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Expression of the *inlAB* Operon by *Listeria monocytogenes* Is Not Required for Entry into Hepatic Cells In Vivo

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***Listeria monocytogenes* injected intravenously into mice is taken up in the liver, where hepatocytes serve as the principal site of intracellular replication. The factors effecting entry of *L. monocytogenes* into hepatic cells remain to be determined. Others have shown that the protein products of the *inlAB* (internalin) operon are required for maximum entry of *L. monocytogenes* into a number of cell lines in vitro. Likewise, we report here that expression of the *inlAB* operon was required for maximum uptake of *L. monocytogenes* by primary cultures of mouse hepatocytes. Uptake of an *inlAB* mutant strain of *L. monocytogenes* was approximately 10-fold less than that of the isogenic wild-type control. In contrast, *inlAB* expression was not a factor in (i) clearance of *L. monocytogenes* injected intravenously into mice and taken up in the liver, (ii) the distribution of *L. monocytogenes* among hepatocytes and nonparenchymal cells in the liver, or (iii) internalization of *L. monocytogenes* by hepatic cells in vivo. These latter findings suggest that infection of hepatic cells by *L. monocytogenes* in vivo does not require the protein products of the *inlAB* operon.**

Listeria monocytogenes is a gram-positive, facultative intracellular bacterium capable of causing severe infections in humans (15, 25). Listeriosis in mice is an experimental model used widely to study cell-mediated host defenses against intracellular pathogens (16). *L. monocytogenes* can replicate within a variety of cell types including macrophages, hepatocytes, fibroblasts, and epithelial cells (2). In the case of macrophages, the binding and uptake of *L. monocytogenes* are mediated by complement receptors expressed on the cell surface and by the C1q and C3b components of complement bound to microorganisms (1, 4, 5). In the absence of complement, type I scavenger receptors expressed by macrophages facilitate *Listeria* binding (7). In the latter case, lipoteichoic acid, a component of most gram-positive bacteria, appears to be the ligand recognized.

The uptake of *L. monocytogenes* by nonprofessional phagocytes, e.g., hepatocytes and epithelial cells, is less well understood. A number of recent studies have focused on the *inlAB* operon and the role of two genes, *inlA* and *inlB*, in the uptake of *L. monocytogenes* by cells in culture (3, 8, 14, 18). *inlA* encodes an 88-kDa protein, internalin (InlA), which is expressed on the surface of *L. monocytogenes* and is required for entry into several epithelial cell lines. *inlB*, located downstream from *inlA*, encodes a protein (InlB) which is similar to internalin in both amino acid sequence and function. *Listeria* mutants unable to generate functional products of the *inlAB* operon are diminished in their capacity to infect a number of cultured cell lines. Although *inlAB* expression is critical for maximum uptake of *L. monocytogenes* by cells in vitro, the function of InlA and InlB in vivo remains to be demonstrated.

The bulk of *L. monocytogenes* injected intravenously (i.v.) into mice is cleared rapidly from the bloodstream and can be recovered in the liver (10, 19). At 6 h postinfection, >93% of the listeriae recovered are located within hepatocytes, which serve as the principal site of listerial replication (9, 10). By employing mutants bearing lesions in the *inlAB* operon, a series of experiments was undertaken to determine the role of internalin in the uptake of *L. monocytogenes* by hepatocytes. The results of these experiments indicate that the product of the *inlB* gene exerts a significant effect on the uptake of *L. monocytogenes* by primary cultures of mouse hepatocytes. Neither *inlAB* expression nor the production of InlB was a factor, however, in (i) the clearance of *L. monocytogenes* from the bloodstream, (ii) the distribution of *L. monocytogenes* among the parenchymal cells and nonparenchymal cells (NPCs) of the liver, or (iii) the internalization of *L. monocytogenes* by hepatic cells in vivo.

MATERIALS AND METHODS

Bacteria. The wild-type strain (EGD-Sm^r) and the isogenic transposon Tn*1545*-induced internalin (*inlAB*) mutant strain (BUG5) of *L. monocytogenes* were obtained from Pascale Cossart (Institut Pasteur, Paris, France); neither *inlA* nor *inlB* is expressed by BUG5 (8). The ESL12 mutant strain, characterized by an in-frame deletion in the *inlA* gene, and the 10403S strain from which it was derived were obtained from Hao Shen (UCLA School of Medicine, Los Angeles, Calif.). Stock cultures of each strain were prepared and stored at −70°C in accordance with methods previously reported (27). Bacteria prepared from broth cultures growing exponentially were used in the experiments described below.

