Host Resistance to an Intragastric Infection with *Listeria monocytogenes* in Mice Depends on Cellular Immunity and Intestinal Bacterial Flora

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Suckling and adult mice were infected intragastrically with different doses of viable *Listeria monocytogenes*. The 50% lethal dose for the intragastric infection was $10^{3.7}$ CFU for suckling mice, while adult mice were highly resistant and the 50% lethal dose was more than $10^{9.3}$ CFU. When adult mice were infected intragastrically with 5×10^8 CFU of *L. monocytogenes*, no mice died. However, 35% of adult mice died when they were treated with cyclosporin A 1 day before infection. Although mice did not die when treated with an *L. monocytogenes*-resistant broad-spectrum cephalosporin, sodium cefbuperazone, before and during infection, the number of *L. monocytogenes* bacteria increased in the feces. The sodium cefbuperazone treatment of mice resulted in superinfection, i.e., a marked decrease of *Escherichia coli* and an increase of *Enterococcus* spp. in the intestines. Furthermore, host resistance against the intragastric infection markedly decreased when the mice were treated with both drugs. The growth of *L. monocytogenes* was augmented in the spleens, mesenteric lymph nodes, Peyer's patches, and feces, and the mortality of the mice was 65%. These results suggest that both cellular immunity and the intestinal bacterial flora are required for host resistance against oral *L. monocytogenes* infection.

Listeria monocytogenes is a gram-positive, facultative intracellular bacterium that is ordinarily nonpathogenic to healthy persons; however, it is important as an opportunistic pathogen. The individuals at highest risk are pregnant women and their fetuses, newborn infants, debilitated elderly persons, and immunocompromised hosts. Recent epidemiologic investigations provided evidence that *L. monocytogenes* may be transmitted as an enteric pathogen by contaminated foods, e.g., vegetables, milk, and dairy products (6, 10, 26), suggesting that natural *L. monocytogenes* infection occurs by the oral route.

Host resistance against *L. monocytogenes* infection is affected by T-cell-dependent mechanisms (14) and the administration of cyclosporin A (CsA), which inhibits many T-cell-dependent functions (22) and decreases antilisterial resistance in mice (7, 17, 25, 28). On the other hand, it has been reported that the intestinal bacterial flora is important in the interference of the colonization of *L. monocytogenes* in the intestinal tracts (4, 31), suggesting that the fate of infection by the oral route depends on the intestinal bacterial flora. Therefore, we focused our studies on the effect of CsA and an *L. monocytogenes*-resistant broad-spectrum cephalosporin, sodium cefbuperazone (CBPZ), on the susceptibilities of specific-pathogen-free mice to an intragastric infection with *L. monocytogenes*. In this report, we provide evidence that a combination of immunosuppression and interference of intestinal bacterial flora results in the marked augmentation of susceptibility of mice to an intragastric infection with *L. monocytogenes*.

MATERIALS AND METHODS

Mice. Specific-pathogen-free female ddY mice (obtained from SLC, Hamamatsu, Shizuoka, Japan), 5 weeks old and weighing 24 to 26 g, were used. In some experiments, 16- to 18-day-old suckling mice (weighing 7 to 11 g) were used. The suckling mice were kept with their mothers and handled as little as possible (3).

Bacteria. *L. monocytogenes* 1b 1684 cells (16) were maintained in tryptic soy broth (Difco Laboratories, Detroit, Mich.) at $-70°C$. In each experiment, an aliquot was thawed, inoculated into 200 ml of tryptic soy broth, and incubated at 37°C for 15 to 16 h. The organisms were collected by centrifugation and resuspended in pyrogen-free saline. The concentration of the cell suspension was adjusted spectrophotometrically at 550 nm. Mice were infected intragastrically with 0.2 ml of a solution containing 5×10^8 CFU of viable *L. monocytogenes* in 0.01 M phosphate-buffered saline (PBS; pH 7.4). For intragastric feeding, we slipped an animal-feeding needle (0.9 by 70 mm; Natsume Factory, Inc., Tokyo, Japan) past the pharynx into the stomach, where the inoculum was injected. The mice were not anesthetized for this procedure.

