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Hemolysin Supports Survival But Not Entry of the Intracellular Bacterium *Listeria monocytogenes*

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The gram-positive bacterium *Listeria monocytogenes* is a facultative intracellular pathogen. The only known property of *L. monocytogenes* which has been shown to be involved in virulence is a hemolysin, listeriolysin (J. L. Gaillard, P. Berche, and P. Sansonetti, Infect. Immun. 52:50–55, 1986; S. Kathariou, P. Metz, H. Hof, and W. Goebel, J. Bacteriol. 169:1291–1297, 1987). Using our previously obtained transposon Tn*916*-induced hemolysin-negative mutants of *L. monocytogenes* Sv1/2a (Mackaness strain), we demonstrated that the loss of hemolysin reduced significantly the rate of survival of the bacteria in mouse peritoneal macrophages but did not reduce their uptake. It was further shown that virulent *L. monocytogenes* strains could invade the mouse embryo fibroblast 3T6 cell line, i.e., mammalian cells which are nonprofessional phagocytes. This uptake was inhibited by cytochalasin B and hence seems to be accomplished by parasite-induced endocytosis. Hemolysin was not essential for this step. Strains of other *Listeria* species could not efficiently penetrate the 3T6 cells.

Listeria monocytogenes, a facultative intracellular gram-positive bacterium, can cause severe infections, mainly in newborns and immunocompromised patients (6, 27). The major symptomatic manifestations of *Listeria* infections are septicemias and meningitis. The elegant studies of Mackaness (18, 19) have shown that *L. monocytogenes* can survive and multiply within macrophages. The tracing of epidemics to food contaminated with *L. monocytogenes* (4, 26) and experimental evidence (22, 23) suggest that the natural route of infection of these bacteria may be primarily the gastrointestinal tract and that intestinal epithelial cells may become invaded by the bacteria at the onset of infection (24). Experimental evidence has indeed indicated that *L. monocytogenes* can enter and multiply in nonphagocytic cells (9). The potential of these bacteria to penetrate nonprofessional phagocytes, however, had not been extensively investigated.

All virulent strains of *L. monocytogenes* produce an extracellular SH-activated hemolysin, listeriolysin. The finding that nonhemolytic transposon mutants were avirulent in the mouse infection model (5, 13) suggests that the expression of this hemolysin is necessary for virulence of *L. monocytogenes*. However, the mechanism of involvement of the hemolysin in the virulence of the bacteria remains unclear.

Using the Tn*916*-induced nonhemolytic mutants of *L. monocytogenes*, we demonstrated that the lack of listeriolysin synthesis reduced significantly the rate of survival of the bacteria in mouse peritoneal macrophages but did not reduce their uptake. We further provide evidence that active uptake of unopsonized *L. monocytogenes* by mouse fibroblast cells is possible and that such uptake is accomplished by parasite-induced endocytosis. Uptake did not require the production of listeriolysin. Only virulent *Listeria* strains were able to enter the fibroblasts, a finding which suggests that the ability to enter nonprofessional phagocytes may represent a component of the virulence of these bacteria.

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MATERIALS AND METHODS

Bacterial strains and growth media. The *Listeria* strains used in this study were derived from the *Listeria* strain collection of H. P. R. Seeliger, Würzburg, Federal Republic of Germany, except strain EGD, which was provided by S. H. E. Kaufmann, Max-Planck-Institute for Immunology, Freiburg, Federal Republic of Germany. The hemolytic and nonhemolytic transposon Tn*916* mutants used in this investigation have been described (13). The *Listeria* strains were grown in brain heart infusion broth (Difco Laboratories) at 37°C with aeration. The bacteria were obtained in the logarithmic phase of growth and were stored at −70°C in phosphate-buffered saline (PBS) with 20% (vol/vol) glycerol until used.