Mice. Specific-pathogen-free female C57BL/6J mice were purchased from The Jackson Laboratories (Bar Harbor, Maine). The mice were housed and cared for in accordance with the guidelines set forth by the Institute of Laboratory Animals Resources, National Research Council. Eight- to 16-week-old mice were used throughout the study reported. The numbers of listeriae recovered in the livers and spleens of infected animals were estimated from the colonies that grew on Trypticase soy agar plates inoculated with an aliquot of organ homogenate (26).

Preparation of hepatic cells. The parenchymal cells (hepatocytes) and NPCs were obtained following perfusion of the liver with collagenase by the two-step method we reported previously (9, 12, 13). The liver was dissected, the gall bladder and extraneous tissues were removed, the liver was teased apart, and the resultant cell suspension was centrifuged at 30 × *g* for 4 min at 4°C. The cell population recovered in the pellet was washed once by low-speed centrifugation and was composed of ≥95% viable hepatocytes as judged on the basis of morphology and the exclusion of trypan blue. The NPCs remaining in suspension after the first low-speed centrifugation were purified by centrifugation on a 16% metrizamide gradient (11).

The hepatocyte- and NPC-associated listeriae were quantified by lysing 10^6 purified cells with 0.05% Triton X-100, inoculating agar plates with aliquots of diluted cell lysate, and counting the colonies that grew. Estimations of the numbers of organisms associated with the total hepatocyte and NPC populations composing the liver are based upon our findings that normal livers or livers at 10 min postinfection contain approximately 1.0×10^8 hepatocytes and 2.75×10^7 NPCs. The size of the NPC population was ≈10% larger at 6 h postinfection, i.e., 3×10^7 NPCs per liver, largely because of the influx of neutrophils (10). Extracellular (antibiotic-sensitive) and intracellular (antibiotic-resistant) listeriae were

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TABLE 1. *inlAB* expression affects the uptake of *L. monocytogenes* by hepatocytes in vitro[a]

Cell population	Strain	Genotype	No. of listeriae (log_{10}/well)[b]	% Entry
Hepatocyte	EGD-Sm^r	Wild type	4.98 ± 0.09	4.77
	BUG5	*inlAB*	3.92 ± 0.06[c]	0.42
	10403S	Wild type	4.56 ± 0.06	1.82
	EJL12	*inlA*	4.62 ± 0.07	2.08
Kupffer cell	EGD-Sm^r	Wild type	3.92 ± 0.04	0.42
	BUG5	*inlAB*	4.12 ± 0.10	0.66
	10403S	Wild type	3.39 ± 0.06	0.12
	EJL12	*inlA*	3.67 ± 0.05	0.23

[a] Mouse hepatocytes and adherent NPCs were infected with the *Listeria* strain indicated (2×10^6 bacteria per well), centrifuged, and incubated for 1 h prior to the addition of gentamicin.

[b] Bacteria surviving 2 h of incubation in the presence of 5 μg of gentamicin per ml. The data are means ± standard deviations derived from four wells treated identically in a single experiment; two additional experiments yielded comparable results.

[c] Significantly less than the value for EGD-Sm^r ($P < 0.001$).

readily differentiated by incubating purified hepatic cells for 2 h in the presence of 5 μg of gentamicin per ml. Lysis of hepatic cells with 0.05% Triton X-100 rendered the associated *L. monocytogenes* completely sensitive to antibiotic treatment. Triton X-100 alone had no effect on the viability of *L. monocytogenes*.

For in vitro studies, the Kupffer cells were separated from other NPC types by attachment to plastic according to methods we described previously (11). Ninety-six-well tissue culture plates were inoculated with 10^5 NPCs per well, and the plates were incubated for 15 min at 37°C. The nonadherent cells were removed by washing the culture substratum six times with warm medium. Kupffer cells constituted ≥85% of the adherent cell population as judged by a number of criteria (11).

