ROLE OF HEMOLYSIN FOR THE INTRACELLULAR GROWTH OF *LISTERIA MONOCYTOGENES*

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Listeria monocytogenes is a Gram-positive bacterial pathogen that occurs free-living in nature as well as in association with a variety of warm-blooded animals (1–4). The oral route is likely the natural mode of transmission and two recent outbreaks were traced to contaminated milk products (5–7). In most human cases, severe disease occurs in pregnant women or in individuals whose immune system has been compromised.

L. monocytogenes is a facultative intracellular pathogen that has been extensively used in a murine model for the study of cell-mediated immunity (8–12). The host cells most generally considered to support the growth of *L. monocytogenes* during infection are of the mononuclear phagocyte lineage (8, 9). Resident macrophages present in the liver and spleen may provide a conducive environment for listerial intracellular growth while activated or elicited macrophages which migrate from the blood are listericidal (10, 12). However, there is evidence that *L. monocytogenes* is also capable of proliferation in nonprofessional phagocytes. For example, histological studies of infected tissues reveal that *L. monocytogenes* is present within liver parenchymal cells (10, 11). Also, after oral challenge, *L. monocytogenes* has been observed within epithelial cells lining the small intestine (13). Finally, *L. monocytogenes* has been shown to infect primary fibroblasts in vitro and multiply intracellularly (14).

The immune response to *L. moncytogenes* has received a great deal of attention, while the cell biology of intracellular growth and bacterial determinants of pathogenicity have yet to be fully evaluated. One likely determinant of pathogenicity is the elaboration of a sulfhydryl-activated hemolysin (15–17). The *L. monocytogenes* hemolysin is a member of a family of bacterial pore-forming cytolysins of which streptolysin O is the prototype (17). Recently, two groups of investigators have isolated nonhemolytic mutants of *L. monocytogenes* that were shown to be avirulent (18, 19). In this report, we address whether hemolysin is required for intracellular growth of *L. monocytogenes* in professional as well as nonprofessional phagocytes.

Materials and Methods

Bacterial Strains and Growth Conditions. *L. monocytogenes* strain 10403S was the primary strain used in this study (20). Strain 10403S belongs to serotype 1, is resistant to 1 mg/ml of streptomycin, and its LD_{50} for mice is 3.3×10^4. *L. monocytogenes* strain BM4140 was provided by P. Courvalin (Institute Pasteur, Paris, France) and was used as a donor of Tn*1545* (18). *Streptococcus faecalis* CG110 was provided by D. Clewell (University of Michigan, Ann Arbor, MI) and was used as a donor of Tn*916* (19).

Bacteria were grown on brain heart infusion agar and broth (BHI; Difco Laboratories, Inc., Detroit, MI) at either 30°C or 37°C as required. Stock cultures were kept as suspensions of cells at −70°C in 50% glycerol. Streptomycin was used at 1 mg/ml and tetracycline was used at 12.5 μg/ml.

Tissue Culture Cells and Growth Medium. The macrophage-like cell line J774 (21), obtained from J. Unkeless (Mount Sinai Medical School, New York, NY) and the embryonic mouse fibroblast cell line CL.7 (ATCC No. TIB 80) were grown in DME supplemented with 5% FCS. The human epithelial cell line Henle 407 (ATCC NO. CCL 6) and the human fetal fibroblast cell line WS1 (ATCC No. CRL 1502) were grown in α-modified Eagle's medium supplemented with 10% FCS. All cell lines were maintained in the presence of penicillin (100 U/ml) and streptomycin (10 μg/ml). Primary cultures of bone marrow–derived macrophages were established from specific pathogen–free female CD-1, ICR mice (Charles River Laboratories, Wilmington MA) (22). Cells were grown for 7 d before use in 100-mm petri dishes (Lab-Tek; American Scientific Products, McGaw Park, IL) in DME supplemented with 20% FCS and 30% L-cell supernatant (source of CSF-1). We routinely derived 8×10^6 macrophages from two femurs.