Collection and cultivation of peritoneal macrophages. Peritoneal macrophages from approximately 3-month-old BALB/c mice were harvested and purified by a modification of the procedure of Harrington-Fowler et al. (8). The peritoneal cells were washed in PBS and suspended in RPMI 1640 medium (Seromed) supplemented with 10% fetal calf serum and L-glutamine. A total of 5×10^5 macrophages were allowed to adhere to petri dishes for 1 h in a 5% CO_2 atmosphere at 37°C. The macrophages were then washed three times with PBS to remove nonadherent cells. Macrophage viability was routinely confirmed by trypan blue exclusion.

Infection and enumeration of cultures. A total of 5×10^5 macrophages were infected with 5×10^6 bacteria. After 40 min of phagocytosis, the cultures were washed five times with 5 ml of PBS to remove excess extracellular bacteria and reincubated for 40 min in medium containing gentamicin (Serva) at a bactericidal concentration (50 μg/ml) to kill residual or adherent bacteria not removed by the washing procedure. The antibiotic gentamicin cannot penetrate mammalian cells (3), and therefore bacteria that escape killing can be regarded as intracellular.

After the gentamicin treatment the cultures were washed three times with 5 ml of PBS to remove the antibiotic. The macrophages were then lysed by the addition of 2 ml of 1% Triton X-100, and the number of intracellular bacteria was

determined by plate counts of the lysates on brain heart infusion agar plates. This determination represented the intracellular count at time zero. The subsequent fate of intracellular bacteria was determined in separate cultures infected as described above. After removal of the antibiotic, the medium was changed every hour for the duration of the experiment, and intracellular bacterial counts were performed at 3 and 6 h. The washing buffer was routinely cultured to monitor the presence of any residual, free, or exocytosed bacteria.

Invasion assays. Invasion assays were performed essentially as described previously (11). 3T6 cells (10^6 per well, 12-well dishes; Costar 3512) in RMPI 1640 medium supplemented with 10% fetal calf serum and L-glutamine were infected with 10^7 bacteria grown and stored as described above. After centrifugation of the bacteria onto the monolayer for 10 min at 1,000 rpm at 20°C in a Sorvall RT6000 centrifuge as described previously (31), the infected cultures were incubated at 37°C for 3 h in a 5% CO_2 atmosphere. During this time the bacteria were allowed to bind to and become endocytosed by the 3T6 cells. Nonadherent bacteria were removed by three washes with 3 ml of PBS, and the cultures were incubated further for 2 h in RPMI 1640 medium containing 50 μg of gentamicin per ml. The cells were then washed twice with 3 ml of PBS, and intracellular bacteria were released by the addition of 2 ml of 1% Triton X-100. Viable cells were determined by plating the lysate.

RESULTS

Hemolysin supports survival of *L. monocytogenes* in macrophages. A nonhemolytic (Hly^-) avirulent *L. monocytogenes* strain was generated by Tn*916* mutagenesis of a streptomycin-resistant (Sm^r) mutant of the hemolytic (Hly^+) virulent strain Mackaness SLCC 5764 (13). The Hly^+ Sm^r strain and the Hly^- Sm^r derivative were compared with respect to their uptake by mouse peritoneal macrophages and their ability to survive within these cells. In vitro studies showed that the two strains were indistinguishable in their growth rates and extent of sensitivity to gentamicin.

Upon incubation with the macrophages, both strains were capable of becoming internalized. Noticeable differences emerged, however, in their relative ability to survive and multiply intracellularly. The Hly^+ bacteria were able to multiply, and after 6 h a 10-fold increase in their numbers was observed. The Hly^- bacteria, on the other hand, seemed unable to multiply intracellularly, and their numbers declined steadily (Fig. 1). These differences in intracellular survival between Hly^+ and Hly^- bacteria were also observed when the original wild-type strain (SLCC 5764) was compared with the Hly^- mutant. Furthermore, similar differences were observed when these bacteria were mixed 1:1 and used to infect a single monolayer and the intracellular bacteria were plated on sheep blood agar (data not shown).

