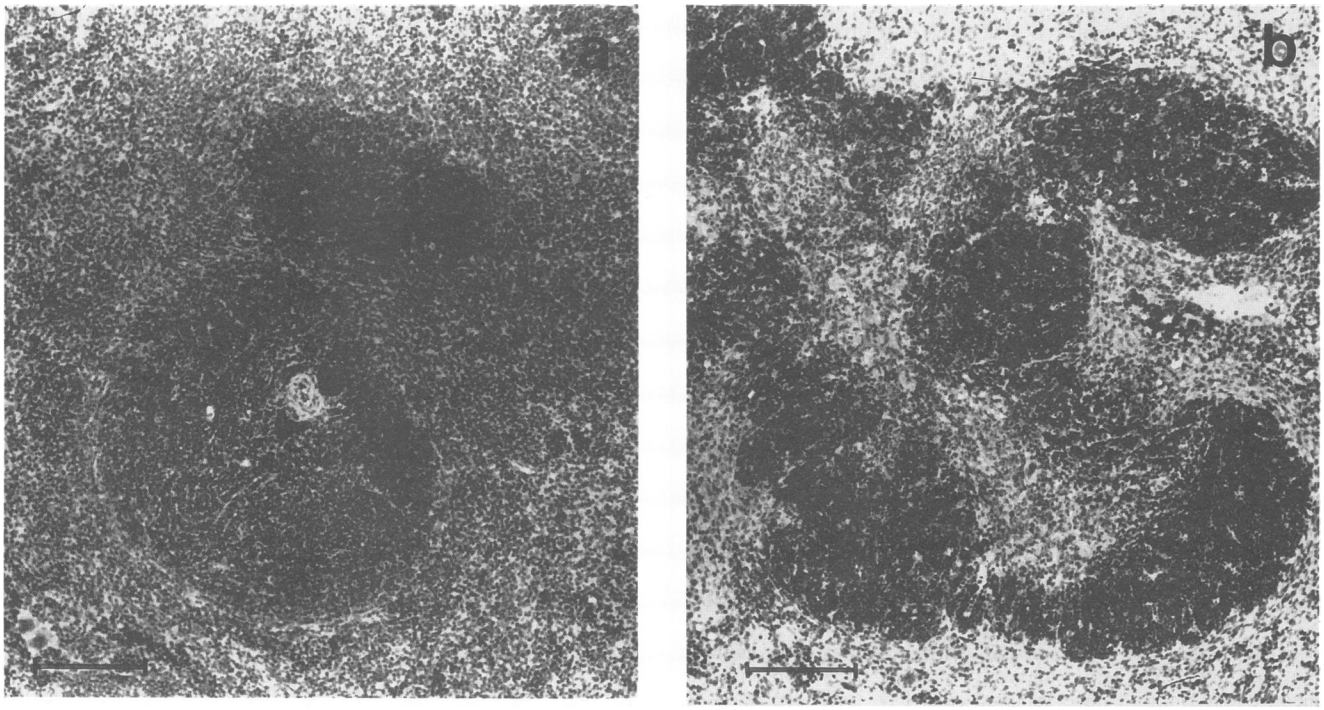

FIG. 8. (a) The spleen of a control mouse with normal follicle formation. (b) The spleen at 3 days after inoculation with *L. monocytogenes* SAR with a "moth-eaten" appearance due to cellular necrosis and an increase in inflammatory cells. HE stain was used. Bar = 150 μm.