Isolation of Hemolysin-negative Mutants. Transposon-mutagenesis of *L. monocytogenes* 10403S was achieved using transposon Tn*1545* as described by Gaillard et al. (18), and with Tn*916* as described by Kathariou et al. (19). In both cases, mutants were selected on tryptic soy agar (Gibco Laboratories, Madison WI) containing 5% defibrinated sheep blood (Gibco Laboratories) plus streptomycin and tetracycline. Mutants were identified by visual inspection of hemolysis surrounding bacterial colonies. 8/3,000 *L. monocytogenes* colonies harboring Tn*1545* were nonhemolytic (hly⁻), while 10/40,000 harboring Tn*916* were hly⁻.

Intracellular Growth Assay. Cell monolayers were grown on 12×1 mm round cover slips (Propper Manufacturing Co. Inc., Long Island City, NY) in 60-mm petri dishes containing 5 ml of the appropriate medium. J774 cells (10^6) grown in spinner were deposited onto the cover slips the evening before use. Henle cells, CL.7 cells, and WS1 cells were passed 1/5 from a confluent monolayer to the coverslips 2 d before use. Bone marrow–derived macrophages (2×10^6 of day 7 cells) were deposited onto the cover slips the evening before use and were cultivated in the absence of L-cell supernatant. In all cases, antibiotics were omitted from the cell culture.

L. monocytogenes was grown overnight in BHI broth at 30°C to a density of 2×10^9/ml. 1 ml of culture was sedimented in a microfuge tube (14,000 g) for 1 min, the supernatant was discarded and the pellet was washed once in 1 ml of PBS, pH 7.4. Monolayers of J774 cells and bone marrow–derived macrophages were infected with 10^6 bacteria per petri dish for 30 min, while Henle cells were infected with 2×10^7 bacteria per petri dish for 60 min, and CL.7 cells were infected with 10^8 bacteria per petri dish for 60 min. After the initial infection (30 or 60 min), monolayers were washed three times with 37°C PBS followed by the addition of 5 ml of prewarmed medium. After 30 min, gentamicin sulfate was added to a final concentration of 10 μg/ml. After 1 h, the number of bacteria per cover slip was determined by depositing cover slips, in triplicate, into 5 ml of sterile distilled water in a 15-ml conical tube. After mixing vigorously for 15 s to lyse the infected cells, dilutions were plated onto BHI agar. After growing at 37°C overnight, the number of bacteria per cover slip was deduced from the number of colonies on the agar plate. The data presented in Fig. 2 represent an average of the three coverslips.

SDS-PAGE. Culture supernatants were prepared for SDS-PAGE by precipitation with 10% TCA. The precipitate was collected by centrifugation and dissolved in final sample buffer (1% SDS, 0.03 M Tris, pH 6.8, 5% glycerol, 0.01% bromophenol blue, and 2.5%

2-ME. After boiling for 5 min, an amount equivalent to 1 ml of culture supernatant was subjected to SDS-PAGE in a discontinuous buffer system (23) using slab gels of 7% acrylamide. Electrophoresis was performed at a constant current of 20 mA for 1 h and 30 mA for an additional 4 h. Gels were stained for 1 h in 0.05% Coomassie brilliant blue R, and destained in 30% methanol and 7.5% acetic acid. Protein molecular weight standards (Bio-Rad Laboratories, Richmond, CA) were used to calibrate apparent molecular weights.

Assay for Hemolytic Activity. Hemolysin activity was assayed essentially as described by Kingdon and Sword (24). Briefly, twofold serial dilutions of samples were made in PBS containing 6 mM cysteine (final pH 5.8). After a 30-min incubation at 37°C, a 1/10 volume of a 10% solution of sheep red blood cells was added to the sample. After an additional 30-min incubation at 37°C the tubes were subjected to centrifugation and were scored for visible hemolysis. Hemolytic units were expressed as the reciprocal of the highest dilution showing complete hemolysis. Complete hemolysis was observed by adding 10% Triton X-100 (1/100 volume) to the sample.

Animal Studies. 6–7-wk-old female BALB/c mice (Simonsen Laboratories, Gilroy, CA) were used for all LD_{50} determinations. Mice were provided feed and water ad libitum. For LD_{50} determinations, groups of four mice were injected intravenously with fivefold dilutions (in PBS) of log-phase cultures of *L. monocytogenes* strains grown in BHI broth. Colony forming units per milliliter were determined by plating dilutions of the cultures on BHI agar plates. Cultures of strains DP-L208, DP-L215, and DP-L224 were concentrated 10-fold by centrifugation before injection into mice. Mice were observed for 10 d, after which time no more deaths occurred. The LD_{50} of the strains were calculated by the method of Reed and Muench (25).