The inability of the Hly^- bacteria to multiply intracellularly appeared to be associated with the Hly^- phenotype itself and was not due to a nonspecific effect of the presence of Tn*916*. Two randomly chosen Hly^+ Tn*916*-carrying derivatives of this strain were capable of intracellular multiplication (Fig. 1). It can be seen, however, that their rate of multiplication was to some extent lower than that of the Hly^+ strain which lacked Tn*916*. This may have been due to a side effect of the resistance to tetracycline conferred by Tn*916*.

In such experiments it often appeared that the uptake of the Hly^- mutant by the macrophages was higher than that of the Hly^+ bacteria. It is possible that the extracellular hemolysin produced by the latter had a cytotoxic effect on the cells, so that fewer macrophages were available for the internalization of these bacteria. This was supported by the finding that in mixed infections, in which one macrophage culture was available for the internalization of the two types of bacteria, no differences in uptake efficiency were observed (data not shown).

***L. monocytogenes* can enter nonprofessional phagocytes, but the hemolysin is not involved in this step.** Peritoneal macrophages were capable of internalizing the Hly^+ and Hly^- strains of *L. monocytogenes*, as well as *L. innocua* (serotype 6a). To determine whether the uptake was due not only to the phagocytic activity of the host cell but also depended on parasite-dependent functions, we performed invasion assays using the mouse embryonic fibroblast cell line 3T6. The Hly^+ virulent *L. monocytogenes*, described above, and its Hly^- derivative were taken up by the 3T6 cells with equally high efficiency (Fig. 2). Two additional virulent strains of *L.*

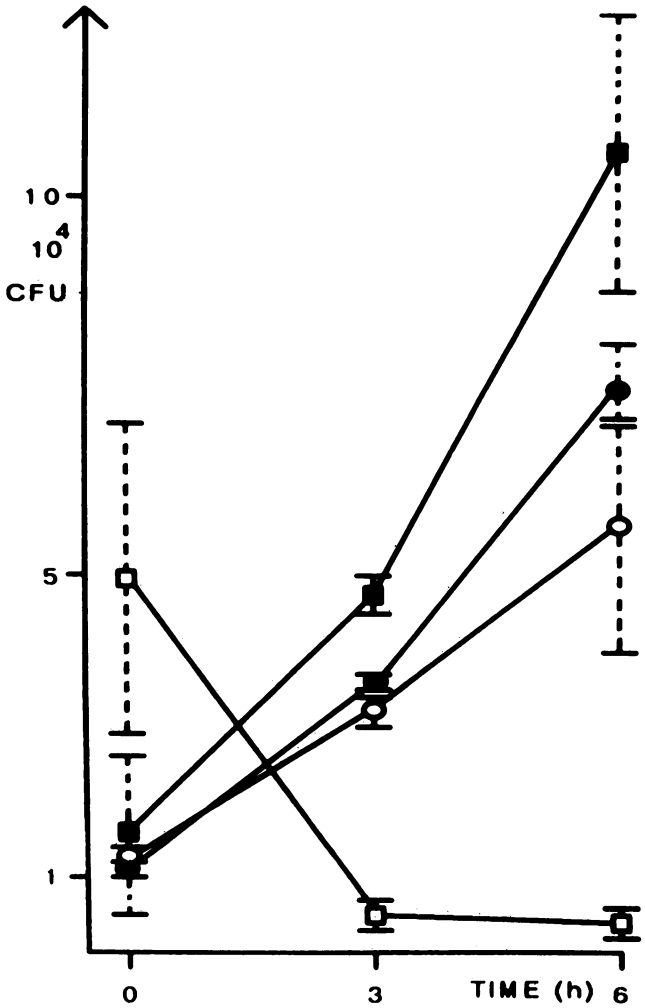

FIG. 1. Intracellular survival of Hly^+ and Hly^- *L. monocytogenes* within mouse peritoneal macrophages. A total of 5×10^5 macrophages were infected with 5×10^6 bacteria: the Sm^r Hly^+ strain (■), the Tn*916*-carrying strains WT1 (●) and WT2 (○), and the Hly^- mutant M3 (□). The uptake and survival of the bacteria were determined as described in the text. Each datum point represents the average of results from two experiments.