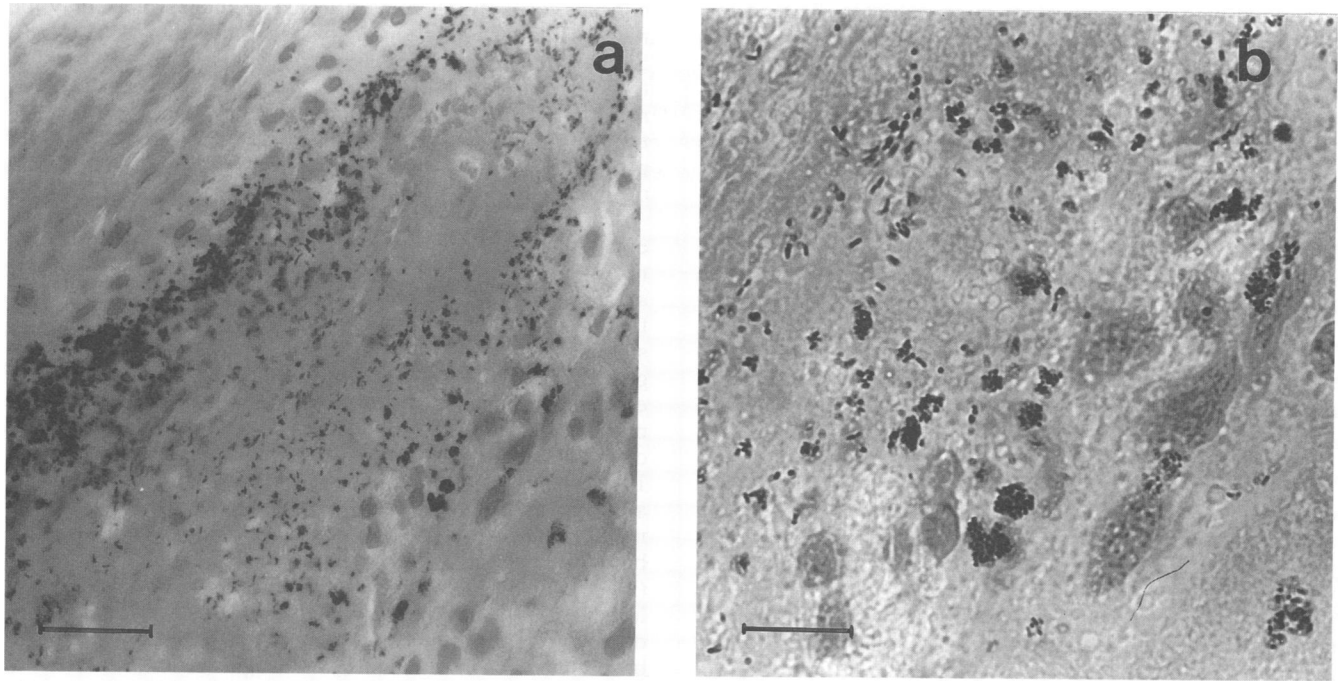

FIG. 9. Gram stain (modified Lilly's stain) of placental tissues of a pregnant mouse 5 days after peroral inoculation with *L. monocytogenes* SA. No immune response was apparent in these tissues. (a) Gram-positive organisms are concentrated in the necrotic zone of the spongiotrophoblast. Bar = 150 μm. (b) At a higher magnification, the listeriae are seen both within cells and freely dispersed in the tissues of the spongiotrophoblast. Bar = 15 μm.

DISCUSSION

The use of a perorally inoculated animal model to study listeric infection is a relevant assay for the evaluation of virulence of a food-borne pathogen. In this study, the course of peroral listeric infection of pregnant BALB/c mice was monitored by microbiologic and histopathologic analyses. This model was used to compare the pathogenesis of several strains of *L. monocytogenes* with that of one strain of *L. innocua*. Two strains of *L. monocytogenes*, SA and SAR, which were of similar virulence to mice when administered i.p., differed in their ability to infect placental and fetal tissues when administered perorally. A third strain, ATCC 19113, did not colonize the gastrointestinal tract, nor did it produce systemic infection. A comparison of listeric infection in conventional pregnant mice with infection in gnotobiotic pregnant mice revealed that infection of the tissues

