

The *inlAB* Locus Mediates the Entry of *Listeria monocytogenes* into Hepatocytes In Vivo

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Summary

The intracellular parasite *Listeria monocytogenes* is able to induce its internalization by cultured mammalian cells that are not normally phagocytic. This process requires the expression of the chromosomal locus *inlAB*. We studied the virulence of an *inlAB* mutant and of its parent in murine listeriosis. Irrespective of the route of inoculation, the *inlAB* mutant was severely attenuated for growth in the liver. The livers of mice inoculated with the *inlAB* mutant displayed much smaller infectious foci than the parent as early as 24 h after infection. Electron microscopy showed that these foci consisted of a few inflammatory cells, with few bacteria; bacteria were rarely found within hepatocytes. In contrast, foci in livers of mice inoculated with the parent consisted of islets of heavily infected hepatocytes that were infiltrated by numerous neutrophils; bacteria seemed intact within hepatocytes and damaged within neutrophils. A direct role of *inlAB* for the entry of *L. monocytogenes* into hepatocytes was confirmed in a cell infection system using the murine embryonic hepatocyte cell line TIB73. The *inlAB* mutant was ~20-fold less invasive than its parent and recovered a full invasive phenotype when *inlAB* was provided in trans. The “invasion locus” *inlAB* contributes to protect *L. monocytogenes* from the host’s innate defense mechanisms by promoting its entry into hepatocytes.

Various bacterial pathogens are able to trigger their uptake by cultured mammalian cells that are not normally phagocytic, such as epithelial cells or fibroblasts (1). Much work in recent years has addressed the bacterial factors involved in the process of entry. Loci required for invasion of nonprofessional phagocytic cells have thus been identified in the genomes of Gram-negative and Gram-positive bacteria, including *Yersinia* (2, 3), *Salmonella* (4, 5), *Shigella* (6), and *Listeria* (7). It is a seductive idea that these “invasion loci” may contribute to virulence by giving the bacteria access to an intracellular niche protected from the host’s phagocytes (8). However, to date, there is no experimental evidence for this hypothesis.

Listeria monocytogenes is a particularly attractive model for investigating the role of invasion loci in vivo. This ubiquitous Gram-positive bacterium can infect both macrophages (9) and nonprofessional phagocytes, including epithelial cells, fibroblastic cells, and hepatocytes (10–14). It is a well-adapted intracellular pathogen that can grow within the cytoplasm of its host cell (11, 15) and takes advantage of the host cell machinery to pass directly into the cytoplasm of neighboring host cells (16, 17). Murine listeriosis is a well-documented model of infection and has been widely used for studying cellular immunity.

By analysis of noninvasive transposon insertion mutants, we have recently identified a chromosomal locus that is

absolutely required for the entry of *L. monocytogenes* into cultured epithelial cells (7). This locus is an operon and is controlled by the activator PrfA (18), as are the virulence-related genes of the *hly* region (19, 20). It is composed of two genes, *inlA* and *inlB*. The *inlA* gene is necessary and sufficient to render the normally noninvasive species *Listeria innocua* invasive for cultured epithelial cells. It encodes an 800-amino acid protein, internalin, which is structurally analogous to certain surface proteins in Gram-positive organisms (7, 21). The *inlB* gene is very similar to *inlA* and encodes a 630-amino acid protein.

After entering the host through the gastrointestinal tract, *L. monocytogenes* spreads via the lymph and blood to distant tissues. In murine infection, it accumulates predominantly in the liver, where it replicates until the host mobilizes a protective cellular immune response (9, 22, 23). Until recently, it was generally believed that the growth of *L. monocytogenes* in the liver was due to its ability to grow within the resident macrophages, the Kupffer cells (9). However, several recent works have clearly shown that *L. monocytogenes* replicates in hepatocytes rather than in Kupffer cells (13, 24–26). In mice infected intravenously with a sublethal dose, there is an ~200-fold increase in the number of hepatocyte-associated *Listeria* during the first 3 d, and a <twofold increase in the number of Kupffer cell-associated *Listeria* (26). Hepatocyte invasion is now

considered as a key event in liver infection and is being analyzed in *in vitro* cellular assay systems (14).

We studied the role of *inlAB* in murine listeriosis and provide evidence that this locus allows *L. monocytogenes* to circumvent the host's innate defense mechanisms by promoting its entry into hepatocytes.

Materials and Methods

Bacterial Strains and Growth Conditions. *L. monocytogenes* strains EGD-SmR and BUG8 were used throughout the study. EGD-SmR is a streptomycin-resistant derivative of strain EGD (27). BUG8 is a transposon Tn1545 mutant from EGD-SmR, which is ~50-fold less invasive than its parent for cultured epithelial cells (7). The transposon insertion in BUG8, 417 bp upstream of the start codon of *inlA* (21), prevents the transcription of *inlA* and *inlB* (7). The BUG8-derivative strains JLG101, JLG102, and JLG103 were obtained in this study. JLG102 and JLG103 harbor the pAT28 derivatives pGM4 and pGM2, respectively; JLG101 harbors the vector pAT28. Plasmids pGM2 and pGM4 have been described elsewhere; they carry *inlAB* and *inlA*, respectively (7). Plasmid pAT28 and derivatives were introduced into *Escherichia coli* strain HB101 (pRK212.1) by transformation and transferred from *E. coli* to *L. monocytogenes* BUG8 by conjugation (7). Transconjugants were selected on tryptic soy agar containing spectinomycin (60 mg/liter) and nalidixic acid (50 mg/liter) (Sigma Chemical Co., St. Louis, MO). JLG101, JLG102, and JLG103 were used for *in vitro* but not for *in vivo* experiments, owing to the instability of the vector pAT28 and derivatives in these strains. *L. innocua* BUG263, BUG261, and BUG531 derive from the strain CLIP11254 and harbor the plasmids pAT28, pGM2, and pGM4, respectively (7, 18). *Listeria* strains were grown in tryptic soy broth (Diagnostics Pasteur, Marnes-la-Coquette, France) at 37°C, without shaking. Spectinomycin (60 mg/liter) was added to cultures of strains harboring plasmid pAT28 and derivatives.