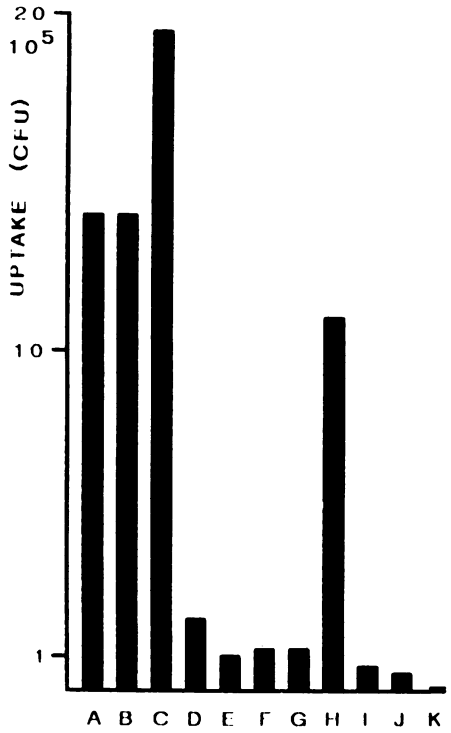

FIG. 2. Uptake of different *Listeria* strains by 3T6 fibroblasts. Bacteria were added to 3T6 monolayers in a ratio of 10 bacteria to 1 fibroblast and treated as described in the text. The strains used were *L. monocytogenes* Mackaness (SLCC 5764) (A), *L. monocytogenes* EGD (B), *L. monocytogenes* ATCC 10527 (serotype 4b) (C), *L. monocytogenes* SLCC 5105 (serotype 3a) (D), *L. innocua* SLCC 3379 (serotype 6a) (E), *L. innocua* SLCC 3423 (serotype 6b) (F), *L. ivanovii* SLCC 2379 (serotype 5) (G), *L. monocytogenes* Hly^- mutant M3 (H), *L. seeligeri* SLCC 18/100 (I), *L. grayi* (J), and *B. subtilis* BR151 (K).

monocytogenes, EGD (serotype 1/2a) and ATCC 10527 (serotype 4b), were internalized with similar efficiency. Reduced efficiency of uptake was observed only for a serotype 3a strain (SLCC 5105) which is hemolytic but has only partial virulence in the mouse infection model (15). The relatively efficient uptake of *L. monocytogenes* is in sharp contrast to what we observed with other *Listeria* species. Several strains of other species were assayed. None of them, including the hemolytic avirulent *L. seeligeri* and the strongly hemolytic *L. ivanovii*, which is pathogenic in mice, was taken up by the 3T6 cells to any appreciable extent. A failure of uptake was observed with *Bacillus subtilis*, a bacterium used in these studies as a noninvasive control.

These results strongly suggest that *L. monocytogenes* possesses a mechanism for active invasion and is therefore able to enter nonprofessional phagocytes. Uptake of the bacteria was abolished by treatment of the 3T6 cells with cytochalasin B at concentrations as low as 5 μg/ml (Fig. 3). This drug inhibits microfilament function and hence endocytosis (2). The observed sensitivity of the uptake to cytochalasin B indicates that parasite-induced phagocytosis was involved in the internalization of the bacteria.

DISCUSSION

It has been conclusively shown that macrophages are of key importance as host cells in infections by *L. monocytogenes* (18, 19). The ability of these bacteria to survive and multiply within macrophages appears to be a prerequisite for proper antigen processing and presentation and for the generation of cell-mediated immunity to *L. monocytogenes* (7). In the present study, we showed that the production of the SH-activated hemolysin of *L. monocytogenes* was correlated with the ability of the bacteria to survive and multiply within peritoneal macrophages.

The mechanism of involvement of the hemolysin in the intracellular survival of the bacteria is not clear. It has been proposed that the hemolysin may lyse the phagosomal membrane and allow the bacteria to escape into the cytoplasm, where they are protected from the action of lysosomal enzymes (1, 14). Electron microscopy of macrophages obtained after intraperitoneal infection of mice with virulent *L. monocytogenes*, however, did provide evidence of fusion of the phagosomal and lysosomal membranes (21). Although our data suggest an involvement of listeriolysin in the survival and intracellular multiplication of *L. monocytogenes*, they do not strictly exclude the possibility that not the hemolysin itself but other factors, the expression of which may be coregulated with hemolysin synthesis, are responsible for this effect.