Uptake of *L. monocytogenes* by hepatic cells in culture. The internalization of *L. monocytogenes* by hepatic cells in culture was assessed by methods previously described by ourselves and others (8, 13). Purified hepatocytes (2×10^4 cells per well) and Kupffer cells in microtiter plates containing HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-buffered RPMI 1640 medium supplemented with 1 mM sodium pyruvate, 10% heat-inactivated fetal bovine serum (Sterile Systems, Inc., Logan, Utah), and 10^{-7} M recombinant human insulin (Humulin R; Eli Lilly Co., Indianapolis, Ind.) were incubated overnight. On the following day, the wells were inoculated with *L. monocytogenes*, the plates were centrifuged for 5 min at 200 × *g*, and the cells were cultured for 1 h at 37°C. Gentamicin (5-μg/ml final concentration) was then added, and the cells were cultured for an additional 2 h in order to kill the extracellular organisms. The cells were then lysed with 0.05% Triton X-100 in Trypticase soy broth. Aliquots of the lysate were transferred to Trypticase soy agar plates, and the number of listeriae per well was calculated from the number of colonies that grew.

Statistical analysis. The results were analyzed by using the SigmaStat statistics program (Jandel Scientific, San Rafael, Calif.). Individual means were compared by using a nonpaired Student *t* test.

RESULTS

***inlAB* expression by *L. monocytogenes* is required for optimal infection of mouse hepatocytes in culture.** The expression of the *inlAB* operon and the production of InlA and InlB affect the binding and subsequent uptake of *L. monocytogenes* by a variety of cell lines (3, 8, 18). Similarly, we found that *inlAB* expression was required for maximum uptake of *L. monocytogenes* by primary cultures of mouse hepatocytes. Relative to that of the wild-type control (EGD-Sm^r), the transposon-induced *inlAB* mutant *Listeria* strain (BUG5) exhibited an 11-fold reduction in its capacity to enter hepatocytes as judged on the basis of its continued sensitivity to gentamicin (Table 1). Entrances by the *inlA* deletion mutant EJL12 and the isogenic wild-type 10403S strains, however, were virtually identical. This latter finding suggests that the expression of *inlB* and the production of InlB, but not InlA, are requirements for maximum uptake of *L. monocytogenes* by mouse hepatocytes in vitro. In contrast to that by hepatocytes, *inlAB* expression was not a factor in the uptake of *L. monocytogenes* by Kupffer cells. Uptake of both the *inlAB* and *inlA* mutant strains was comparable to that of the wild-type strains from which they originated.

TABLE 2. Clearance of *L. monocytogenes* from the bloodstream does not require *inlAB* expression[a]

Time postinfection	Strain	No. of listeriae (CFU/organ)[b] in:	
		Liver	Spleen
10 min	EGD-Sm^r	6.72 ± 0.08 (31.0 ± 6.2)	5.61 ± 0.16 (2.5 ± 0.7)
	BUG5	6.62 ± 0.08 (32.8 ± 6.9)	5.39 ± 0.14 (2.0 ± 0.9)
6 h	EGD-Sm^r	5.89 ± 0.03 (4.6 ± 0.3)	5.24 ± 1.10 (1.1 ± 0.6)
	BUG5	5.76 ± 0.07 (4.6 ± 0.8)	4.46 ± 0.15 (0.2 ± 0.1)

[a] Mice were inoculated i.v. with 7.24 log_{10} units of EGD-Sm^r or 7.11 log_{10} units of BUG5 listeriae. The livers and spleens were dissected at 10 min and 6 h postinfection, and the CFU per organ were determined.

[b] Values are the means ± standard deviations obtained from four mice; comparable results were obtained in a second experiment. Numbers in parentheses indicate percentages of the inoculum; differences between the EGD-Sm^r and BUG5 strains under comparable conditions are not significant.

***inlAB* expression does not affect clearance of *L. monocytogenes* from the bloodstream.** *L. monocytogenes* injected i.v. into mice is cleared rapidly from the bloodstream (19). Clearance of the blood was not affected by expression of the *inlAB* operon. At 10 min postinfection, approximately 30 and 2% of the inoculum were recovered in the livers and spleens, respectively, of mice infected i.v. with either the EGD-Sm^r (*inlAB*$^+$) or the BUG5 (*inlAB* mutant) strain of *L. monocytogenes* (Table 2). Similarly, the bacterial loads of the organs obtained from the mutant and wild-type strains of *L. monocytogenes* at 6 h postinfection were virtually identical.