Analysis of Virulence in Mice. Specific pathogen-free female Swiss mice (Charles Rivers, Saint-Aubin-lès-Elbeuf, France) were used when they were 6–8 wk old. The challenge inoculum was prepared from 18-h cultures in tryptic soy broth, containing antibiotics as appropriate. Bacteria were pelleted by centrifugation, washed once, and diluted appropriately in 0.15 M NaCl. Mice were infected by direct intragastric inoculation with 10⁹ bacteria (in 0.25 ml) by means of a feeding needle for oral challenge, and by intravenous injection of either 5 × 10⁵ or 3 × 10⁶ bacteria (in 0.5 ml) for intravenous challenge. Bacterial growth in liver and spleen was determined by killing groups of five mice at various intervals after infection. Mice were killed by cervical dislocation, in accordance with the policies of the animal welfare committee of the Faculté Necker-Enfants Malades. The liver and spleen were aseptically removed and ground. Samples (0.1 ml) of serial dilutions of whole-organ homogenates were surface plated on tryptic soy agar (Diagnostics Pasteur). Bacterial counts in BUG8-infected mice were determined in parallel on tryptic soy agar containing or not containing kanamycin (25 mg/liter) to assess the presence of Tn1545 in bacteria. Colonies were counted after 24 h of incubation at 37°C, and the results were expressed as the mean log₁₀ bacteria per organ. The minimum detection limit was 2 log₁₀ bacteria per organ. The intravenous LD₅₀ was estimated by the probit method. Groups of eight mice were challenged intravenously with various doses of bacteria, and mortality was observed for 3 wk.

Histology. Mice were challenged intravenously with a dose

of 10⁸ bacteria (in 0.5 ml) and killed by cervical dislocation at intervals of up to 1 wk. Small pieces of liver were removed and processed for Gram staining, immunolabeling, or electron microscopy. Samples to be Gram stained were fixed in 10% formalin and embedded in paraffin; 2–3-μm sections were cut and stained using the Gram-Weigert procedure. Samples to be immunolabeled were embedded in OCT compound (Miles Scientific, Naperville, IL) and frozen in liquid-cooled isopentane (Sigma Chemical Co.). Cryostat sections 5–7-μm thick were cut, mounted onto glass slides, and fixed in acetone. Sections were first incubated with a rabbit anti-*L. monocytogenes* serum (1:100 dilution) for 1 h, and then with peroxidase-conjugated goat anti-rabbit IgG (1:100 dilution) (Sigma Chemical Co.). 3,3'-diaminobenzidine (Sigma Chemical Co.) was used as the chromogenic substrate to reveal bound peroxidase. Samples to be processed for electron microscopy were fixed in 2% glutaraldehyde (Sigma Chemical Co.) in 0.1 M phosphate buffer, pH 7.4, for 2 h at room temperature, postfixed in 2% aqueous osmium tetroxide (Merck, Darmstadt, Germany) for 1 h, dehydrated in graded ethanol solutions, and embedded in Epon 812 (TAA-Jamming, France). Semithin sections were cut and stained with 1% toluidine blue for light microscopy. Ultrathin sections of the appropriate blocks were cut, stained with uranyl acetate and lead citrate, and examined with an electron microscope (model CX200; Jeol, Croissy-sur-Seine, France).

Culture of the Cell Line TIB73. The murine embryonic hepatocyte cell line TIB73 was purchased from the American Type Culture Collection (Rockville, MD). It was cultured in DMEM (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS (Gibco Laboratories). TIB73 cells were seeded at a density of 10⁵ cells/cm² in 35-mm tissue culture plates (Falcon Labware, Becton Dickinson & Co., Lincoln Park, NJ) for gentamicin survival assay and onto 12-mm diameter glass coverslips in 24-well tissue culture plates (Falcon Labware) for fluorescence microscopy. Monolayers were used after 24–48 h of incubation.

Gentamicin Survival Assay. Bacterial inoculum was prepared as described for virulence testing. Cell monolayers grown in 35-mm tissue culture plates were infected for 1 h at 37°C at a multiplicity of infection of ~100 bacteria per cell. After two washings, the cells were reincubated in fresh DMEM containing gentamicin (Sigma Chemical Co.) at concentrations of either 5, 10, or 25 mg/liter. At intervals, they were washed twice and lysed by adding cold water. Viable bacteria released from the cells were titered on agar plates.

Immunofluorescence Microscopy. Cell monolayers grown on glass coverslips were infected as described above. At intervals during the incubation period in the presence of gentamicin, the cells were washed twice with PBS, fixed with 3% paraformaldehyde (wt/vol in PBS) for 30 min at room temperature, and permeabilized for 5 min in 0.1% Triton X-100 (Sigma Chemical Co.) in PBS. Cells were then washed three times with PBS and processed for immunolabeling and actin staining. For immunolabeling, cells were incubated sequentially with appropriate dilutions of a polyclonal rabbit anti-*L. monocytogenes* EGD antibody and of a FITC goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories Inc., Bio/Can Scientific, Mississauga, Canada) in 1% BSA-PBS; incubations were carried out for 30 min at room temperature. Rhodamine-phalloidin (Molecular Probes, Inc., Eugene, OR) was used for F-actin staining. Coverslips were mounted on slides and examined by fluorescence microscopy using a microscope (Axioskop; Carl Zeiss, Inc., Thornwood, NY).

Plaque Assay. Plaque formation by *L. monocytogenes* was assayed in the mouse L2 fibroblast cell line, using a protocol modi-

fied from Sun et al. (28). Briefly, confluent L2 monolayers obtained in 6-well tissue culture dishes (Falcon Labware) were infected with 1–20 μ l of a bacterial inoculum prepared as described above. After 1 h of incubation at 37°C, the monolayers were washed three times and overlaid with culture medium containing 0.75% agarose and 10 μ g of gentamicin per milliliter. After 3 d, plaques were visualized by the addition of 0.1% neutral red (Sigma Chemical Co.).

Results

Virulence of the *inlAB* Mutant in Mice. The *inlAB* mutant BUG8 and its parent EGD-SmR were first studied in mice infected by the oral route. Mice were challenged by intragastric inoculation of 10^9 bacteria, and bacterial spreading to the liver and spleen was followed as indicative of intestinal translocation (Fig. 1). Both strains were isolated from these organs from day 2, thus indicating that the *inlAB* mutant retained the ability to invade the host through the gut. The bacterial growth curves, however, were similar for the two strains in the spleen and dissimilar in the liver. The bacterial counts in the spleen were slightly lower for BUG8 than for EGD-SmR at all time points, but the growth curves were parallel. This was not the case in the liver: There was a 10-fold difference in the bacterial