Treatment with CsA. CsA was donated by Sandoz AG (Basel, Switzerland). It was dissolved in pharmaceutical-grade olive oil with sonication before use. One hundred milligrams of the drug (in 0.2-ml quantities) per kg of body weight was injected intraperitoneally 1 day before infection with *L. monocytogenes* (17). Control mice were injected with drug-free olive oil.

Treatment with an antibacterial agent. CBPZ was obtained from Toyama Chemical Co., Tokyo, Japan. The MIC of CBPZ for *L. monocytogenes* 1b 1684 was more than 60 µg/ml. The MIC was determined by a broth microdilution method (23) by using brain heart infusion broth (Difco). Bacteria were inoculated at a final concentration of 10^6 CFU/ml. For in vivo treatment, mice were administered intraperitoneally 500 mg of

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CBPZ per kg of body weight or saline (control) daily from day −2 of *L. monocytogenes* infection. CBPZ was dissolved in pyrogen-free saline.

Determination of the number of viable *L. monocytogenes* bacteria in the organs and feces. The numbers of viable *L. monocytogenes* bacteria in the spleens and mesenteric lymph nodes of the infected animals were established by plating serial 10-fold dilutions of organ homogenates in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) containing 1% (wt/vol) 3-[(cholamidopropyl) dimethylammonio]-1-propane-sulfate (Wako Pure Chemical Co., Osaka, Japan) on tryptic soy agar (Difco). The number of bacteria in the Peyer's patches and feces was determined on PALCAM *Listeria* selective agar (30) containing 23 g of Bacto Peptone (Difco), 1 g of starch, 5 g of NaCl, 13 g of agar (Nissui Pharmaceutical Co., Tokyo, Japan), 5 g of D-(−)mannitol, 0.5 g of ferric ammonium citrate, 0.8 g of esculin, 0.5 g of glucose, 15 g of LiCl, and 0.08 g of phenol red per liter of distilled water (pH 7.2). The medium also contained selective supplements, including 10 mg of polymyxin B sulfate (Pfizer Pharmaceutical Co., Tokyo, Japan), 5 mg of acriflavine (Sigma Chemical Co., St. Louis, Mo.), and 20 mg of ceftazidime (Japan Glaxo Co., Tokyo, Japan). PALCAM agar plates were incubated in a GasPak CO_2 system (BBL Microbiology Systems, Cockeysville, Md.) at 37°C for 48 h. The numbers of *L. monocytogenes* bacteria were expressed as CFU per gram (wet weight) in the spleens, mesenteric lymph nodes, and Peyer's patches and as CFU per gram (dry weight) in the feces.

Determination of the numbers of *Escherichia coli* and *Enterococcus* spp. in feces. The numbers of *E. coli* and *Enterococcus* spp. were determined on DHL agar (Nissui) and NM agar (31), respectively. NM agar contained 10 g of tryptose (Difco), 3 g of beef extract (Difco), 5 g of NaCl, 1 g of D-(−)mannitol, 0.08 g of bromothymol blue, 0.04 g of nalidixic acid (Sigma), and 15 g of agar (Nissui) per liter of distilled water (pH 7.4). After incubation at 37°C for 48 h, deep gold and opaque colonies were estimated as *Enterococcus* spp. (31).

Statistical evaluation of the data. Data were expressed as means ± standard deviations, and the Wilcoxon rank sum test was used to determine the significance of the differences of bacterial counts in the specimens between control and experimental groups. The generalized Wilcoxon test was used to determine the significance of difference in survival rate. Each experiment was repeated at least twice and accepted as valid only when trials showed similar results.

RESULTS

Susceptibilities of adult and suckling mice to intragastric infection with *L. monocytogenes*. Mice of each group, consisting of 15 to 21 suckling mice from three litters and 5 adults, were infected intragastrically with different doses of viable *L. monocytogenes* cells, and survival rates were observed for 10 days (Fig. 1). The suckling mice were highly susceptible to the infection: 71% of them which were infected with only 100 CFU of *L. monocytogenes* cells died on day 2 to day 7 of infection. In contrast, no adult mice died even after having received 10^9 CFU of the pathogen (data not shown). The 50% lethal doses for intragastric infection with *L. monocytogenes* cells were $10^{3.7}$ CFU for suckling mice and more than $10^{9.3}$ CFU for adult mice.