***inlAB*$^+$ and *inlAB* mutant strains of *L. monocytogenes* taken up in the liver are equally distributed among hepatic cells.** Recently, we reported that *L. monocytogenes* injected i.v. and taken up in the liver was initially distributed equally among the parenchymal cells (hepatocytes) and NPCs (10). Expression of the *inlAB* operon did not affect this distribution. Regardless of whether the *inlAB*$^+$ or *inlAB* mutant strain of *L. monocytogenes* was used for inoculation, 5 to 8% of the organisms were associated with both the hepatocyte and NPC populations at 10 min postinfection (Table 3). Likewise, a comparable percentage of each inoculum was associated with the hepatocytes and NPCs at 6 h postinfection.

TABLE 3. *inlAB* expression does not affect the association of *L. monocytogenes* with hepatic cells in vivo[a]

Time postinfection	Strain	Listeriae (% of inoculum)[b] in:	
		Hepatocytes	NPCs
10 min	EGD-Sm^r	5.0 ± 0.3	6.9 ± 0.6
	BUG5	6.8 ± 1.1	8.7 ± 3.9
6 h	EGD-Sm^r	0.6 ± 0.1	0.3 ± 0.1
	BUG5	0.6 ± 0.1	0.9 ± 0.3

[a] Mice were infected i.v. with 7.58 log_{10} units of the wild-type (EGD-Sm^r) strain or 6.77 log_{10} units of the internalin mutant (BUG5) strain of *L. monocytogenes*. Perfusion of livers and purification of hepatic cells were initiated at the indicated times postinfection.

[b] Values are the means ± standard deviations of the percentages of the inoculum associated with the hepatocyte and NPC populations derived from four mice; a second experiment yielded comparable results.

TABLE 4. Most *inlAB* mutant listeriae recovered in the liver at 6 h postinfection are intracellular[a]

Strain	Cells	No. of listeriae ($\log_{10}$ CFU/10^6 cells)[b] in cultures:		% Gentamicin-resistant listeriae
		Without gentamicin	With gentamicin	
BUG5	Hepatocytes	3.23 ± 0.04	3.16 ± 0.02	85
	NPCs	2.91 ± 0.17	2.79 ± 0.13	62
EGD-Sm^r	Hepatocytes	3.47 ± 0.15	3.35 ± 0.13	76
	NPCs	3.33 ± 0.15	3.14 ± 0.05	65

[a] Perfusion of livers and purification of hepatic cell populations were initiated at 6 h postinfection i.v. with 6.67 $\log_{10}$ units of the BUG5 strain or 7.56 $\log_{10}$ units of the EGD-Sm^r strain of *L. monocytogenes*. Purified cells were subsequently cultured for 2 h in the absence or presence of 5 μg of gentamicin per ml.

[b] Values are the means ± standard deviations for populations obtained from four mice in a single experiment; comparable results were obtained in a second experiment.

Expression of *inlAB* does not affect internalization of *L. monocytogenes* by hepatic cells in vivo. Previously, we reported that the bulk of *L. monocytogenes* injected i.v. and recovered initially in the liver was bound extracellularly and sensitive to gentamicin treatment (10). The organisms recovered in the liver at 6 h postinfection, on the other hand, were located intracellularly and were drug resistant. To determine the effect of *inlAB* expression on the internalization of *L. monocytogenes* by hepatic cells in vivo, mice were infected i.v. with the BUG5 (*inlAB*) strain. The hepatocytes and NPCs were isolated 6 h later, and the effect of gentamicin treatment on associated *L. monocytogenes* was determined. As shown in Table 4, the bulk of *inlAB* mutant *L. monocytogenes* associated with hepatocytes and NPCs isolated at this time was intracellular and gentamicin resistant. Comparable results were obtained when mice were infected with the *inlAB*$^+$ (EGD-Sm^r) strain of *L. monocytogenes*.

DISCUSSION

Listeriae injected i.v. into mice are cleared rapidly from the bloodstream and taken up in the liver where hepatocytes constitute the principal site of replication (9, 10, 24). Recently, we reported that the organisms taken up in the liver are initially distributed equally among the parenchymal cells and NPCs (10). Moreover, the bulk of the listeriae recovered in the liver early during the course of infection are bound extracellularly as indicated by the sensitivity of cell-associated bacteria to gentamicin treatment. In contrast, most organisms associated with hepatic cells isolated later, i.e., at 6 h postinfection, are intracellular and gentamicin resistant. The factors that facilitate the entry of *L. monocytogenes* into hepatic cells remain to be delineated.