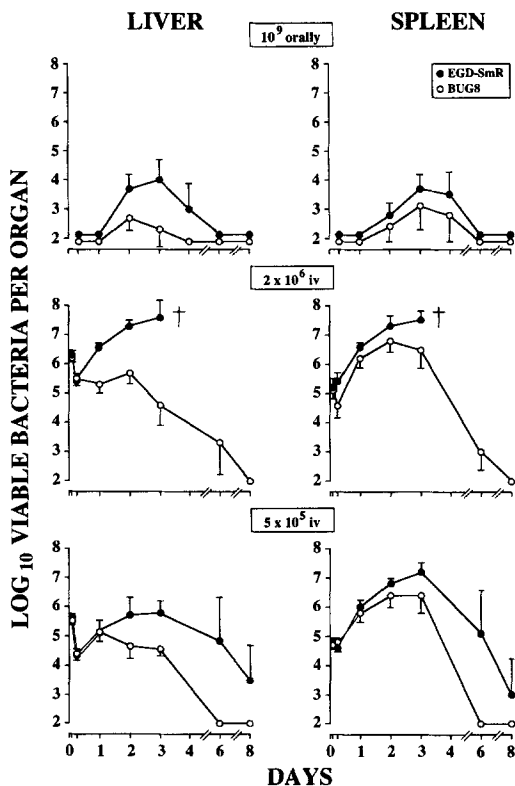


Figure 1. Growth curves for EGD-SmR and BUG8 in liver and spleen of mice after oral and intravenous challenges. Mice were infected by intragastric inoculation of 10^9 bacteria (oral challenge) or by intravenous injection of either 5×10^5 or 2×10^6 bacteria (intravenous challenges). Bacterial growth was followed in liver and spleen by killing groups of mice at intervals. Data points and error bars represent the mean and SD of the \log_{10} bacteria per organ (mean of five mice for each point).

counts of the two strains as early as day 2. BUG8 failed to grow and was totally eliminated from the liver by day 4, whereas EGD-SmR proliferated. Oral LD_{50} experiments were not performed because mice infected orally with EGD-SmR survive challenges as high as 10^{10} bacteria (Gaillard, J.-L., unpublished data).

Thus, BUG8 appeared to be cleared more readily from liver than EGD-SmR. This was confirmed by infecting mice by the intravenous route. Mice were challenged intravenously with 5×10^5 or 3×10^6 bacteria, and bacterial growth was followed in liver and spleen (Fig. 1). With the dose of 3×10^6 bacteria, the bacterial growth curves in the spleen were comparable for the two strains until day 3, when the first EGD-SmR-infected mice began to die. In contrast, the growth curves in the liver were quite different. After a fourfold reduction of the inoculum during the first 6 h of infection, EGD-SmR multiplied rapidly in the liver during the first 2 d of infection (90-fold increase in bacterial counts from 6 to 48 h). Mice began to die on day 3. Of the 10 mice kept to determine bacterial counts on days 8 and 10, three had died by day 4 and two more by day 6. For BUG8, after the initial decrease in bacterial counts, a low rate of bacterial growth was observed until day 2 (1.5-fold increase in bacterial counts from 6 to 48 h). Bacteria were completely eliminated from the liver within 8 d. None of the BUG8-infected animals died.

The dose of 5×10^5 bacteria did not result in the death of any mouse infected with EGD-SmR or BUG8. This allowed us to continue the bacterial growth curves over 8 d for both strains. Again, it was found that EGD-SmR and BUG8 behaved similarly in the spleen and dissimilarly in the liver during the first days of infection. In the latter organ, the number of EGD-SmR bacteria increased 20-fold and the number of BUG8 bacteria only 1.6-fold from 6 to 48 h after the challenge.

The intravenous LD_{50} s were determined and were $10^{7.55}$ for BUG8 and $10^{6.70}$ for EGD-SmR. This difference is consistent with the mortality rates observed during the analysis of bacterial growth in organs after the highest challenge.

Role of *inlAB* for Hepatocyte Invasion In Vivo. Liver sections from mice inoculated intravenously with the *inlAB* mutant BUG8 or EGD-SmR were examined microscopically. A high inoculum (10^8 bacteria/mouse) was used to facilitate visualization of bacteria by optical and electron microscopy. The histopathology of livers from mice infected by EGD-SmR and BUG8 was different. The livers of mice inoculated with EGD-SmR were studded with large infectious foci as early as 24 h (Fig. 2, a and c). Consistent with recent studies of other invasive *L. monocytogenes* strains (13, 24), the foci were constituted of clusters of heavily infected hepatocytes and of inflammatory cells recruited locally, predominantly neutrophils (Fig. 3 a). Electron microscopy visualized bacteria free within the cytoplasm of hepatocytes, sometimes dividing, whereas in neutrophils the bacteria were confined in all cases within phagosomes and were often being destroyed (Fig. 3 b). After 48 h of infection, nearly all mice inoculated with EGD-SmR were