Double Immunofluorescence Assay. The double immunofluorescence assay used determine the percentage of intracellular bacteria was performed similar to the method described by Heesemann and Laufs (26). Monolayers were infected with *L. monocytogenes* for 45 min at a multiplicty of infection to achieve ~10 bacteria/cell (10^7 bacteria/ml). The coverslips were removed, washed (by dipping 5× in PBS), and overlaid with a 1/320 dilution of *Listeria* O rabbit antiserum (Sigma Chemical Co., St. Louis, MO) for 30 min at 4°C. After washing, FITC-conjugated goat F(ab')$_2$ anti–rabbit IgG (Tago Inc., Burlingame CA) was added for 30 min to stain extracellular bacteria. After washing, the cover slips were immersed in methanol for 5 min to make the host cells permeable to subsequent antibody additions. After washing to remove the methanol, *Listeria* O antiserum was again added for 30 min followed by rhodamine isothiocyanate (RITC)–conjugated goat F(ab')$_2$ anti–rabbit IgG (Tago Inc., Burlingame, CA). After washing, the cover slips were air dried and mounted in a solution containing 50% glycerol in PBS. Both intracellular and extracellular bacteria were labeled with RITC while extracellular bacteria were labeled with only FITC. 100 RITC-labeled bacteria were scored for FITC labeling and the percentage of intracellular bacteria was calculated. Microscopy was performed using a Leitz fluorescence photomicroscope equipped with epiflouresence.

Isolation of Revertants. Monolayers of J774 cells on cover slips were infected with the hly⁻ mutants so that there were ~10 bacteria/cell. After 1 h, extracellular bacteria were removed by washing three times with warm PBS, followed by the addition of 10 μg/ml of gentamicin. After 24 h, the cover slips were washed once in sterile PBS to remove the gentamicin, and the cells were lysed by mixing in 1 ml of distilled water. The number of bacteria per cover slip was determined by plating dilutions on BHI agar. The phenotype of 100 colonies was determined by plating on blood agar to assay for hemolysin production and on BHI containing tetracycline to assay for the presence of the transposon. The bacteria were grown overnight by adding 1 ml of 2X BHI to the lysed monolayer and were used to infect a fresh monolayer of J774 cells and the procedure was repeated.

Results

Construction and Characterization of Hemolysin-negative Mutants. To ascertain the role of hemolysin (hly) for intracellular growth, hly⁻ mutants were isolated

TABLE I

Hemolytic Activity and LD$_{50}$ of Hly$^+$ and Hly$^-$ L. monocytogenes

Strain and description	Hemolytic titer*	LD$_{50}$[‡]
10403S (parental strain)	80	3.3×10^4
DP-L216 (10403S::Tn*1545*)	80	5.8×10^4
DP-L208 (10403S::Tn*1545*)	5	2.1×10^9
DP-L215 (20403S::Tn*1545*)	UD§	1.9×10^9
DP-L224 (10403S::Tn*916*)	UD	2.2×10^9
DP-L290 (hly$^+$ revertant of DP-L208)	80	4.9×10^4
DP-L291 (hly$^+$ revertant of DP-L215)	80	4.3×10^4
DP-L292 (hly$^+$ revertant of DP-L224)	80	3.6×10^4

* Hemolytic titer is expressed as the reciprocal of the highest dilution at which complete lysis of the erythrocytes was observed.

[‡] The LD$_{50}$ was determined by the method of Reed and Muench (25) after intravenous injection of *L. monocytogenes* into mice.

§Undetectable.

using the conjugative transposons Tn*1545* and Tn*916* as described by Gaillard et al. (18) and Kathariou et al. (19). Three mutants were chosen for study. Two of these mutants (DP-L215 and DP-L224) showed no detectable hemolysin activity in bacterial supernatant fluid, while one mutant (DP-L208) did exhibit some hemolytic activity in culture supernatant fluid (5 hemolytic units/ml of culture supernatant compared with 80 U/ml for the hly$^+$ strain). The three mutants will be referred to as hly$^-$ mutants.