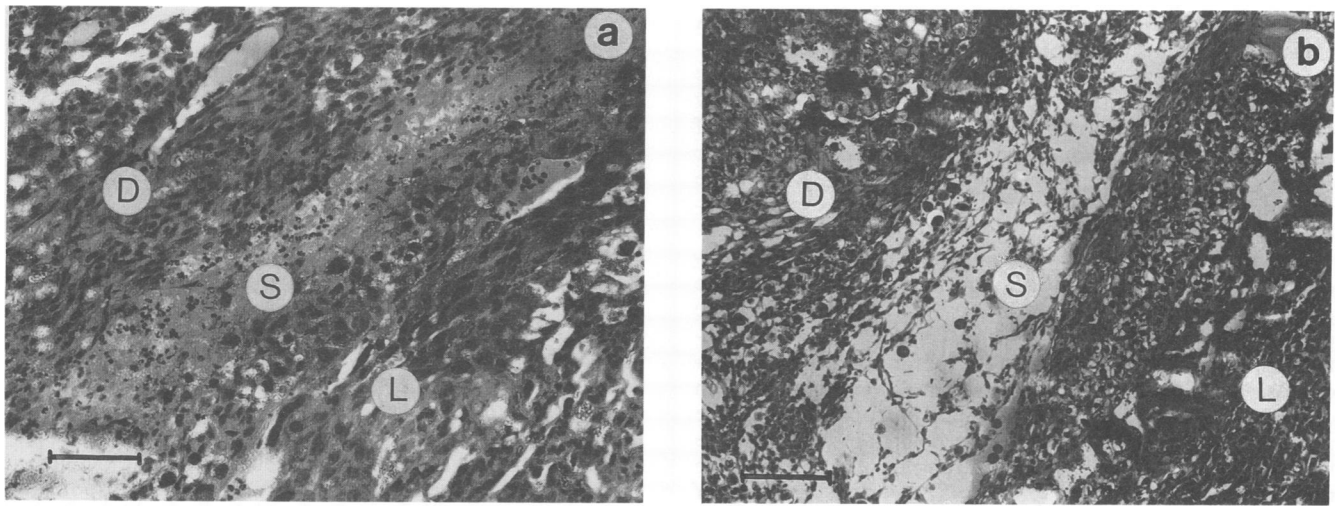

FIG. 10. (a) Normal cellular morphology of placental decidua (D), spongiotrophoblast (S), and labyrinth (L) in a gnotobiotic pregnant control mouse. (b) Disruption of the spongiotrophoblast region 5 days after peroral inoculation with *L. monocytogenes* SA. Large populations of SA (approximately 10^9 CFU/g) were isolated from the pooled placental tissues of this mouse. Abbreviations are the same as for panel a. HE stain was used. Bar = 40 μm.

examined was more consistent in gnotobiotes. The normal microflora of the mouse gastrointestinal tract interfere with colonization by *L. monocytogenes* (15, 53). Also, gnotobiotic animals have altered physiological reactions and impaired immunological responses compared with animals raised under conventional conditions and thus are a more sensitive model for detecting differences in virulence (4).

With the exception of ATCC 19113, all strains of *L. monocytogenes* colonized the gastrointestinal tract of gnotobiotic pregnant BALB/c mice throughout the 5-day study. Histopathology of the cecum/colon of gnotobiotic mice revealed a mild to moderate subacute inflammatory reaction; however, necrosis of the epithelium was not prominent. Although not determined, it is possible that the damage to the intestinal epithelium resulted from a host-induced immune response involving release of lytic enzymes from the inflammatory cells phagocytizing the listeriae rather than from a cytotoxic effect of the organism itself (15, 33, 41, 53).

The invasive phase of listeric infection occurred soon after oral dosing. Strain SA was detected within macrophages in the interstitium of the jejunum and ileum of the small intestine by 12 h postinoculation. This was consistent with the findings of Rácz et al. (41), who demonstrated the presence of *L. monocytogenes* within epithelial cells in the small intestine of guinea pigs as early as 9 h after peroral inoculation and in macrophages of the submucosa by 22 h postinfection.