Kinetics of growth of *L. monocytogenes* in the organs of adult mice which received intragastric infection. Adult mice were infected intragastrically with 5×10^8 CFU of viable *L. monocytogenes*, and the number of bacteria in the spleens, mesenteric lymph nodes, Peyer's patches, and feces was determined
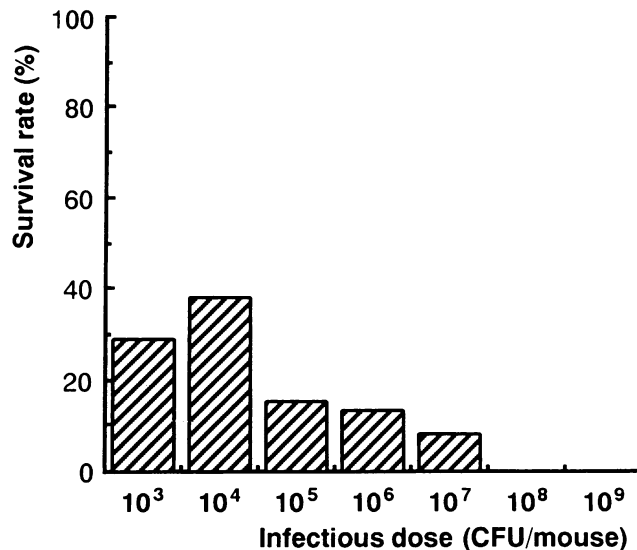

FIG. 1. Susceptibilities to *L. monocytogenes* infection of suckling mice. Fifteen to 21 suckling mice (7 to 11 g) were infected intragastrically with different doses of bacteria. The survival rates were observed for 10 days.

until day 10 of infection (Fig. 2). Similar patterns were observed in the numbers of bacterial cells between the spleens and mesenteric lymph nodes and between Peyer's patches and feces. No increase in the number of *L. monocytogenes* bacteria in the Peyer's patches and feces was seen, and the number fell to undetectable levels before day 5 of infection. In contrast, the number of bacteria in the mesenteric lymph nodes increased from day 1 to day 3 of infection and then decreased. *L. monocytogenes* appeared in the spleens on day 2 of infection. Thereafter, the kinetics of the number of bacterial cells was similar to that of the mesenteric lymph nodes.

Effect of CsA and CBPZ on susceptibilities to intragastric infection in adult mice. Adult mice were infected intragastrically with 5×10^8 CFU of *L. monocytogenes*, and they were divided into the following four groups. Mice of the first group were injected intraperitoneally with 100 mg of CsA per kg 1 day before infection. Under these conditions, almost all of the CsA-treated mice died by intravenous infection with a 0.1 50% lethal dose of *L. monocytogenes* (17). Mice of the second group were injected intraperitoneally with a 500-mg/kg dose of CBPZ, which showed no antibacterial activity against the strain of *L. monocytogenes* used herein, daily from day −2 to day 9 of infection. The treatment disturbed the normal bacterial flora of the intestines of the mice: the number of *E. coli* in the feces decreased to less than 100 CFU/g; however, the number of *Enterococcus* spp. increased to almost 100-fold in the feces of the CBPZ-treated mice (data not shown). Mice of the third group were treated with both CsA and CBPZ. Mice of the fourth group were treated with drug-free olive oil and PBS. These mice were used as a control. The survival rate for each group is shown in Fig. 3. All control mice and almost all CBPZ-treated mice did not die, whereas a significant decrease in the survival rate was observed in CsA-treated mice ($P < 0.01$). Furthermore, the mortality was accelerated by treatment with a combination of CsA and CBPZ, and most of the mice in this group died from day 2 to day 5 of infection.