The three hly$^-$ mutants, the hly$^+$ parental strain, and hly$^+$ revertants (see below), were tested for their LD$_{50}$ in mice (Table I). All of the hly$^+$ strains were virulent with LD$_{50}$ values of ~4 × 10^4. In contrast, all of the hly$^-$ strains were avirulent with LD$_{50}$ values of > 10^9. These results are similar to those described by others for hly$^-$ mutants of *L. monocytogenes* (18, 19, 28).

The secreted protein profile of the hly$^-$ mutants was analyzed using SDS-PAGE. Four major bands and a number of minor bands were observed in Coomassie blue–stained gels of TCA precipitates of *L. monocytogenes* culture supernatant fluid derived from the hly$^+$ strain (Fig. 1). All of the hly$^-$ mutants
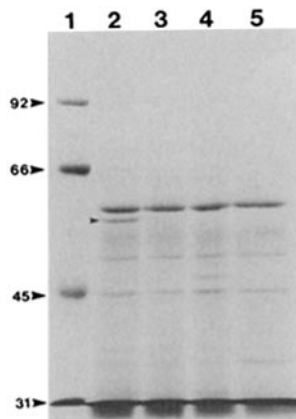

FIGURE 1. SDS-PAGE of the secreted polypeptides of hly$^+$ and hly$^-$ *L. monocytogenes*. Lanes: *1*, molecular weight markers (× 10^{-3}) *2*, DP-L216 (hly$^+$); *3*, DP-L208 (hly$^-$); *4*, DP-L215 (hly$^-$); *5*, DP-L224 (hly$^-$). The small arrowhead points to the 58-kD hemolysin.
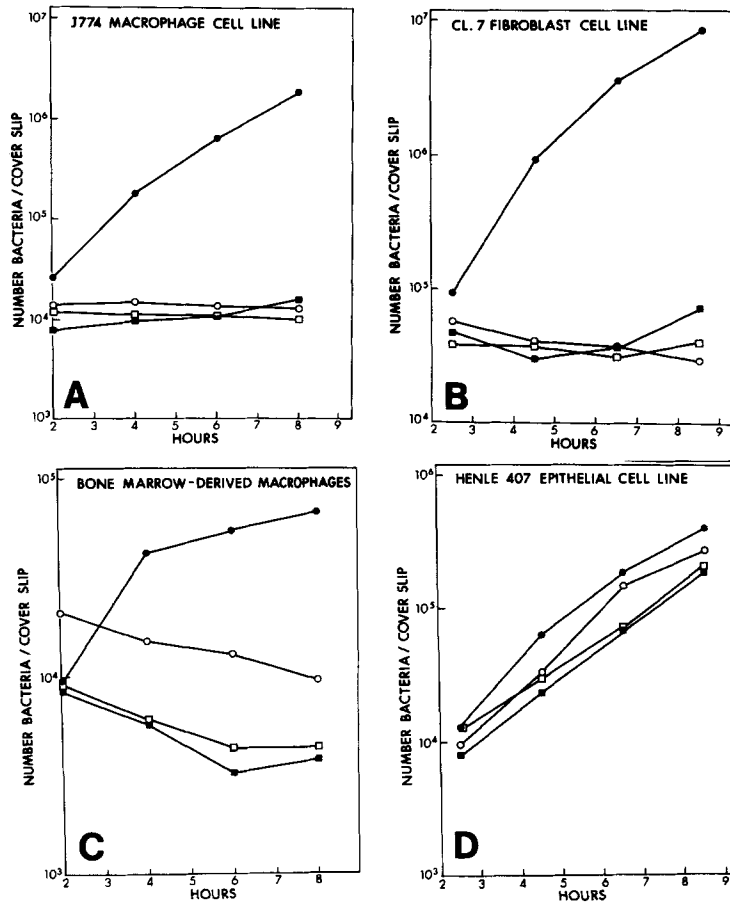
FIGURE 2. Intracellular growth of hly$^+$ and hly$^-$ *L. monocytogenes*. The intracellular growth assays were performed as described in Materials and Methods. The cell type infected is indicated on top of each panel. *(Filled boxes)* strain DP-L208; *(open boxes)* strain DP-L215; *(Filled circles)* strain DP-L216; *(open circles)* strain DP-L224.

lacked a 58-kD polypeptide. Strain DP-L224 lacked the 58-kD band but showed a new protein species of 35-kD, while strain DP-L215 lacked the 58-kD polypeptide and did not exhibit any additional polypeptide bands. The identity of the lower molecular weight polypeptides secreted by strains DP-L215 and DP-L224 has not been established, but they may represent truncated forms of the hemolysin as described by Kathariou et al. (19).

