Internalin B Promotes the Replication of *Listeria monocytogenes* in Mouse Hepatocytes

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The uptake of *Listeria monocytogenes* by a variety of cell types in vitro is facilitated by the protein products of the *inlAB* (internalin) operon expressed by the organism. In the case of mouse hepatocytes, the extent to which *inlAB* expression influenced the uptake of *Listeria* in vitro was markedly dependent upon the ratio of bacteria to cells. At a ratio of 100:1, greater than 40-fold fewer transposon-induced *inlAB* mutant listeriae entered hepatocytes compared to the isogenic wild-type control; the difference was only fourfold, however, in cultures inoculated at a 1:1 ratio. Similarly, the uptake of in-frame *inlB* or *inlAB* deletion mutants differed only fourfold from the uptake of wild-type or *inlA* mutant *Listeria* at a 1:1 multiplicity of infection. Mutations affecting *inlB* or *inlAB*, on the other hand, resulted in a marked decrease in the capacity of *Listeria* to proliferate within mouse hepatocytes in vivo and in vitro. Electron micrographs of *Listeria*-infected hepatocytes demonstrated the impaired capacity of *inlB* mutants to escape from endocytic vacuoles and to enter the cytoplasm where proliferation occurs. These findings indicate that the protein product of *inlB* exerts a significant effect on the intracellular replication of *Listeria*.

*Listeria monocytogenes* is a gram-positive bacterium capable of replicating intracellularly and causing severe, sometimes fatal infections in humans (14, 21). Listeriosis in mice is an experimental model used widely to study infections by intracellular pathogens and the factors that effect host resistance (15). Virulence of the organism is dependent upon a cluster of six genes: *hly*, *plcA*, *plcB*, *actA*, *mpl*, and *prfA* (18). The products of these genes mediate escape from phagolysosomes and the intracellular and intercellular movement of *Listeria*. In addition to these virulence factors, two cell-wall-associated proteins (internalin A [InlA]) and internalin B [InlB]) encoded by genes located within the *inlAB* (internalin) operon facilitate the uptake of *Listeria* by nonprofessional phagocytes. *Listeria* mutants unable to produce functional InlA and InlB exhibit a reduced capacity to infect a variety of cell types in vitro (3, 5, 13, 16). Recently, several new members of the internalin multigene family were identified and characterized: *irpA* (also called *inlC*), *inlC2*, *inlD*, *inlE*, and *inlF* (2, 4). The protein products of these genes bear strong amino acid sequence homology to InlA and InlB. Surprisingly, in-frame deletion mutations affecting these genes had no demonstrable effect on the capacity of *Listeria* to infect a number of cell lines. *inlC* mutants did exhibit a decrease in virulence, leading others to suggest that InlC may play a role in the dissemination of infection rather than the uptake of *Listeria* by nonprofessional phagocytes (2).

Most listeriae injected intravenously (i.v.) into mice are cleared rapidly from the bloodstream and taken up in the liver, where the parenchymal cells, i.e., hepatocytes, serve as the principal site of intracellular replication (8, 9, 12, 19). Recently, we reported that expression of the internalin operon does not affect the uptake of *Listeria* by hepatocytes (10). *inlAB* expression, however, was not required for uptake of *Listeria* by hepatocytes. Rather, wild-type and *inlAB* mutant strains of *Listeria* exhibited equivalent capacities to infect the parenchymal and nonparenchymal cells of the liver. While expression of the *inlB* operon does not affect the uptake of *L. monocytogenes* by hepatic cells in vivo, we report here that *inlB* expression and the production of InlB promote escape from endocytic vacuoles and replication of *Listeria* within the cytoplasm of mouse hepatocytes.