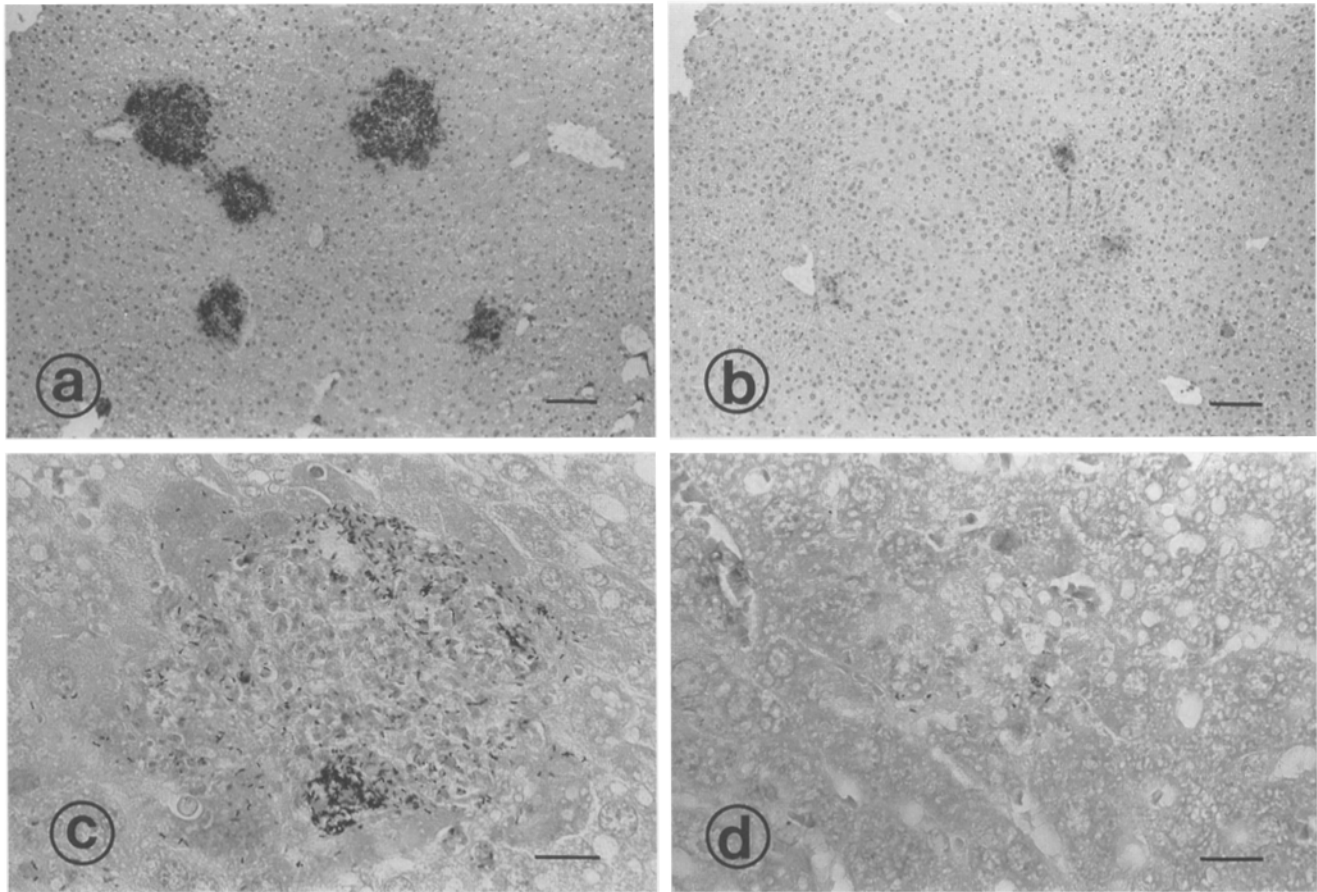


Figure 2. Histological analysis of mouse liver 24 h after infection with EGD-SmR and BUG8. Mice were challenged intravenously at a dose of 10^8 bacteria, and liver samples were obtained 24 h later for microscopic examination. (a and b) Immunoperoxidase labeling using an anti-*L. monocytogenes* serum as a primary antibody. (a) EGD-SmR: Very large foci of infection are seen. (b) BUG8: The foci of infection are less numerous and much smaller. (c and d) Gram staining. (c) EGD-SmR: Many bacteria can be seen, mainly at the periphery of the lesion; note the major inflammatory reaction. (d) BUG8: A small number of bacteria are seen; the inflammatory reaction is barely visible. Bars: (a and b) 25 μm ; (c and d) 5 μm .

dead. Very large infectious foci were found in liver sections of surviving animals (Fig. 4 a). The centers of the lesions were necrotic. Bacteria were abundant in peripheral hepatocytes, suggesting that they had multiplied unrestrictedly while propagating radially from hepatocyte to hepatocyte. No animal survived after 72 h of infection.

The livers of mice infected with the same dose of BUG8 gave a very different aspect. By 24 h, the infectious foci were scarce and much smaller than those for EGD-SmR (Fig. 2, b and d). A typical lesion was populated by a few inflammatory cells and contained no more than 15–25 bacteria. The infectious foci were analyzed by electron microscopy. They were small and rare, and thus their detection required many more grids than for EGD-SmR. The foci contained a few inflammatory cells, which had moved out of the sinusoids and had accumulated locally (Fig. 3 c). Neutrophils and mononuclear cells were present in equal proportion, in close physical contact with hepatocytes. Most bacteria were seen in inflammatory cells. In mononuclear cells but not in neutrophils, some appeared free within the cytoplasm and surrounded by a dense microfibrillar mate-

rial, presumably actin. Thus, some of the bacteria within mononuclear cells appeared to be capable of intracellular movements in the in vivo setting. A few bacteria were found inside hepatocytes adjacent to infected mononuclear cells, suggesting that the hepatocytes might have been infected through bacterial spreading from mononuclear cell to hepatocyte. The rare bacteria inside hepatocytes replicated freely within the cytoplasm, sometimes appearing as a dividing organism coated with actin. Some ultrathin liver sections from BUG8-infected mice displayed a different pattern (Fig. 3 d): Rare bacteria were confined in a K upffer cell, without any adjacent inflammatory cell; the bacteria were located within vacuoles, and most appeared to be in the process of destruction.

All mice were alive after 48 h of infection with BUG8. The infectious foci in liver had increased in size by local recruitment of inflammatory cells but remained much smaller than those observed with EGD-SmR after the same time period (Fig. 4 b). Bacteria were detected inside inflammatory cells and hepatocytes. Infection of hepatocytes remained restricted to a very few individual cells, and each