Intracellular Growth of L. monocytogenes. Tissue culture models for listerial intracellular growth were developed using mouse bone marrow–derived macrophages, the mouse macrophage-like cell line J774 (21), the primary mouse fibroblast line CL.7, and the human epithelial cell line Henle 407 (27) (Fig. 2). The tissue culture cells, grown as monolayers on cover slips, were infected with a suspension of *L. monocytogenes*. The extracellular bacteria were killed with gentamicin (14), which had no measurable effect on the viability of intracellular bacteria (data not shown). The number of bacteria internalized by the cells var-

ied considerably among the different cell types. Bone marrow–derived macrophages and the J774 macrophage-like cell line were infected ~50 times more efficiently than the fibroblast and epithelial cell lines (data not shown). Accordingly, the number of bacteria used during infection of the different cell types was varied to achieve multiplicities of infection of ~1 bacteria/10 host cells.

Subsequent to internalization the intracellular doubling times of *L. monocytogenes* was ~60 min in all cell types examined (Fig. 2). However, listerial infection of Henle 407 cells, J774 cells, and CL.7 cells at any multiplicity resulted in continuous intracellular growth until there were ~100 bacteria/cell (Fig. 2, and data not shown). This is consistent with an observation that *L. monocytogenes* spreads cell-to-cell after infection of primary fibroblasts (14). Thus, monolayer infection eventually results in each cell being maximally infected. In contrast, growth appeared to have ceased after three bacterial generations within bone marrow–derived macrophages (Fig. 2 *C*). The difference in the extent of intracellular growth observed within the cell lines and the primary mouse bone marrow–derived macrophages is not clear.

The hly⁻ mutants were analyzed for growth in the four cell types (Fig. 2). All of the hly⁻ mutants were internalized by the tissue cell monolayers to a similar extent as the hly⁺ parent strain as determined by the number of gentamicin protected bacteria/monolayer. However, all of the hly⁻ mutants were deficient for proliferation in J774 macrophage-like cells, bone marrow–derived macrophages, and CL.7 fibroblasts. The weakly hemolytic strain 208 did continue to grow intracellularly in J774 and CL.7 cells, but its doubling time was increased to ~4 h. Surprisingly, all of the hly⁻ mutants grew in Henle 407 human epithelial cells. This may reflect the fact that Henle 407 cells are of human origin since the mutants also grew in the WS1 primary human fibroblast line (data not shown).

To rule out the possibility that the failure of hly⁻ mutants to grow intracellularly was due to differences in susceptibility to gentamicin, bone marrow–derived macrophages were infected with the parental strain or the hly⁻ mutants
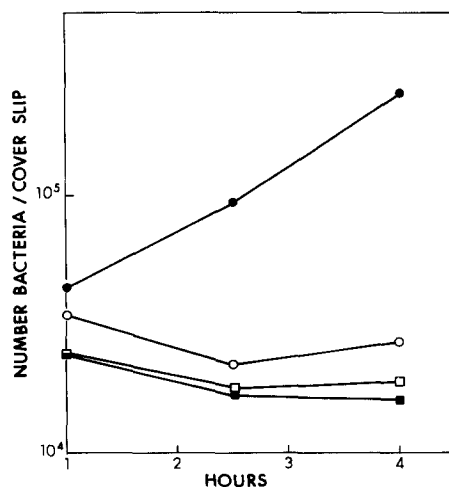