Effect of CsA and CBPZ on bacterial growth in the organs of adult mice. The numbers of *L. monocytogenes* bacteria in the spleens, mesenteric lymph nodes, Peyer's patches, and feces of
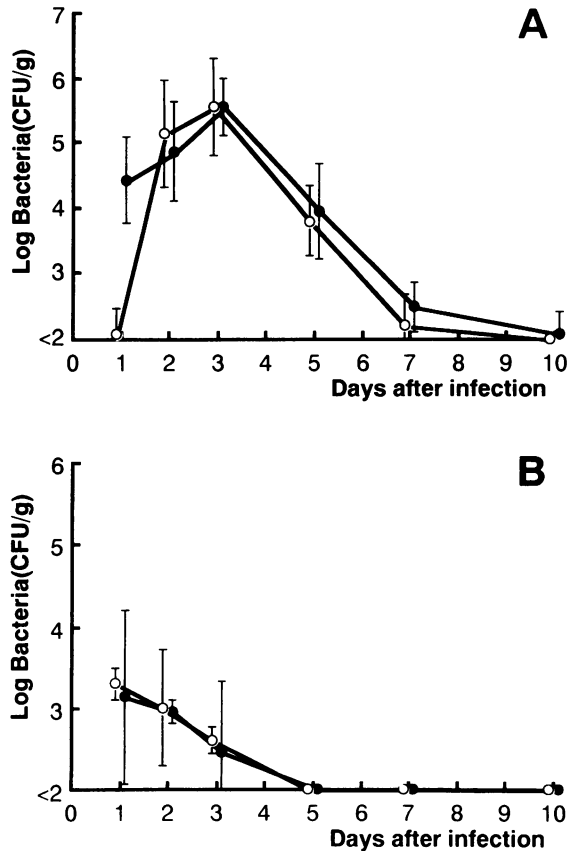
FIG. 2. (A) Growth of *L. monocytogenes* in spleens (O) and mesenteric lymph nodes (●) of adult mice; (B) growth of *L. monocytogenes* in Peyer's patches (O) and feces (●) of adult mice. Mice were infected intragastrically with 5×10^8 CFU of *L. monocytogenes*, and the number of bacteria in the specimens on the indicated days was determined. Each point represents the mean ± standard deviation for a group of five mice.

FIG. 3. Effect of CBPZ and CsA on susceptibilities to an intragastric infection with *L. monocytogenes* in adult mice. Mice were infected with 5×10^8 CFU of bacteria. They were injected intraperitoneally with CBPZ from day −2 to day 9 (●), with CsA on day −1 of infection (□), or with both (■). The control group was treated with drug-free olive oil and PBS (O). Each result represents a group of 10 mice from two experiments. An asterisk indicates a significant difference from the value for the control group at a P of <0.01.

6A), mesenteric lymph nodes (Fig. 6B), and feces (Fig. 6C) depended on the infectious doses. The bacterial growth in the spleens and mesenteric lymph nodes was observed in both the CsA- and CBPZ-treated mice, which were infected with only

the four groups were compared on day 1 (Fig. 4) and day 3 (Fig. 5) of infection. In the spleens, only the lower concentration of bacteria was found in the control mice on day 1, whereas significantly augmented growth of bacteria was observed in the other three groups ($P < 0.01$) (Fig. 4A). On day 3 (Fig. 5A), the number of bacteria in mice which received CsA only or both CsA and CBPZ was still significantly more than that in the control mice ($P < 0.01$ and $P < 0.05$, respectively). Similar results were obtained for the mesenteric lymph nodes (Fig. 4B and 5B), although the bacterial growth in CBPZ-treated mice was comparable to that in the control mice. In the Peyer's patches (Fig. 4C and 5C), mice which received the combined treatment showed a significant increase in the bacterial numbers on either day 1 or day 3 ($P < 0.01$). In the feces (Fig. 4D and 5D), the number of bacteria in both CsA- and CBPZ-treated mice was significantly more than that in the control mice ($P < 0.01$).