Listeriae were not consistently isolated from the maternal blood by direct culture; however, it is probable that phagocytized listeriae were present in the bloodstream in populations too small to detect by direct plating. Similarly, Miller and Burns (37) failed to recover *L. monocytogenes* by direct plating of blood from perorally inoculated mice, whereas the organism has been consistently isolated from blood when broth enrichment is used (15). From women with listeriosis complicating pregnancy, positive cultures of the organism from blood are made in only approximately 50% of cases (36), suggesting that bacteremia is a transient feature of the infection in the pregnant host.

The proliferation of the *L. monocytogenes* strains in the livers and spleens and the histopathology of these tissues reflected the typical course of listeric infection and development of a cellular immune response by 3 to 4 days postinoculation (1, 8, 20, 26, 28, 52). The larger populations of SA in the livers than in the spleens are characteristic of listeriosis in pregnant mice (32), whereas in nonpregnant mice larger populations of *L. monocytogenes* are consistently recovered from the spleen (2, 20, 32). The development of early-stage pyogranulomas by day 5 provided histologic evidence that the infection induced a maternal immune response (26, 28, 34) despite the immunosuppression associated with pregnancy (51).

In contrast to the liver and spleen, there was little evidence of inflammation or a cellular immune response in the placental and fetal tissues. Strain SA progressed to the placental tissues by day 3 postinoculation, preceding infection of the fetuses at days 4 and 5. Gram and immunoperoxidase staining revealed that SA proliferated initially in degenerating regions within the spongiotrophoblast of the placental tissues. This necrosis is considered part of the normal remodelling that occurs during development of the mouse fetus (12) and may provide a favorable medium for the growth of *L. monocytogenes*. Localized immunosuppression in the mouse uteroplacental region during listeric infection has been shown (42, 43). In human full-term placentas, the predominant mononuclear cell population consists of anti-inflammatory macrophages that may be responsible for inhibition of specific immune responses (38). Similar mechanisms may have allowed the relatively uncontrolled proliferation of *L. monocytogenes* reaching this site, resulting in overt infection. The presence of large populations (10^9 CFU/g) of SA in the placental tissues would probably cause disruption of the normal placental morphology, leading to loss of the fetus.

The rough strain SAR was clearly invasive and translocated to and multiplied within the maternal organs but failed to attain the levels of population found for strains SA, V7, and 316 in gnotobiotic mice. SAR was cleared more rapidly from inoculated mice and infected placentas and fetuses significantly less consistently than SA. These findings suggested that SAR was less virulent than SA, a difference not evident by i.p. inoculation of normal and immunocompromised mice. The conversion from smooth to rough morphology is a spontaneous event occurring in approximately 1 in 10,000 colonies (29). Rough strains produce chains of cells and appear to be defective in forming separated discrete rods. The basis for this phenotype is not clearly understood, but it has been associated with the loss of cell wall proteins (21) and the reduced production of a major 60-kDa extracellular protein named p60 (27). One function of p60 may be the division of daughter cells during cell division; p60 may also be involved in invasiveness, as rough mutants have a decreased ability to invade cell line 3T6 mouse fibroblasts (29). It is possible that deficiencies in cell division and invasiveness may lead to a decrease in the organism's resistance to host defense mechanisms or hinder its cell-to-cell invasiveness (17). Nevertheless, when SAR was administered i.p. it was as successful as the smooth parent strain SA in producing a lethal outcome, suggesting that i.p. inoculations bypass the natural host and microbial processes that determine the outcome of a food-borne infection.

L. monocytogenes ATCC 19113 was not invasive, nor did it colonize the gastrointestinal tract of even gnotobiotic perorally inoculated pregnant mice. *L. monocytogenes* serogroup 3 strains are infrequently associated with human clinical illness (17, 35), although they are isolated from foods as often as strains of serogroup 4 (13, 24). Although more strains of serogroup 3 should be tested, their inability to survive in the gastrointestinal tract may in part explain the low prevalence of this serotype in human illness.