**MATERIALS AND METHODS**

**Bacteria.** Stock cultures of the following strains of *L. monocytogenes* (obtained from Pascale Cossart, Institut Pasteur, Paris, France) were prepared, stored, and used in accordance with methods previously reported (3, 10): BUG5, an isogenic transposon Tn545-induced internalin (inlAB) mutant; BUG947, BUG1047, and BUG949, in-frame deletion mutants of *inlA*, *inlB*, and *inlAB*, respectively; and EGD-Sm” and BUG600, wild-type controls. Bacteria derived from broth cultures growing exponentially were used in the experiments described.

**Mice.** Specific-pathogen-free female C57BL/6J mice, purchased from the Jackson Laboratories (Bar Harbor, Maine), were cared for in accordance with the guidelines set forth by the Institute of Laboratory Animals Resources, National Research Council. Mice 8 to 12 weeks of age were used in the experiments reported. The number of listeriae recovered in the livers of infected animals was estimated from the colonies that grew on Trypticase soy agar plates inoculated with an aliquet of organ homogenate (23).

**Hepatocyte culture.** Purified hepatocytes were obtained from the livers of mice following perfusion with collagenase by the two-step method we reported previously (10). Purified hepatocytes were cultured in microtiter plates (2 × 10³ cells/well) containing HEPES-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.).

The infection and replication of *Listeria* within hepatocytes in vitro were assessed by methods also reported previously (5, 10, 11). The number of intracellular listeriae that survive culture for ≥ 1 h in the presence of 5 μg of gentamicin per ml (final concentration) was determined by lysing infected hepatocytes with 0.05% Triton X-100. Aliquots of the cell lysate were transferred to Trypticase soy agar plates, and viable listeriae were calculated from the number of colonies that grew.

**Electron microscopy.** Mouse hepatocytes (10⁶) were seeded into 10-cm tissue culture dishes, and the cells were incubated overnight. On the following day, the plates were inoculated with 10⁷ listeriae and incubated for an additional 4 h. The cell monolayers were then washed four times with Hank's balanced salt solution, and the cells were fixed in situ with 20% paraformaldehyde–2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. The fixed cells were scraped into suspension, postfixed with 1% osmium tetroxide, and embedded in epoxy resin. Thin sections were cut, stained with lead citrate and uranyl acetate, and examined by electron microscopy (Philips Model 201).
RESULTS

Concentration-dependent effect of internalin on the uptake of Listeria by mouse hepatocytes. Expression of the inlAB operon and the production of InlA and InlB affect the capacity of Listeria to enter a variety of cell types in vitro (3, 5, 10, 13, 16). Indeed, in agreement with results reported previously (10), primary mouse hepatocyte cultures inoculated at a high (100:1 ratio of bacteria to cells) multiplicity of infection (MOI) internalized >40-fold more wild-type (EGD-Sm') than inlAB mutant (BUG5) listeriae (Fig. 1). At a lower MOI, however, internalin exerted far less of an effect on the uptake of Listeria. The capacities of the EGD-Sm' and BUG5 strains to infect hepatocytes differed only fourfold when cell cultures were inoculated with bacteria at a 1:1 ratio.

inlAB promotes the proliferation of Listeria in the liver. The parenchymal cells serve as the principal site of listerial replication in the livers of mice. At ≥6 h postinfection i.v., essentially all of the listeriae recovered in the liver are located within the hepatocyte population (8, 9). As we reported previously (10), inlAB expression did not influence the recovery of Listeria in the liver at 6 h postinfection; the numbers of wild-type (EGD-Sm') and inlAB mutant (BUG5) listeriae (Fig. 1). At a lower MOI, however, internalin exerted far less of an effect on the uptake of Listeria. The capacities of the EGD-Sm' and BUG5 strains to infect hepatocytes differed only fourfold when cell cultures were inoculated with bacteria at a 1:1 ratio.

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InlB promotes the proliferation of Listeria within hepatocytes. The diminished ability of BUG5 to proliferate within murine hepatocytes is documented by the in vitro experiment shown in Fig. 3. During a 10-h interval, the number of inlAB mutant strain of Listeria to proliferate in the liver, presumably within the hepatocyte population.