Effect of CsA and CBPZ on host resistance to intragastric infection with different doses of *L. monocytogenes*. Both CsA- and CBPZ-treated mice and the control mice were infected intragastrically with different doses of viable *L. monocytogenes*, and the numbers of bacterial cells in the spleens, mesenteric lymph nodes, and feces were estimated on day 3 of infection. The numbers of *L. monocytogenes* bacteria in the spleens (Fig.
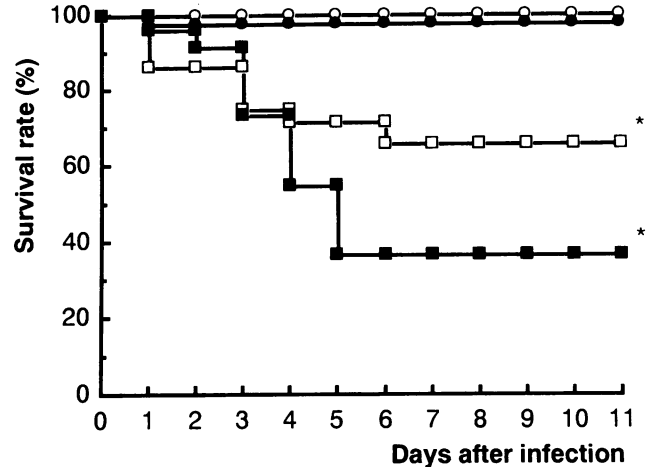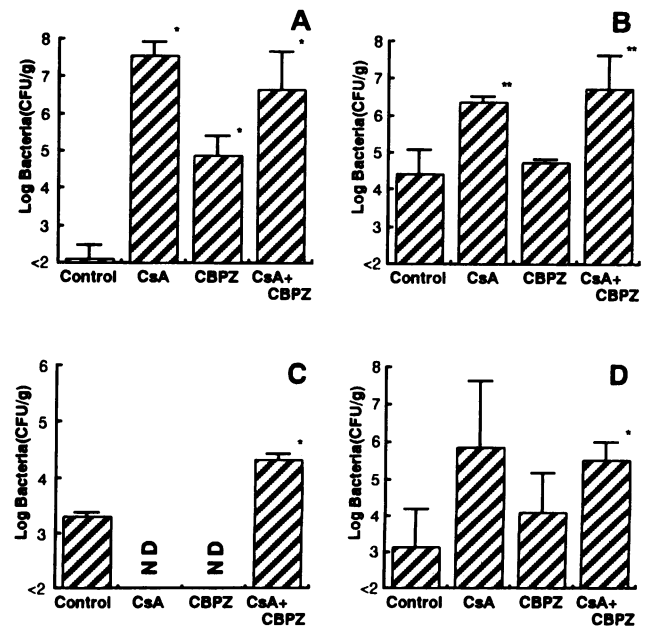