By peroral inoculation, *L. innocua* 404 was invasive and translocated from the gastrointestinal tract but failed to proliferate in the livers and spleens or cause overt infection in placental and fetal tissues. Similarly, *L. innocua* is recovered from the livers and spleens of mice inoculated by i.p. and intravenous injection but is rapidly eliminated without evoking an immune response (20, 52).

It is recognized that the secretion of a sulfhydryl-based hemolysin, although a crucial virulence factor, is not the sole diagnostic measure of virulence for differentiating strains of *L. monocytogenes* (7, 14, 25, 30). Determinants that offer an explanation for the prevalence of only certain serotypes in human listeriosis have not yet been identified. The results of this study suggest that the gnotobiotic pregnant mouse is a useful model for detecting intraspecies differences in virulence relating to colonization, invasiveness, and uteroplacental infection that cannot be detected by i.p. inoculation.

The nature of the underlying factors that predispose the host for listeric infection ultimately determines the outcome of disease. In a pregnant, healthy host, the mother rarely suffers severe symptoms and the maternal immunity is sufficiently intact to overcome infection. Progression of the

infection to the fetus, where the organism can proliferate, results in stillbirth, abortion, or birth of an infected infant. By contrast, in the nonpregnant, immunocompromised host, especially those with T-cell dysfunctions, the outcome is most often meningitis, meningoencephalitis, or life-threatening bacteremia (46). Therefore, it must be kept in mind that the model described here does not reflect the pathogenesis of listeric infection in severely immunocompromised hosts but rather measures behavior in a host that is capable of mounting an immune response. Although cost and availability of gnotobiotic animals limit the use of this assay in routine diagnostic testing, gnotobiotic mice offer the advantage of promoting gastrointestinal colonization and perhaps invasiveness that is usually difficult to obtain by peroral inoculation with *L. monocytogenes* (2, 53) without severely altering the immune response.

Differentiating *L. monocytogenes* on the basis of behavior in vivo can identify strain variations that require further investigation. Such research is needed to fully understand the virulence mechanisms of *L. monocytogenes* and its significance in foods and potential for causing food-borne disease. Current regulatory actions result in recalls of foods, some of which may contain only small populations of *L. monocytogenes* strains that pose little risk to the consumer. Identifying intraspecies markers for highly virulent strains may contribute to a rational basis for regulatory actions and allow emphasis of controls on foods likely to be contaminated with strains of heightened virulence.

ACKNOWLEDGMENTS

This investigation was funded by the International Life Sciences Institute-Nutrition Foundation Committee on Food Microbiology and the Wisconsin Center for Dairy Research at the University of Wisconsin—Madison. A.M.L. gratefully acknowledges the financial support of the Health of Animals Division of Agriculture Canada.

We thank C. Fischer and J. P. Ford for technical assistance and J. Cerveny of the Oscar Mayer Foods Corp., Madison, Wis., for advisory contributions on behalf of ILSI-NF.