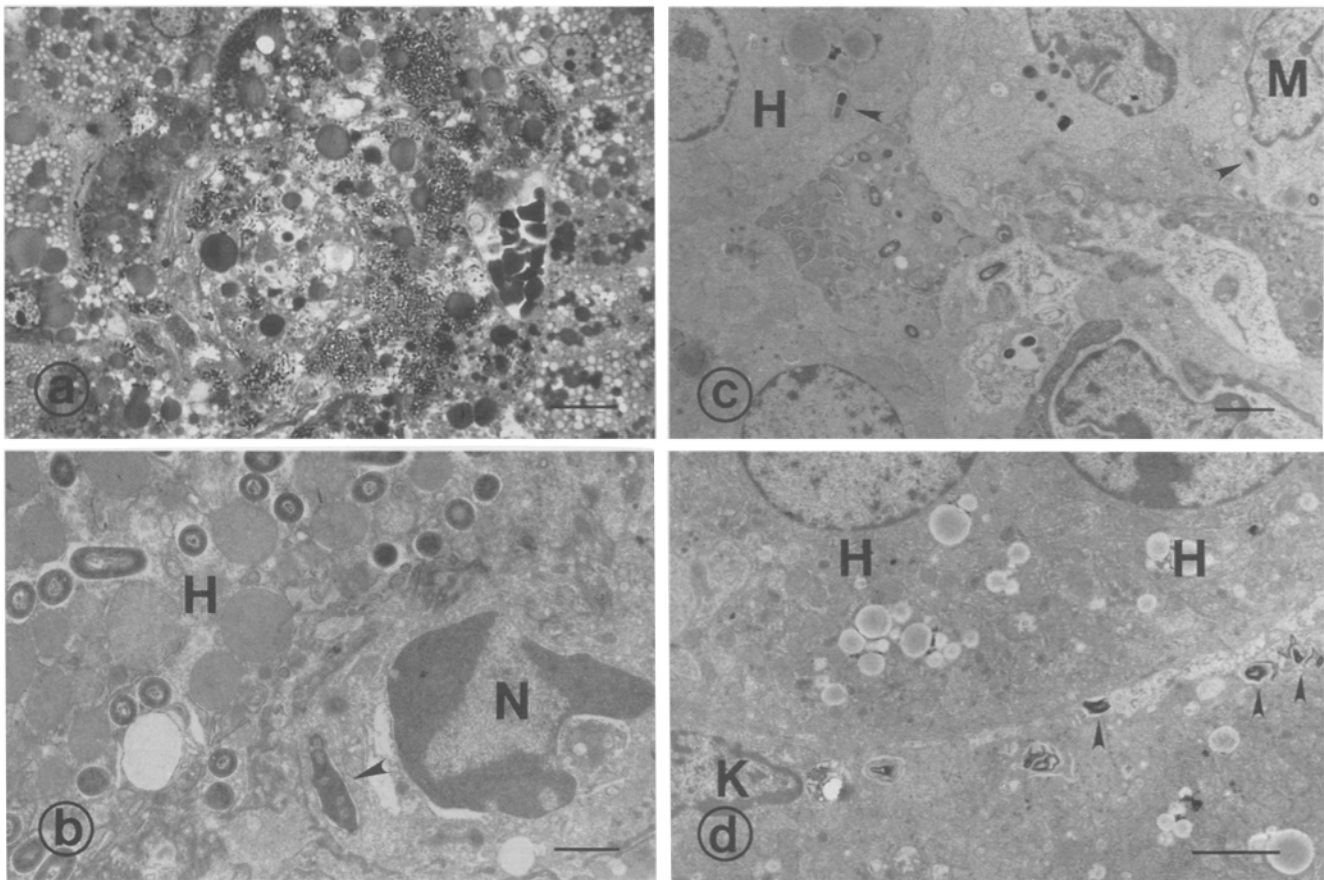


Figure 3. Semithin and ultrathin sections of the infectious foci at 24 h. Semithin (a) and ultrathin (b–d) liver sections were obtained from mice infected as described in the legend to Fig. 2 and examined by light microscopy after staining with toluidine blue and by electron microscopy, respectively. (a) EGD-SmR: The infectious foci are constituted of hepatocytes filled with closely packed *Listeria* and of inflammatory cells. (b) EGD-SmR: A neutrophil (N) can be seen in close physical proximity to a heavily infected hepatocyte (H); note that bacteria in the hepatocyte are free within the cytoplasm and appear as intact organisms, whereas those in the neutrophil are confined to vacuoles and in the process of being killed (arrowhead). (c) BUG8: A typical infectious focus containing a small number of inflammatory cells; the few visible bacteria are seen within inflammatory cells rather than within hepatocytes; note that some bacteria located within mononuclear cells (M) and hepatocytes (H) are coated with a dense microfibrillar material, presumably actin (arrowheads). (d) BUG8: A less common picture, in which bacteria are being destroyed in a Kupffer cell (K); note that no inflammatory cell is seen in the vicinity of the Kupffer cell and that adjacent hepatocytes (H) do not contain any bacterium. Bars: (a) 5 μm ; (b) 1 μm ; (c and d) 2.5 μm .

hepatocyte contained a small number of bacteria. Only a small proportion of BUG8-inoculated mice died between day 3 and day 7, confirming the weak virulence of BUG8. Microscopic examination of liver sections from the surviving mice showed a rapid disappearance of bacteria, with a complete healing of foci by day 7 (not shown).

Role of *inlAB* in Bacterial Entry into Hepatocytic Cells In Vitro. The histological data suggested that *inlAB* promotes the early entry of *L. monocytogenes* into hepatocytes and that this is a prerequisite for bacterial growth and dissemination in the hepatic parenchyma. To investigate the role of *inlAB* for the invasion of murine hepatocytes, we infected the murine hepatocyte cell line TIB73 with EGD-SmR and BUG8, complemented or not in trans with *inlA* or *inlAB*. Bacterial entry was assessed using a gentamicin survival assay with an antibiotic concentration of 5 mg/liter. It was found that BUG8 was ~ 20 -fold less invasive than EGD-SmR (Table 1). We checked that presumably intracellular BUG8 bacteria were not invasive revertants. 20 independent colonies were randomly selected and assayed for entry.

In each case, the bacteria exhibited the same phenotype as BUG8. The introduction of a plasmid carrying *inlAB* into BUG8 restored completely the ability of this mutant to invade TIB73 cells, whereas the introduction of the vector without insert or carrying *inlA* had no effect on entry (Table 1). Overall, the levels found with EGD-SmR, BUG8, and its derivatives in the TIB73 model were very low compared with other cell systems (e.g., the Caco-2 cell line) (7). However, the numbers of bacteria recovered after gentamicin treatment with these strains were much higher than those found with the *L. innocua* strains (Table 1) and thus could not be regarded as background levels due to the presence of surviving extracellular bacteria. This was consistent with BUG8, suggesting that this mutant was only partially entry defective (see below). Also, in contrast with previous findings in the Caco-2 model (7), neither the expression of *inlAB* nor that of *inlA* was sufficient to promote the entry of *L. innocua* in TIB73 cells.

Infection of TIB73 monolayers with EGD-SmR and BUG8 was followed for 18 h in the presence of gentamicin