FIG. 4. Effect of CBPZ and CsA on the number of *L. monocytogenes* cells in the spleens (A), mesenteric lymph nodes (B), Peyer's patches (C), and feces (D) on day 1 of infection. Mice were infected intragastrically with 5×10^8 CFU of bacteria. They were injected with CBPZ from day −2 to day 0, CsA on day −1 of infection, or both. The control group was treated with drug-free olive oil and PBS. Each result is for a group of five mice. Single and double asterisks indicate significant differences from the value for the control group at P values of <0.01 and <0.05, respectively. ND, not determined.
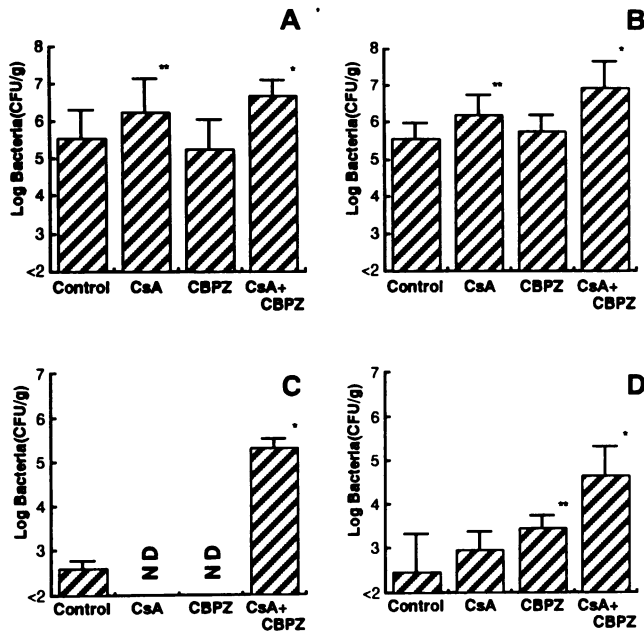
FIG. 5. Effect of CBPZ and CsA on the numbers of *L. monocytogenes* bacteria in the spleens (A), mesenteric lymph nodes (B), Peyer's patches (C), and feces (D) on day 3 of infection. Mice were infected intragastrically with 5×10^8 CFU of bacteria. They were injected with CBPZ from day -2 to day 2, CsA on day -1 of infection, or both. The control group was treated with drug-free olive oil and PBS. Single and double asterisks indicate significant differences from the value for the control group at P values of <0.01 and <0.05, respectively. ND, not determined.
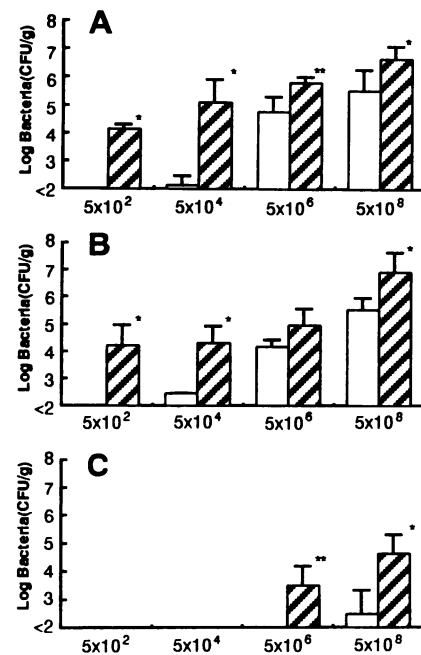
FIG. 6. Effect of CBPZ and CsA on susceptibilities to an intragastric infection with different doses of *L. monocytogenes* in adult mice. Mice, which were infected with different doses of bacteria, were injected with CBPZ from day -2 to day 2 and with CsA on day -1 of infection (hatched bars). The control group was treated with drug-free olive oil and PBS (open bars). The numbers of bacteria in the spleens (A), mesenteric lymph nodes (B), and feces (C) were determined on day 3 of infection. Each result is for a group of five mice. Single and double asterisks indicate significant differences from the value for the control group at P values of <0.01 and <0.05, respectively.

500 CFU of the bacterium, although this observation was not made for the control mice. The bacterial cells were detected in the feces when both CsA- and CBPZ-treated mice were infected with more than 5×10^6 CFU of the bacteria. In the experiments, the survival of the remaining mice was observed until day 10 of infection. The mortalities of mice which had received 5×10^8, 5×10^6, 5×10^4, or 5×10^2 CFU were 70, 20, 10, and 10%, respectively.

DISCUSSION

The studies presented here demonstrated that both cellular immunity and intestinal bacterial flora are required for host resistance against an intragastric infection with *L. monocytogenes*.

Adult mice except those that are germfree reportedly show high resistance against intragastric *L. monocytogenes* infection (13, 21, 31), although Pine et al. (20) reported that intragastric and intraperitoneal 50% lethal doses of *L. monocytogenes* were comparable. In the present study, adult mice were highly resistant to the intragastric infection compared with the systemic infection; i.e., the 50% lethal dose of an intragastric infection with the *L. monocytogenes* strain used herein was more than $10^{9.3}$ CFU (Fig. 1), while that of an intravenous infection with the strain was $10^{5.3}$ CFU (18). In contrast, suckling mice were highly susceptible to intragastric infection. It has been reported that suckling mice are markedly susceptible to systemic infection with *L. monocytogenes* (11, 19) and that intracellular killing activity against *L. monocytogenes*, antigen-presenting abilities of macrophages, and generation of *L. monocytogenes*-specific memory T cells are reduced in

suckling mice (3, 11, 12, 19). Furthermore, it is known that the intestinal bacterial flora differs markedly between suckling and adult mice (24). From the results, therefore, we noticed the effects of both immunological and bacterial changes on susceptibilities to an intragastric infection with *L. monocytogenes*.