In the experiments reported here, the transposon-induced *inlAB* mutant, BUG5, exhibited a reduced capacity to infect primary cultures of mouse hepatocytes. This finding correlates with the results of others demonstrating the importance of InlA, InlB, and the expression of the *inlAB* operon in the uptake of *L. monocytogenes* by a variety of cell lines in vitro (3, 8, 14, 18). In contrast to BUG5, the *inlA* mutant (EJL12) was comparable to the isogenic wild-type strain in its capacity to infect mouse hepatocytes. Thus, *inlA* expression was not required for the entry of *L. monocytogenes* into mouse hepatocytes in vitro. Rather, entry appeared to be dependent upon the protein product of *inlB*, i.e., InlB. Other investigators reported a similar requirement, i.e., for the expression of *inlB* but not *inlA*, for optimal penetration of a murine hepatocyte cell line (ATCC TIB73) by *L. monocytogenes* (3). While this result correlates directly with our own, it is pertinent that some doubt exists in the literature concerning the relationship of the TIB73 cell line (characterized originally as a mouse fibroblast cell line derived from embryonic liver) to authentic mouse hepatocytes (22). Nonetheless, our findings support the suggestion of Dramsi et al. (3) that the family of genes composing the *inlAB* operon may contribute to the tropism exhibited by *L. monocytogenes* for cells in culture.

While internalin is a critical factor in the uptake of *L. monocytogenes* by a variety of cell types in culture, a number of studies indicate the existence of an internalin-independent pathway(s) of entry into cells. The uptake of *L. monocytogenes* by human endothelial (6) and mouse fibroblast (20) cell lines, for example, is significant even in the absence of *inlAB* expression. In the experiments reported here, *inlAB* expression was not a factor in the uptake of *L. monocytogenes* by Kupffer cells. The ability of Kupffer cells to internalize *inlAB*$^+$ and *inlAB* mutant strains of *L. monocytogenes* at comparable rates undoubtedly reflects the variety of receptors or mechanisms that are known to mediate phagocytosis by macrophages (21).

While expression of the *inlAB* operon exerted a significant influence on the capacity of *L. monocytogenes* to infect mouse hepatocytes in culture, it had no detectable effect on (i) clearance of *L. monocytogenes* from the bloodstream, (ii) the distribution of *L. monocytogenes* among the parenchymal cells and NPCs of the liver, or (iii) internalization of *L. monocytogenes* by hepatic cells in vivo. In this regard, it is relevant that internalization of both the *inlAB*$^+$ and *inlAB* mutant strains of *L. monocytogenes* used in this study was small relative to internalization of the wild-type strain of *L. monocytogenes* used in our laboratory for many years. While <1.0% of either the *inlAB*$^+$ or the *inlAB* mutant strain inoculated i.v. was recovered within the hepatocyte population at 6 h postinfection (Table 3), *L. monocytogenes* equivalent to >20% of the inoculum was recovered within hepatocytes at 6 h in experiments reported previously (10).

Recently, Hess et al. (14), studying the role of internalin in the uptake of *L. monocytogenes* in vivo, reported findings compatible with the results reported here. Mice inoculated per os with wild-type (EGD-Sm^r) and isogenic *inlAB* mutant (BUG8) *Listeria* strains exhibited comparable bacterial loads in their livers at 1 and 5 days postinfection. In contrast, other investigators reported the retarded growth of *Listeria inlAB* mutants in the livers of mice inoculated intragastrically (3). No significant difference was found, however, in the bacterial loads of the spleens and mesenteric lymph nodes of these same animals. This latter finding led the authors to query the relevance of internalin to listerial infections in vivo and to suggest that *L. monocytogenes* may have evolved multiple strategies for invading host tissues (3). Indeed, recent experimental evidence indicates that a major extracellular protein (p60) secreted by *L. monocytogenes* also affects the uptake of organisms by mammalian cells in vitro (17, 23). Moreover, mutants impaired in their capacity to synthesize p60 are noninvasive and avirulent (17, 23).

Recent experiments in our laboratory indicate the capacity of hepatocytes to bind and internalize a variety of bacteria in addition to *L. monocytogenes*, e.g., *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella pneumoniae* (unpublished observation). The factors that facilitate the binding and uptake of bacteria by the parenchymal cells of the liver are a subject of ongoing investigation.

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