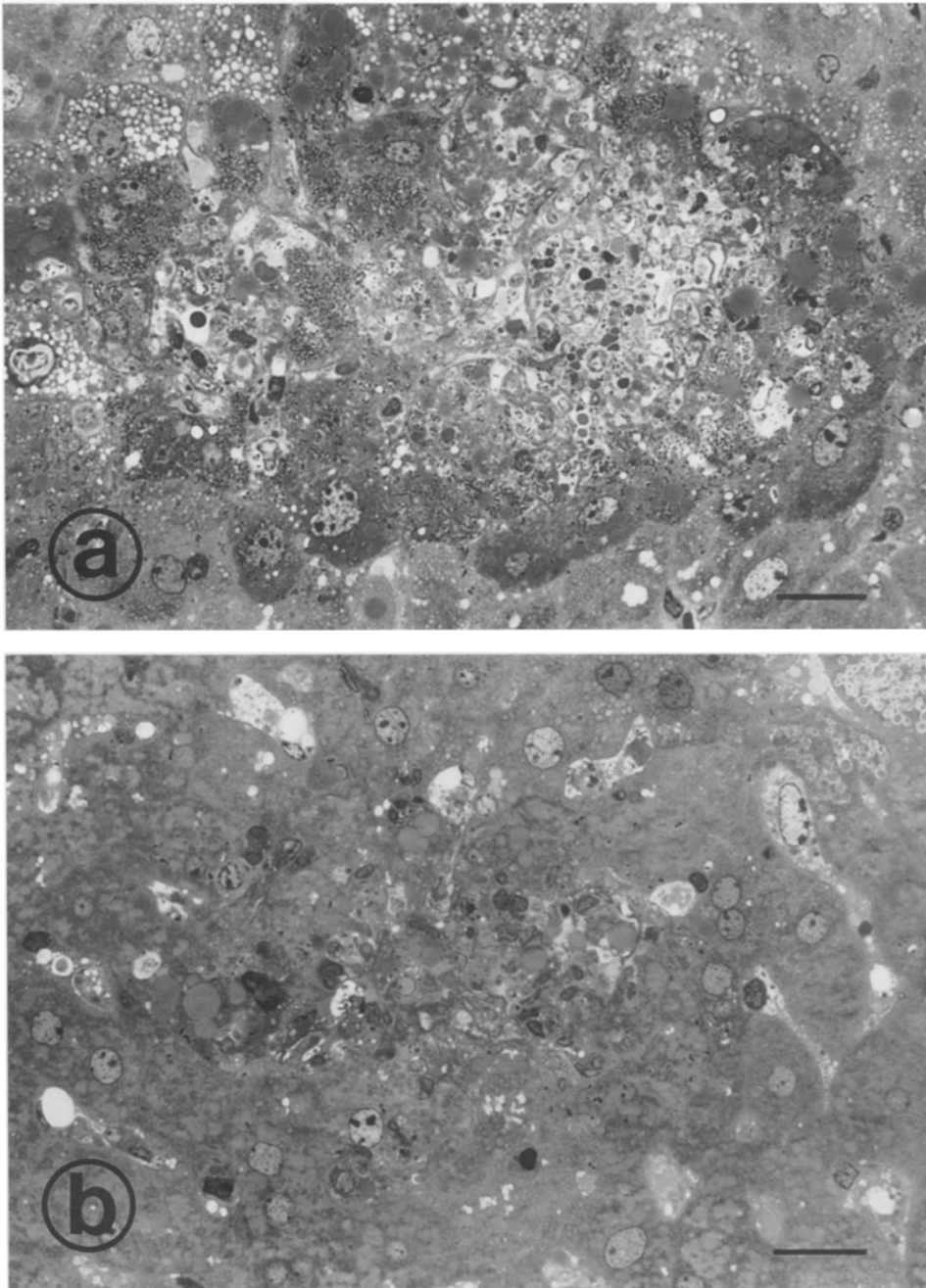


Figure 4. Semithin sections of the infectious foci at 48 h. Liver semithin sections were obtained after 48 h of infection from mice inoculated as described in the legend to Fig. 2 and examined by light microscopy after toluidine blue staining. (a) EGD-SmR: A typical lesion is shown, consisting of a large area of necrosis surrounded by a ring of heavily infected hepatocytes. (b) BUG8: The lesion is much smaller; inflammatory cells and bacteria are small in number; only a few bacteria can be seen within hepatocytes. Bars: 2.5 μ m.

at different concentrations. At given times, cells were washed and either lysed for enumerating viable bacteria or processed for fluorescence microscopy. Similar results were observed for the different concentrations of gentamicin tested (Fig. 5). BUG8 entered the cells with a low efficiency, but its intracellular growth rate was comparable to that of EGD-SmR (generation time of ~ 1 h during the first 8 h of infection for both strains). Microscopic examination of monolayers after immunolabeling of bacteria and F-actin staining showed that BUG8 behaved like the parental strain inside cells. After 18 h of infection, the foci of bacterial replication formed by both strains were identical (Fig. 6); however, they were 10–20-fold less numerous

with BUG8, because of the lower invasive capacity of this strain compared with its parent. The ability of BUG8 to grow intracellularly and to propagate from cell to cell was also evaluated in a plaque assay using the mouse L2 fibroblast cell line (28). Consistent with the data obtained in the TIB73 model, plaques formed by BUG8 were 10–20-fold less numerous than those formed with EGD-SmR, but their morphology was strictly normal (not shown).

Discussion

The aim of this work was to characterize the role of the *inlAB* locus in infection of a living host by *L. monocytogenes*.

Table 1. Entry of *Listeria* Strains into TIB73 Cells

Strains	Number of bacteria
<i>L. monocytogenes</i>	
EGD-SmR	$8 (\pm 2) \times 10^3$
BUG8	$4 (\pm 3) \times 10^2$
JLG101	$3 (\pm 4) \times 10^2$
JLG102	$4 (\pm 4.5) \times 10^2$
JLG103	$6 (\pm 3) \times 10^3$
<i>L. innocua</i>	
CLIP11254	<10
BUG263	<10
BUG261	<10
BUG531	<10

TIB73 monolayers were infected for 1 h at 37°C with 100 bacteria per cell. After washing, the cells were overlaid with fresh culture medium containing gentamicin (5 mg/liter) and reincubated for 2 h at 37°C. The cells were then washed again and lysed, and viable bacteria were titered on agar plates. The results are expressed as the mean number (\pm SD) of viable bacteria per well (three determinations).

This was addressed by studying the virulence of an *inlAB* mutant and of its parent in murine listeriosis. Human listeriosis usually occurs by the intestinal route, after ingestion of *Listeria*-contaminated food products (29). *InlAB* was found to be required for the penetration of *L. monocytogenes* into an enterocytic cell line (7), and we therefore postulated that this locus was necessary for *L. monocytogenes* translocation through the gut mucosa.

Analysis of oral infections in mice showed that the *inlAB* mutant remained able to cause a systemic infection after oral challenge, with bacterial spread to liver and spleen.

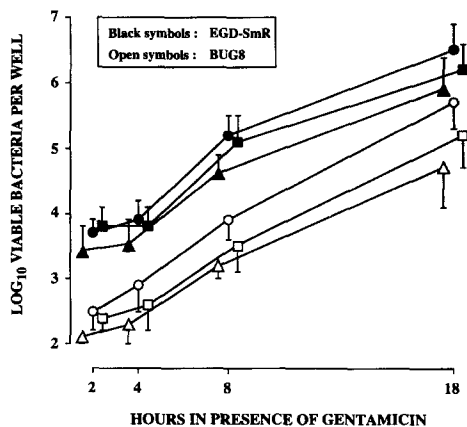


Figure 5. Growth of EGD-SmR and BUG8 in TIB73 cells. TIB73 monolayers were infected for 1 h at 37°C with 100 bacteria per cell. After washing, the cells were reincubated for 18 h in fresh culture medium containing gentamicin at a concentration of 5 (○), 10 (□), and 25 (△) mg/liter. At intervals, the cells were washed again and lysed, and viable bacteria were titered on agar plates. Data points and error bars represent the mean and SD of the number of bacteria per well (mean of three determinations). BUG8 was ~20-fold less invasive than EGD-SmR but multiplied intracellularly to the same extent as its parent.

This is in agreement with results of previous experimental studies showing that *Listeria* given orally to rodents penetrate into the Peyer's patches and not into the intestinal villi (23). As reported for other bacterial pathogens (30–33), *L. monocytogenes* might thus use the M cells within the Peyer's patches as entry portals. This is consistent with analyses of the invasion of cultured enterocytic cells by *L. monocytogenes*. Indeed, confluent Caco-2 cell monolayers are not permissive for *L. monocytogenes* (11), and, in non-confluent monolayers, the bacteria enter via cells at the periphery of Caco-2 islets (17). This suggests that *L. monocytogenes* enters enterocytic cells by the basolateral domain of the cell surface, as demonstrated for *Shigella* (34). Thus, *L. monocytogenes* may be unable to invade enterocytes by their apical pole in the intestine and require M cell transport for translocating from the intestinal lumen.