FIGURE 3. Intracellular growth of hly⁺ and hly⁻ *L. monocytogenes* in bone marrow–derived macrophages in the absence of gentamicin. Intracellular growth assay was performed as described in Materials and Methods but without the addition of gentamicin. The symbols are the same as Fig. 2.
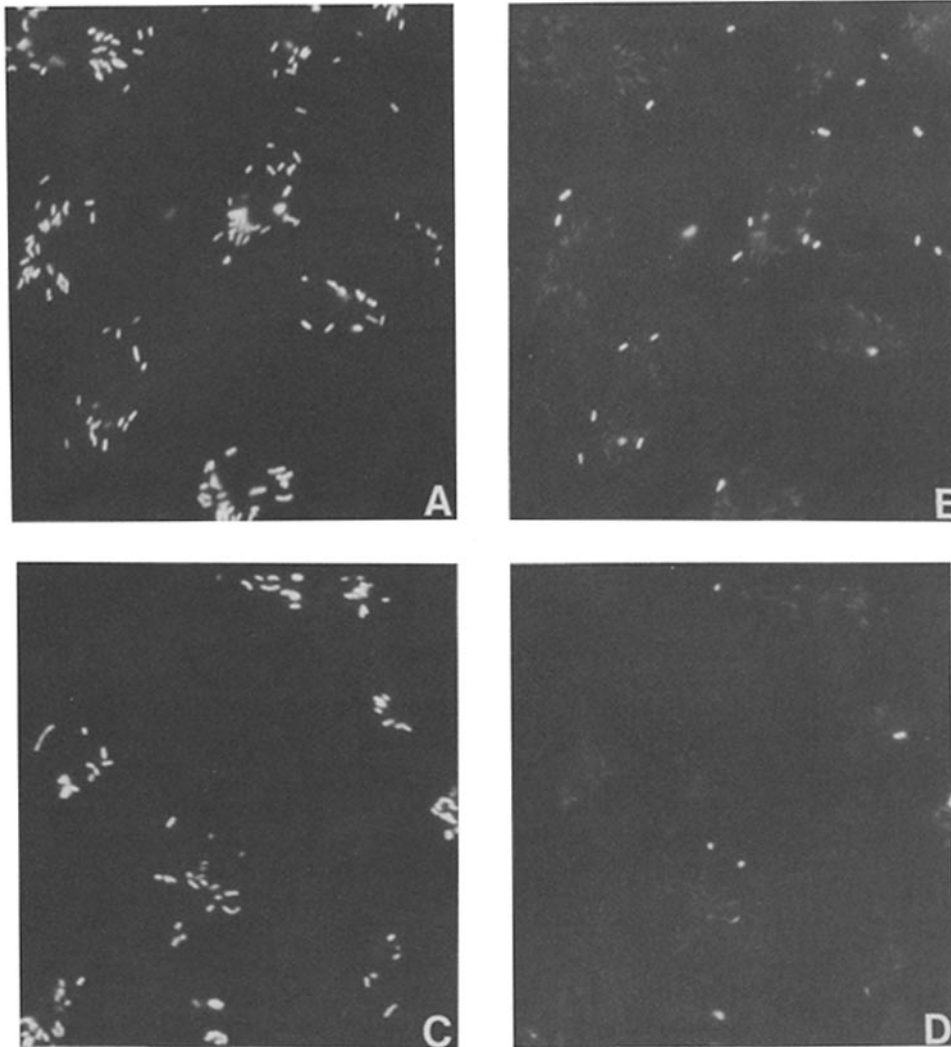
FIGURE 4. Intracellular localization of hly⁺ and hly⁻ *L. monocytogenes* in bone marrow–
derived macrophages. Monolayers were infected for 45 min with 10^7 bacteria/ml. *Panels* (*A*),
DP-L216 (hly⁺)-infected macrophages labeled with RITC (intracellular and extracellular bac-
teria); (*B*) same as *A* but labeled with FITC (extracellular bacteria); (*C*) DP-L215 (hly⁻)-
infected macrophages labeled with RITC; (*D*) same as *C* but labeled with FITC. The back-
ground flourescence seen in *B* and *D* is due to an overlap of RITC flourescence.

in the absence of gentamicin (Fig. 3). Extracellular bacteria were not a problem
in this cell type because the vast majority of cell-associated bacteria were intra-
cellular (Fig. 4). The hly⁻ mutants exhibited no multiplication even after 4 h in
the absence of gentamicin (Fig. 3). Furthermore, the hly⁻ bacteria were clearly
intracellular, as determined by indirect fluorescence microscopy (Fig. 4). We
concluded that hly⁻ *L. monocytogenes* are internalized, but cannot multiply in the
murine cells tested.