The finding that unopsonized *L. monocytogenes* can be internalized not only by professional phagocytes but also by fibroblasts suggests that the virulent bacteria may be actively involved in their own uptake. It was interesting to discover that the only strain (SLCC 5105) which was not efficiently internalized by the 3T6 cells has been determined to have only partial virulence (15). This strain was able to survive but not multiply in host tissues (15). All of the other *Listeria* species which were surveyed, i.e., *L. innocua*, *L. grayi*, *L. seeligeri*, and *L. ivanovii* appeared to be unable to mediate their own endocytosis in 3T6 cells. The first three species are

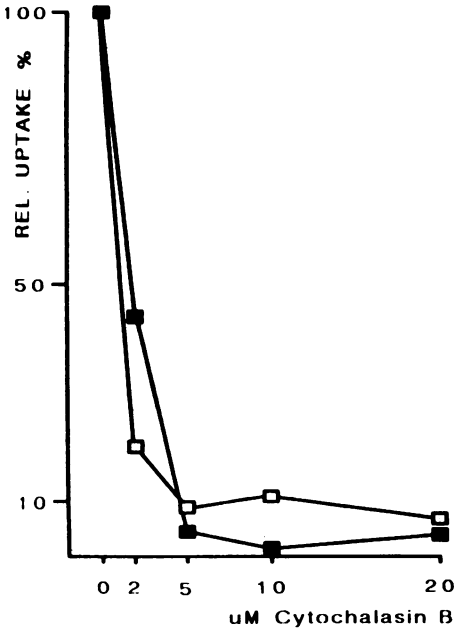

FIG. 3. Cytochalasin B inhibition of 3T6 cell invasion by *L. monocytogenes*. An invasion assay was performed with 3T6 embryo mouse fibroblast cells as described in the text. Cytochalasin B (Sigma Chemical Co.) was added to the cell culture 30 min before infection. The cytochalasin B was dissolved in dimethyl sulfoxide at a concentration of 2 mM. The inhibitory effect of cytochalasin B was determined with the Hly^+ Sm^r strain (■) and the Hly^- mutant (□).

avirulent. *L. ivanovii* is experimentally pathogenic for mice (25) but differs clearly from *L. monocytogenes* in pathogenic potential, disease symptomatology, and epidemiology of infections (10, 12, 20, 29, 30). Our data suggest that the ability of the bacteria to mediate their own endocytosis is a factor necessary, but not sufficient, for the expression of virulence. The efficient internalization of the Hly^- avirulent mutant of *L. monocytogenes* indicates that uptake is not dependent on the production of the SH-activated hemolysin.

The lack of uptake of the *L. innocua* strains is meaningful in the context of the special status of this species: *L. innocua* is indistinguishable from *L. monocytogenes* in morphology and fermentative characteristics but differs in being nonhemolytic and avirulent in the mouse infection model (25, 28). This report provides the first evidence suggesting that the lack of virulence of *L. innocua* may be due to the absence of not only the hemolysin but also additional virulence factors, such as the ability to enter nonprofessional phagocytes.

The ability of *L. monocytogenes* to enter nonprofessional phagocytes (invasiveness) may be especially important in the early phase of infection. Recent epidemics of listeriosis have been traced to food contaminated with *L. monocytogenes* (4, 15), and intestinal infections by these bacteria have been demonstrated experimentally (17, 20, 22, 23). It is possible that after their ingestion the virulent bacteria are able to mediate their own uptake by intestinal epithelial cells. In experimental systemic infections the bacteria are rapidly sequestered within the resident macrophages of the spleen and liver (18). It is still to be determined whether in vivo the ability of the bacteria to mediate their own uptake is involved in their internalization and subsequent processing by these macrophages and by the immigrant macrophages with high bactericidal potential (16).

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