Although our results demonstrate that *inlAB* is not essential to *L. monocytogenes* for crossing the gut mucosa, they do not exclude a role for this locus at the intestinal step of infection. It has been suggested that *Shigella* infection of M cells is followed by basolateral infection of enterocytes (34). A similar process could occur during *Listeria* infection, involving *inlAB*-dependent mechanisms. Enterocytes may thus be the first site of bacterial replication in the host. This view is supported by a previous electron microscopic study in which *Listeria* given orally to guinea pigs could be seen dividing inside enterocytes of the small intestine within 3 h of inoculation (35). Furthermore, our results show that the initial counts in liver and spleen of orally challenged mice were substantially lower for the *inlAB* mutant than for its parent.

Our data provide evidence that the invasion of hepatocytes by *L. monocytogenes* in mice is promoted by the *inlAB* locus. First, the *inlAB* mutant inoculated into mice by the oral and the intravenous routes was severely attenuated for growth in the liver. Bacterial counts increased 1.5-fold for the *inlAB* mutant and 90-fold for the parental strain from 6 to 48 h after an intravenous challenge of 2×10^6 bacteria (*inlAB* mutant 0.1 LD₅₀). Second, histological examination of the livers showed that the *inlAB* mutant failed to accumulate in hepatocytes. 24 h after an intravenous challenge of 10^8 *inlAB* mutant bacteria, the infectious foci were composed of a small number of inflammatory cells, with few visible bacteria; bacteria were rarely found within hepatocytes by electron microscopy. In contrast, after 24 h of infection, the foci in the livers of mice challenged with the parental strain consisted of islets of heavily infected hepatocytes that were infiltrated by numerous inflammatory cells, predominantly neutrophils.

The role of *inlAB* for the invasion of hepatocytes in vivo was strengthened by the results of in vitro experiments using the murine hepatocyte cell line TIB73. The *inlAB* mutant was ~20-fold less invasive for TIB73 cells than the parental strain and recovered its invasive capability when *inlAB* was provided in trans. In contrast to the results of a previous study using the colon carcinoma cell line Caco-2 (7), we found that the presence of *inlA* in trans was not sufficient to restore the invasive capability of the *inlAB* mutant

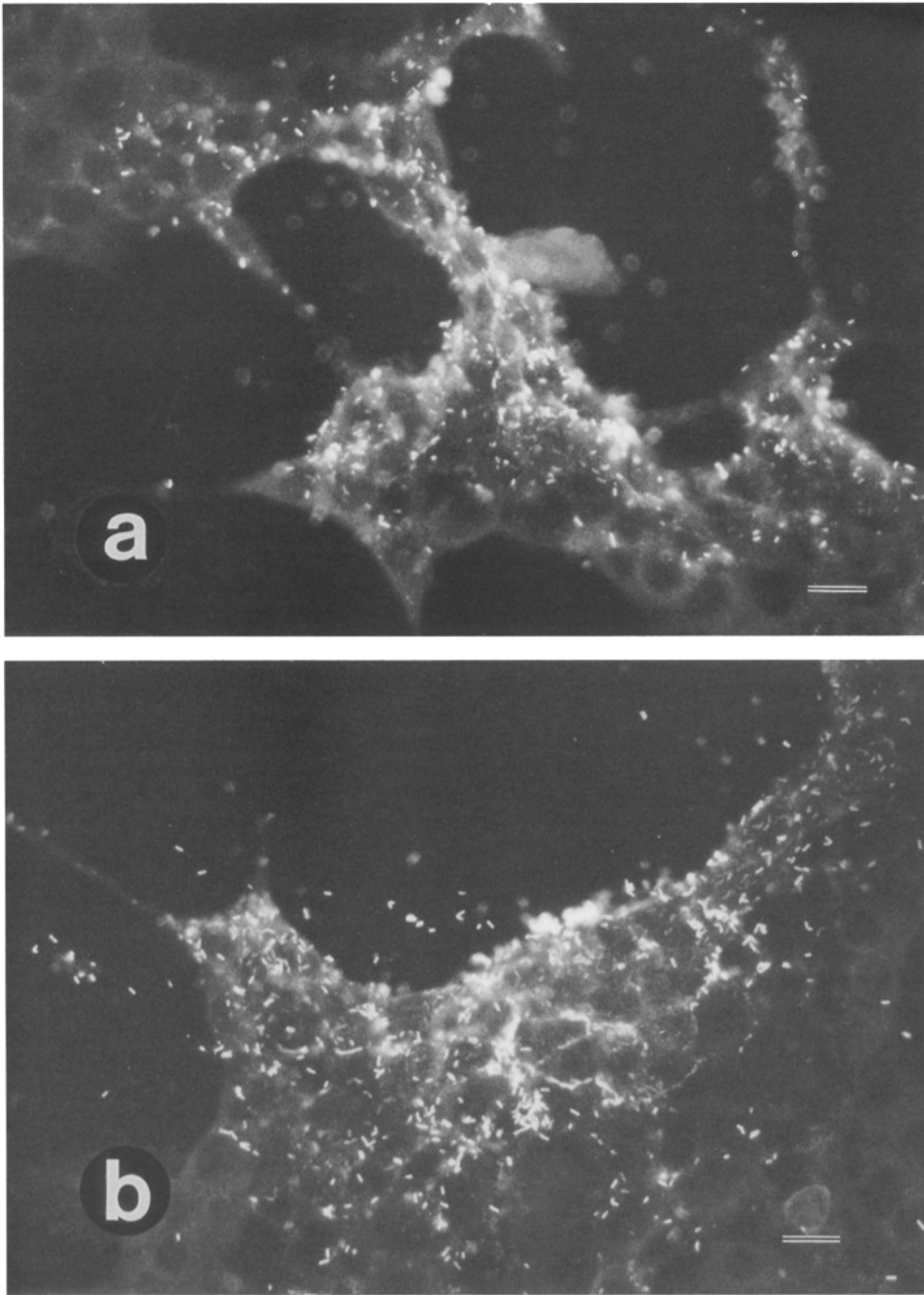


Figure 6. Microscopic examination of TIB73 monolayers 18 h after infection with EGD-SmR and BUG8. TIB73 monolayers were infected for 18 h as described in the legend to Fig. 5. The cells were then washed, fixed, permeabilized, and processed for immunolabeling of bacteria using anti-*L. monocytogenes* antibody. Plaques of infection observed with EGD-SmR (a) and BUG8 (b) shared the same features. Bars: 5 μ m.

for TIB73 cells. This is in full agreement with a recent study from Dramsi et al., in which different cell lines were infected with *inlA*, *inlB*, and *inlAB* deletion mutants from strain EGD (36). These authors have shown that *inlB* is necessary to the entry of *L. monocytogenes* into the cell line TIB73 and, to a lesser extent, into the human hepatoma cell line HepG2. Thus, the entry of *L. monocytogenes* into hepatocytes appears to require the coordinate expression of *inlA* and *inlB*. Moreover, since the expression of *inlAB* is not sufficient to promote the entry of *L. innocua* into cultured hepatocytes (36; this study), other *L. monocytogenes*-

specific genes, possibly belonging to the *inl* gene family, may also be necessary for the completion of this process.