   Isolation of Revertants in J774 Cells.   J774 cell monolayers were infected with

each of the hly$^-$ mutants for 24 h and resulting bacteria were characterized with respect to hemolysin production and the presence of Tn916 (tet^r). As previously shown (Fig. 2 A) the mutants were unable to grow in these cells. Therefore, passage of hly$^-$ bacteria through J774 cells in the presence of gentamicin selects for a rare revertant capable of intracellular growth. Revertants of strain 215 were selected after a single 24-h pass through J774 cells. 75% of the colonies were hly$^+$ tet^s while the remainder were hly$^-$ tet^r. Therefore, passage through the cell line selected for hly$^+$ *L. monocytogenes*. The hly$^-$ tet^r bacteria presumably represent bacteria present in the original inoculum. These data suggested that Tn1545 had excised resulting in reacquisition of the hemolytic phenotype. Revertants of strain 208 were isolated after two passes through J774 cells. Of these, 88% of the colonies were hly$^+$ tet^s while the remainder were hly$^-$ tet^r. Revertants of strain 224 were isolated after two passes through J774 cells of which 85% of the colonies were hly$^+$ tet^r, 4% of the colonies were hly$^+$ tet^s, and the remainder were hly$^-$ tet^r. The latter result suggested that the growth of the revertants was associated with reacquisition of the hemolytic phenotype, but the majority of the revertants retained a copy of Tn916.

Each of the hly$^+$ revertants was fully virulent in mice (Table I). Furthermore, all of the hly$^+$ revertants showed the presence of a 58-kD polypeptide on SDS-PAGE (data not shown). Taken together, these data indicated a complete correlation between expression of the 58-kD hemolysin, intracellular growth, and mouse virulence.

Discussion

There are a number of reports that nonhemolytic (hly$^-$) *L. monocytogenes* are avirulent and fail to multiply in the tissues of infected mice (18, 19, 28). Our results corroborate the results of others in which transposable elements were used to insertionally inactivate hemolysin gene expression (18, 19). In the present study, three different hly$^-$ mutants were shown to be avirulent in a mouse model, while hly$^+$ revertants were fully virulent. The failure of hly$^-$ mutants to multiply in infected tissues has led to speculation that hemolysin is essential for listerial intracellular multiplication (29). In the present study we directly demonstrated that mutants lacking hemolysin activity were unable to multiply within murine cells, including macrophages and fibroblasts. Thus, we presume that the hemolysin is required for intracellular growth in murine cells. In contrast to our observation in murine cells, hly$^-$ mutants still grew within the human epithelial cell line Henle 407 or human fibroblast cell line WS1. These data may suggest a fundamental difference in the cell biology of listerial infection in mouse cells compared with human cells. Thus, hemolysin appears to be an essential determinant of listerial pathogenicity in an in vivo mouse model and in vitro in murine-derived cells, but its role, if any, in human infection remains unknown.

The results of this study strongly suggest that the *L. monocytogenes* hemolysin is an essential determinant of pathogenicity. However, we have not directly demonstrated that the transposable elements used to generate hly$^-$ mutations are located within the structural gene for hemolysin. Indeed, mutant DP-L208 secreted reduced but detectable hemolytic activity, which suggests that this

strain may have suffered a mutation in a gene other than hemolysin, possibly a regulatory gene. In contrast, hly$^-$ mutants DP-L215 and DP-L224 secreted no detectable hemolytic activity. However, it is possible that transposon insertions responsible for the hly$^-$ phenotype in these mutants may have also caused polar mutations. For example, nonhemolytic insertion mutations in *Bordetella pertussis* also lack extracellular adenylate cyclase a separate virulence determinant (30). Absolute proof for a role of hemolysin in the pathogenicity of *L. monocytogenes* still awaits introduction of the cloned hemolysin gene into a hly$^-$ strain.

The precise intracellular growth defect of the hly$^-$ mutants is still unclear. However, our results suggest that it occurs subsequent to internalization since hly$^-$ mutants were localized intracellularly. Kingdon and Sword (31) reported that the hemolysin was responsible for lysis of macrophage lysosomes. Geoffroy et al. (32) have shown that the pH optimum of the hemolysin is 5.5, which approximates the pH of a phagolysosome. It is thus tempting to speculate that the role of hemolysin is to lyse the phagolysosomal membrane, consequently liberating bacteria to the host cell cytoplasm. Alternatively, hemolysin may play a nutritional role, possibly by liberating intracellular iron to afford intracellular growth (33).