REFERENCES

1. **Armstrong, B. A., and C. P. Sword.** 1966. Electron microscopy of *Listeria monocytogenes*-infected mouse spleen. J. Bacteriol. **91**:1346–1355.
2. **Audurier, A., P. Pardon, J. Marly, and F. Lantier.** 1980. Experimental infection of mice with *Listeria monocytogenes* and *Listeria innocua*. Ann. Microbiol. Inst. Pasteur (Paris) **131B**:47–57.
3. **Bennett, R. W.** 1988. Production of flagellar ("H") and somatic ("O") subfactor antibodies to *Listeria monocytogenes*, abstr. 176, p. 123. Abstr. 48th Annu. Meet. Inst. Food Technol. 1988.
4. **Berg, R. D., and A. W. Garlington.** 1979. Translocation of certain indigenous bacteria from the gastrointestinal tract to the mesenteric lymph nodes and other organs in a gnotobiotic mouse model. Infect. Immun. **23**:403–411.
5. **Bernàth, S., and M. Pitron.** 1989. Haemolysin producing capacity and mouse-pathogenicity of *Listeria monocytogenes*. Acta Microbiol. Hung. **36**:373–376.
6. **Bibb, W. F., B. G. Gellin, R. Weaver, B. Schwartz, B. D. Plikaytis, M. W. Reeves, R. W. Pinner, and C. V. Broome.** 1990. Analysis of clinical and food-borne isolates of *Listeria monocytogenes* in the United States by multilocus enzyme electrophoresis and application of the method to epidemiologic investigations. Appl. Environ. Microbiol. **56**:2133–2141.
7. **Bielecki, J., P. Youngman, P. Connelly, and D. A. Portnoy.** 1990. *Bacillus subtilis* expressing a haemolysin gene from *Listeria monocytogenes* can grow in mammalian cells. Nature (London) **345**:175–176.
8. **Bortolussi, R., D. D. McGregor, P. A. L. Kongshavn, S. Galsworthy, W. Albritton, J. W. Davies, and H. P. R. Seeliger.** 1984. Host defense mechanisms for perinatal and neonatal *Listeria monocytogenes* infection. Surv. Synth. Pathol. Res. **3**:311–332.
9. **Bortolussi, R., W. F. Schlech III, and W. L. Albritton.** 1985. *Listeria*, p. 205–208. *In* E. H. Lennette, A. Balows, W. J. Hausler, Jr., and H. J. Shadomy (ed.), Manual of clinical microbiology, 4th ed. American Society for Microbiology, Washington, D.C.
10. **Bourne, J. A.** 1989. Handbook of immunoperoxidase staining methods. DAKO Corporation, Santa Barbara, Calif.
11. **Chakraborty, T., and W. Goebel.** 1988. Recent developments in the study of virulence in *Listeria monocytogenes*. Curr. Top. Microbiol. Immunol. **138**:41–58.
12. **Cinquetti, R.** 1983. Histological changes in the chorioallantoic placenta of the mouse with increasing gestational age. Arch. Ital. Anat. Embriol. **88**:267–287.
13. **Conner, D. E., V. N. Scott, S. S. Sumner, and D. Bernard.** 1989. Pathogenicity of foodborne, environmental and clinical isolates of *Listeria monocytogenes* in mice. J. Food Sci. **54**:1553–1556.
14. **Cossart, P., M. F. Vicente, J. Mengaud, F. Baquero, J. C. Perez-Diaz, and P. Berche.** 1989. Listeriolysin O is essential for virulence of *Listeria monocytogenes*: direct evidence obtained by gene complementation. Infect. Immun. **57**:3629–3636.