Statistical analysis. The results were analyzed by the SigmaStat statistics program (Jandel Scientific, San Rafael, Calif.).
InlB facilitates escape of *Listeria* from endocytic vacuoles. An examination of thin sections derived from primary mouse hepatocyte cultures infected with wild-type *Listeria* revealed bacteria located free within the cytoplasm, i.e., unrestricted by a lipid membrane (Fig. 6A). These organisms were surrounded by a halo characterized by others as polymerized actin (22). In contrast, bacteria contained within membrane limiting vacuoles were evident in sections prepared from hepatocyte cultures infected with inlB mutant *Listeria* under comparable conditions (Fig. 6B). These findings demonstrate the impaired capacity of inlB mutants to escape from endocytic vacuoles and to enter the cytoplasm where proliferation occurs.

**DISCUSSION**

The cell-wall-associated protein products of the inlAB operon facilitate the entrance of *Listeria* into a variety of cell types in vitro (3, 5, 10, 13, 16). In the case of mouse hepatocyte cultures, the role of internalin was influenced by the concentration of bacteria added and was diminished markedly at lower bacteria-to-hepatocyte ratios. Thus, at a 1:1 ratio, wild-type and inlAB mutant strains of *Listeria* differed only fourfold in their capacities to infect hepatocytes; in the case of the mutants, e.g., BUG5, ~0.5% of the inoculum penetrated the cells during a 1-h incubation period. This latter finding demonstrates the ability of *Listeria* to enter mouse hepatocytes in the absence of internalin and supports the proposed existence of internalin-independent pathways for the uptake of *Listeria* by eukaryotic cells (1, 3, 5, 6, 13).

Rather than affecting entry, inlAB expression appeared to exert its greatest effect on the replication of *Listeria* within hepatocytes both in vivo and in vitro. Relative to the wild-type control (EGD-Smr), proliferation of the transposon-induced inlAB mutant (BUG5) in primary cultures of mouse hepatocytes was reduced substantially. Similar findings were obtained with a mutant (BUG949) that expressed an in-frame deletion inlAB and inlA, on listerial replication in mouse hepatocytes. This negates the possibility existing in the case of BUG5 that the reduced capacity to proliferate was due to a polar mutation affecting some gene product other than internalin. Experiments comparing the inlB and inlA mutant strains of *Listeria* demonstrate the positive influence of InlB, but not InlA, on listerial replication in mouse hepatocytes. This correlates with the specific role of InlB in mediating the uptake of *Listeria* by mouse hepatocytes in cultures inoculated at a high MOI (10).

Our results demonstrating the decreased capacities of inlAB and inlB mutants to replicate within mouse hepatocytes contradict a previous study reporting the comparable growth rates of inlAB mutant and wild-type *Listeria* cells in a purported mouse hepatocyte cell line, TIB73 (characterized originally in...
the literature as a mouse fibroblast cell line derived from embryonic liver [17]) (7). The uncertain relationship of the TIB73 cell line to authentic mouse hepatocytes could readily account for the disparity in our findings. Our results do agree, however, with a recent report demonstrating the role of internalin in promoting the proliferation of Listeria within a macrophage cell line (20). In the latter case, the number of wild-type listeriae in J774A.1 macrophages increased approximately 2.5 log10 during a 4-h incubation period; an inlAB mutant, on the other hand, failed to proliferate intracellularly.

A number of studies in addition to the one reported here have demonstrated the decreased capacity of internalin mutants to replicate in vivo (3, 4, 7, 16, 20). Gaillard et al., for example, reported 1 to 2 log10 fewer organisms recovered in the livers of mice on day 2 postinfection i.v. with inlB mutant, relative to wild-type control, listeriae (7). This difference is most often attributed to a reduction in the number of mutants that enter permissive cells, e.g., the parenchymal cells of the liver (hepatocytes), where they can proliferate (3, 7). Alternatively, it has been suggested that the protein products of the internalin genes may exert their greatest effect on the proliferation of intracellular organisms.

REFERENCES

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