Some in vitro results may explain the behavior of the *inlAB* mutant in the liver of mice. First, this strain was only partially entry defective, presumably because *L. monocytogenes* possesses other invasion determinants than *inlAB*. This might account, at least partially (see below), for the low degree of hepatocyte invasion observed in mice inoculated with the *inlAB* mutant. Another interesting result arising from in vitro studies was the demonstration that, once inside host cells, the *inlAB* mutant behaves in the same man-

ner as its parent. After 18 h of infection, large plaques containing hundreds of intracellular bacteria were observed with both strains in TIB73 monolayers. Moreover, the *inlAB* mutant formed normal plaques in mouse L2 fibroblast monolayers, confirming that this strain retained its ability to spread from cell to cell.

Together, these data suggest the existence of two distinct pathways of hepatocyte invasion by *L. monocytogenes* (Fig. 7). The first steps of infection are common to both pathways. The bacteria circulating in the liver sinusoids are trapped by Kupffer cells, where most of them are destroyed rapidly. A variable proportion of bacteria, depending on the inoculum size, is able to escape from the phagolysosomes of Kupffer cells and replicate. In the first pathway of hepatocyte invasion (Fig. 7 a), the bacteria might be released from damaged Kupffer cells into the extracellular environment and enter hepatocytes by a phagocytic-like process induced by *InlAB*. It is likely that this pathway is greatly favored by heavy bacterial burdens as it requires the destruction of Kupffer cells (compare the bacterial growth curves in the livers of mice challenged with 5×10^5 and 3×10^6 EGD-SmR bacteria in Fig. 1).

Data from some electron micrographs suggest that a second pathway may occur involving the actin-based propagation of the bacteria from Kupffer cells (or, at later stages, from monocytes recruited locally) to adjacent hepatocytes (Fig. 7 b). This pathway is probably much less sensitive to the inoculum size and may play a major role at the low doses occurring in natural infections. The expression of *actA* is essential to the completion of this process but also to the propagation of *L. monocytogenes* from hepatocyte to hepatocyte. Thus, an *actA* mutant can enter hepatocytes via the first pathway but cannot disseminate in the liver parenchyma. This explains how an *actA* mutant (37) is much less virulent than an *inlAB* mutant, which enters hepatocytes

via the second pathway and subsequently is able to spread from hepatocyte to hepatocyte. Although the *inlAB* mutant behaves normally in terms of cell-to-cell spreading, it gives infectious foci of small size in the livers of mice compared with its parental strain. The most likely explanation is that this mutant enters hepatocytes less efficiently as it uses only the second pathway and is therefore controlled more easily by the host's immune system.

To establish inside a mammalian host, an invasive pathogen must resist the host's innate defense mechanisms. The most common and direct way is to synthesize surface molecules with antiphagocytic activity, for example, polymers of repeated sugar residues (e.g., polysaccharide capsules) or various proteins (8). An indirect and more subtle way is to invade cells that are not armed to destroy bacteria, such as epithelial or parenchymal cells (8). These nonprofessional phagocytic cells constitute a sanctuary where bacteria can multiply until a specific immune response develops. *L. monocytogenes* appears to have evolved the latter strategy.

Using *L. monocytogenes* as a model, we report the first evidence that the expression of invasion loci allows a pathogen to evade the host's innate defense mechanisms by taking shelter in nonprofessional phagocytes. The timing of this evasion is important. In murine listeriosis, the hepatocytes are invaded early in the course of infection, before neutrophils populate the sites of bacterial implantation (24). The bacteria can then multiply and propagate in the host tissues by cell-to-cell spreading, preceding phagocytes that are recruited locally. Infection of liver parenchyma by *L. monocytogenes* can thus be seen as a race between the microorganism and the immune cells. *L. monocytogenes* must enter the hepatocytes and multiply to numbers capable of overwhelming the host's immune response. Kupffer cells, neutrophils, and monocytes must hold infection to a level that can be resolved subsequently by a T cell-mediated response.

L. monocytogenes is commonly regarded as a facultative intracellular pathogen whose trademark is to survive and replicate inside host macrophages (9, 38). The resolution of *L. monocytogenes* infection is therefore believed to depend on a T cell-mediated activation of macrophages, enhancing their microbicidal activity (38–40). This study and other reports (13, 24–26) demonstrate that the principal site of replication of *L. monocytogenes* in a living host is the hepatocyte and not the macrophage. By analogy, this may also be true for other facultative intracellular pathogens. It has been shown recently that *Salmonella typhimurium* and *Francisella tularensis* also multiply extensively in hepatocytes in mice (41). An intriguing question is whether the immune system interacts with infected nonphagocytic cells as it does with professional phagocytes to control the bacterial multiplication. Some relevant data have been reported. *Listeria*-infected mouse hepatocytes are selectively lysed by neutrophils, which are recruited in the infectious foci (13, 24). It has also been suggested that hepatocytes could be induced to kill *Listeria* by IFN- γ (42). This fascinating aspect of bacterial immunity is just starting to be explored.

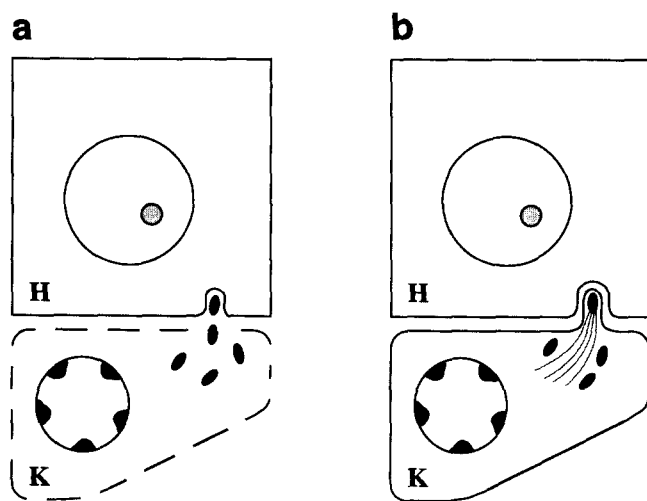


Figure 7. Models of hepatocyte invasion by *L. monocytogenes*. (a) *inlAB*-mediated invasion by bacteria released from damaged Kupffer cells. (b) Actin-based passage from Kupffer cells to hepatocytes.

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