15. **Czuprynski, C. J., and E. Balish.** 1981. Pathogenesis of *Listeria monocytogenes* for gnotobiotic rats. Infect. Immun. **32**:323–331.
16. **Davis, B. D., R. Dulbecco, H. N. Eisen, H. S. Ginsberg, and W. B. Wood, Jr. (ed.).** 1973. Microbiology, 2nd ed., p. 663–664. Harper & Row, Publishers, Inc., New York.
17. **Farber, J. M., and P. I. Peterkin.** 1991. *Listeria monocytogenes*, a food-borne pathogen. Microbiol. Rev. **55**:476–511.
18. **Federal Register.** 1988. Bacteriological analytical manual, chapter 29—*Listeria* isolation; revised method of analysis. Fed. Regist. **55**:44153–44158.
19. **Fleming, D. W., S. L. Cochi, K. L. MacDonald, J. Brondum, P. S. Hayes, B. D. Plikaytis, M. B. Holmes, A. Audurier, C. V. Broome, and A. L. Reingold.** 1985. Pasteurized milk as a vehicle of infection in an outbreak of listeriosis. N. Engl. J. Med. **312**:404–407.
20. **Heymer, B., C. H. Wirsing von König, H. Finger, H. Hof, and P. Emmerling.** 1988. Histomorphology of experimental listeriosis. Infection **16**(Suppl. 2):106–111.
21. **Hof, H., and S. Chatzipanagiotou.** 1987. The role of surface structures of *Listeria* spp. for pathogenicity. Ann. Microbiol. Inst. Pasteur (Paris) **138**:268–273.
22. **Hof, H., and P. Hefner.** 1988. Pathogenicity of *Listeria monocytogenes* in comparison to other *Listeria* spp. Infection **16**(Suppl. 2):141–144.
23. **Isobe, Y., S.-T. Chen, P. K. Nakane, and W. R. Brown.** 1977. Studies on translocation of immunoglobulins across intestinal epithelium. I. Improvements in the peroxidase-labeled antibody method for application to study of human intestinal mucosa. Acta Histochem. Cytochem. **10**:161–171.
24. **Johnson, J. L., M. P. Doyle, and R. G. Cassens.** 1990. *Listeria monocytogenes* and other *Listeria* spp. in meat and meat products: a review. J. Food Prot. **53**:81–91.
25. **Kathariou, S., J. Rocourt, H. Hof, and W. Goebel.** 1988. Levels of *Listeria monocytogenes* hemolysin are not directly proportional to virulence in experimental infections of mice. Infect. Immun. **56**:534–536.
26. **Kaufmann, S. H. E.** 1988. Immunity against intracellular bacteria: biological effector functions and antigen specificity of T lymphocytes. Curr. Top. Microbiol. Immunol. **138**:141–176.
27. **Köhler, S., A. Bubert, M. Vogel, and W. Goebel.** 1991. Expression of the *iap* gene coding for protein p60 of *Listeria monocytogenes* is controlled on the posttranscriptional level. J. Bacteriol. **173**:4668–4674.
28. **Kongshavn, P. A. L.** 1986. Genetic control of resistance to *Listeria* infection. Curr. Top. Microbiol. Immunol. **124**:67–85.
29. **Kuhn, M., and W. Goebel.** 1989. Identification of an extracellular protein of *Listeria monocytogenes* possibly involved in intracellular uptake by mammalian cells. Infect. Immun. **57**:55–61.
30. **Kuhn, M., S. Kathariou, and W. Goebel.** 1988. Hemolysin supports survival but not entry of the intracellular bacterium