The administration of CsA to humans and animals results in the inhibition of many T-cell-dependent functions (22). An important effect of CsA is its ability to inhibit production of cytokines, including gamma interferon, interleukin-1, and interleukin-2. Host resistance against systemic infection with *L. monocytogenes* is inhibited in CsA-treated mice (7, 25, 28). We demonstrated previously that a decreased production of endogenous gamma interferon, which is crucial in antilisterial resistance (2), is involved in the CsA effect (17). In the present study, antilisterial resistance against the intragastric infection decreased in the spleens and mesenteric lymph nodes of CsA-treated adult mice and some of the mice finally died (Fig. 3), whereas CsA did not affect the growth of *L. monocytogenes* cells in the intestines, i.e., the primary site of the oral infection (Fig. 4 and 5).

Zachar and Savage (31) reported that an intragastric inoculation of only 100 CFU of *L. monocytogenes* cells resulted in colonization in the gastrointestinal tracts of germfree mice, whereas colonization of the bacterium was not observed in those of specific-pathogen-free mice which were inoculated with as much as 5×10^7 CFU of the bacterium. Czuprynski and Balish (4) also observed that *L. monocytogenes* colonized the gastrointestinal tracts of germfree rats but not those of conventional animals. Furthermore, they demonstrated that *L.*

monocytogenes colonies were cleared from the gastrointestinal tracts of germfree rats after conventionalization. These results suggested that the intestinal bacterial flora is important in the interference of the colonization of *L. monocytogenes* in the gastrointestinal tracts. We investigated the effect of an *L. monocytogenes*-resistant broad-spectrum cephalosporin, CBPZ, on the fate of the intragastric infection. CBPZ treatment of adult mice resulted in superinfection, i.e., a marked decrease of *E. coli*, which is sensitive to CBPZ, and an increase of *Enterococcus* spp., which are resistant to the drug, were observed. The growth of *L. monocytogenes* was enhanced in CBPZ-treated mice (Fig. 5), whereas the bacterial growth in the spleens and mesenteric lymph nodes or the survival rates were not affected (Fig. 3 to 5). In contrast, treatment with a combination of CsA and CBPZ resulted in enhancement of the growth of *L. monocytogenes* in all of the organs examined (Fig. 4 and 5). Furthermore, acceleration of death from listeriosis was observed in these animals (Fig. 3). The results suggest that both cellular immunity and the intestinal bacterial flora might be essential in the host resistance against oral *L. monocytogenes* infection. When *L. monocytogenes* is given orally, the bacteria penetrate into Peyer's patches, disseminate via the efferent lymphocytes to the mesenteric lymph nodes, and finally form the infectious foci in the spleens and livers (31). In the present study, we showed that CBPZ might disturb the intestinal bacterial flora, leading easily to *L. monocytogenes* colonization of the intestinal tracts. On the other hand, CsA might suppress the functions of T cells in the Peyer's patches, mesenteric lymph nodes, spleens, and livers to disseminate *L. monocytogenes* and lead it to grow explosively.

Although broad-spectrum cephalosporins are now used in therapies of various bacterial infections, *L. monocytogenes* is consistently resistant to these antibiotics (5, 15, 29). Furthermore, it has been reported that *L. monocytogenes* can be carried easily in the gastrointestinal tracts of humans (1, 8, 9, 27). Our present results suggest that the administration of such drugs might trigger systemic *L. monocytogenes* infection via oral infection in immunocompromised hosts. Furthermore, CsA- and CBPZ-treated mice might be a useful model for analyzing mechanisms of oral *L. monocytogenes* infection and to prevent the infection.

ACKNOWLEDGMENTS

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