Results presented in this study indicate that *L. monocytogenes* invades and grows within epithelial cells and fibroblasts, as well as macrophages. The initial intracellular growth rate was comparable in all cell types examined, although bone marrow–derived macrophages and the J774 macrophage cell line bound and ingested considerably more bacteria than the other cell lines. Mononuclear cells may be a preferred cite for listerial multiplication in vivo, but *L. monocytogenes* apparently grows within liver parenchymal cells as well (10, 11). Furthermore, after oral challenge, which may represent the natural route of infection (34), *L. monocytogenes* invades the Peyer's patches of the small intestine (35) and may grow intracellularly in small intestinal epithelial cells (13). While it is well established that *L. monocytogenes* is an intracellular pathogen of resident macrophages (8, 9), it is also clear that invasion and growth within nonprofessional phagocytes is an essential feature of listerial pathogenicity. Furthermore, hemolysin production appears to be essential for growth in both types of cells. However, hemolysin is incapable of promoting listerial growth in activated or elicited macrophages (9, 36). This may be because bacteria are killed by activated macrophages before hemolysin can exert an effect. Alternatively, the oxygen-labile hemolysin may be rendered impotent by secreted oxidative metabolites of activated macrophages (37).

The precise immunological mechanisms used to clear an intracellular bacterial infection in which bacteria reside within nonprofessional phagocytes are not understood. Immunity to *L. monocytogenes* is T cell mediated (38, 39), but there is controversy in the literature regarding which T cell population(s) is necessary to orchestrate listerial immunity (40–42). Recently, Bishop and Hinrichs (20) have shown that long-lived immunity to *L. monocytogenes* can be adoptively transferred to mice with a Lyt-2$^+$ T cell population. Consistent with this, Kaufmann et al. (43) have shown that CTL are generated during listerial infection in mice, and that CTL clones can lyse listerially infected bone marrow–derived macrophages. Thus, the role of CTL during immunity to *L. monocytogenes* may be to

lyse bacterially infected cells. It remains to be demonstrated, however, that a population of Lyt-2$^+$ T cells can lyse listerially infected nonprofessional phagocytes such as a fibroblast or epithelial cell. A potential role for CTL is consistent with the observation that nude mice become chronically infected with *L. monocytogenes* (44). Also, Bancroft et al. (45) have recently shown that mice with severe combined immunodeficiency (scid), which lack T cells and B cells, harbor *L. monocytogenes* chronically even though they generate high levels of IFN-γ and possess activated macrophages. Thus, the activated macrophage may be poised to kill extracellular bacteria, but without CTL the infection may persist intracellularly especially in nonprofessional phagocytes. Hemolysin appears to be an essential determinant for listerial intracellular growth, and nonhemolytic mutants, like heat-killed *L. monocytogenes,* fail to induce protective immunity (29, 46). Thus, hemolysin-mediated intracellular growth may be required to induce an appropriate T cell response.

Summary

Listeria monocytogenes insertion mutants defective in hemolysin production were generated using the conjugative transposons Tn*916* and Tn*1545*. All of the nonhemolytic mutants (hly$^-$) lacked a secreted 58-kD polypeptide, presumedly hemolysin, and were avirulent in a mouse model. An intracellular multiplication assay was established in monolayers of mouse bone marrow–derived macrophages, the J774 macrophage-like cell line, the CL.7 embryonic mouse fibroblast cell line, and the Henle 407 human epithelial cell line. The hly$^+$ strain grew intracellularly in all of the tissue culture cells with a doubling time of ~60 min. In contrast, the hly$^-$ mutants failed to grow in the murine-derived tissue culture cells, but retained the ability to grow in the human tissue culture cells examined. Hemolytic-positive revertants were selected after passage of the hly$^-$ mutants through monolayers of J774 cells. In each case, the hemolytic revertants possessed the 58-kD polypeptide, were capable of intracellular growth in tissue culture monolayers and were virulent for mice.

Note added in proof: After submission of this manuscript Gaillard et al. (47) reported that hly$^-$ *L. monocytogenes* almost completely lost the ability to grow in the human enterocyte-like cell line Caco-2. Thus, hemolysin is likely essential for normal growth in some, but not all human cell lines.

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