Listeria monocytogenes. Infect. Immun. **56**:79–82.
31. **Lee, W. H., and D. McClain.** 1986. Improved *Listeria monocytogenes* selective agar. Appl. Environ. Microbiol. **52**:1215–1217.
32. **Luft, B. J., and J. S. Remington.** 1982. Effect of pregnancy on resistance to *Listeria monocytogenes* and *Toxoplasma gondii* infections in mice. Infect. Immun. **38**:1164–1171.
33. **MacDonald, T. T., and P. B. Carter.** 1980. Cell-mediated immunity to intestinal infection. Infect. Immun. **28**:516–523.
34. **Mainou-Fowler, T., A. P. MacGowan, and R. Postlewaite.** 1988. Virulence of *Listeria* spp.: course of infection in resistant and susceptible mice. J. Med. Microbiol. **27**:131–140.
35. **McLauchlin, J.** 1990. Distribution of serovars of *Listeria monocytogenes* isolated from different categories of patients with listeriosis. Eur. J. Clin. Microbiol. Infect. Dis. **9**:210–213.
36. **McLauchlin, J.** 1990. Human listeriosis in Britain, 1967–85, a summary of 722 cases. 1. Listeriosis during pregnancy and in the newborn. Epidemiol. Infect. **104**:181–189.
37. **Miller, J. K., and J. Burns.** 1970. Histopathology of *Listeria monocytogenes* after oral feeding to mice. Appl. Microbiol. **19**:772–775.
38. **Mues, B., D. Langer, G. Zwadlo, and C. Sorg.** 1989. Phenotypic characterization of macrophages in human term placenta. Immunology **67**:303–307.
39. **Piffaretti, J.-C., H. Kressebuch, M. Aeschbacher, J. Bille, E. Bannerman, J. M. Musser, R. K. Selander, and J. Rocourt.** 1989. Genetic characterization of clones of the bacterium *Listeria monocytogenes* causing epidemic disease. Proc. Natl. Acad. Sci. USA **86**:3818–3822.
40. **Pinner, R. W., A. Schuchat, B. Swaminathan, P. S. Hayes, K. A. Deaver, R. E. Weaver, B. O. Plikaytis, M. Reeves, C. V. Broome, J. D. Wenger, and the Listeria Study Group.** 1992. Role of foods in sporadic listeriosis. II. Microbiologic and epidemiologic investigation. JAMA **267**:2046–2050.
41. **Rácz, P., K. Tenner, and E. Mérö.** 1972. Experimental *Listeria* enteritis. I. An electron microscopic study of the epithelial phase in experimental *Listeria* infection. Lab. Invest. **26**:694–700.
42. **Redline, R. W., and C. Y. Lu.** 1987. Role of local immunosuppression in murine fetoplacental listeriosis. J. Clin. Invest. **79**:1234–1241.
43. **Redline, R. W., and C. Y. Lu.** 1988. Specific defects in the anti-listerial immune response in discrete regions of the murine uterus and placenta account for susceptibility to infection. J. Immunol. **140**:3947–3955.
44. **Rocourt, J., E. P. Espaze, R. Minck, B. Catimel, B. Hubert, and A. L. Courtieu.** 1989. Cluster of listeriosis with different serovar and phagovar characteristics. Lancet **ii**:1217–1218.
45. **Schuchat, A., K. A. Deaver, J. D. Wenger, B. O. Plikaytis, L. Mascola, R. W. Pinner, A. L. Reingold, C. V. Broome, and the Listeria Study Group.** 1992. Role of foods in sporadic listeriosis. I. Case-control study of dietary risk factors. JAMA **267**:2041–2045.
46. **Schuchat, A., B. Swaminathan, and C. V. Broome.** 1991. Epidemiology of human listeriosis. Clin. Microbiol. Rev. **4**:169–183.
47. **Schwartz, B., D. Hexter, C. V. Broome, A. W. Hightower, R. B. Hirschhorn, J. D. Porter, P. S. Hayes, W. F. Bibb, B. Lorber, and D. G. Faris.** 1989. Investigation of an outbreak of listeriosis: new hypothesis for the etiology of epidemic *Listeria monocytogenes* infections. J. Infect. Dis. **159**:680–685.
48. **Seeliger, H. P. R.** 1988. Listeriosis—history and actual developments. Infection **16**(Suppl. 2):80–84.
49. **Snedecor, G. W., and W. G. Cochran.** 1989. Statistical methods, 8th ed., p. 76–79. Iowa State University Press, Ames.
50. **Stelma, G. N., Jr., A. L. Reyes, J. T. Peeler, D. W. Francis, J. M. Hunt, P. L. Spaulding, C. H. Johnson, and J. Lovett.** 1987. Pathogenicity test for *Listeria monocytogenes* using immunocompromised mice. J. Clin. Microbiol. **25**:2085–2089.
51. **Weinberg, E. D.** 1984. Pregnancy-associated depression of cell-mediated immunity. Rev. Infect. Dis. **6**:814–831.
52. **Wirsing von Koenig, C. H., B. Heymer, H. Hof, and H. Finger.** 1983. Course of infection and development of immunity in experimental infection of mice with *Listeria* serotypes. Infect. Immun. **40**:1170–1177.
53. **Zachar, Z., and D. C. Savage.** 1979. Microbial interference and colonization of the murine gastrointestinal tract by *Listeria monocytogenes*. Infect. Immun. **23**:168–174.