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Listeria monocytogenes Infects Human Endothelial Cells by Two Distinct Mechanisms

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Infection of endothelial cells by bacteria may be an important component of the bacteria's ability to escape host defenses and cause disease. *Listeria monocytogenes* causes sepsis and central nervous system infection in domesticated animals and immunocompromised humans, suggesting that this bacterium interacts with endothelial cells in a significant fashion. The experiments presented here tested the hypothesis that *L. monocytogenes* can invade and replicate within human endothelial cells. We found that *L. monocytogenes* grows readily in umbilical vein endothelial cells and that its intracellular life cycle involves phagosomal escape, F-actin-based motility, and cell-to-cell spread. We found that *L. monocytogenes* invaded endothelial cells by cell-to-cell spread from adherent mononuclear phagocytes which were previously infected by this bacterium. Interestingly, *L. monocytogenes* mutants lacking the invasion protein, internalin, bound less well to endothelial cells than did wild-type bacteria in the absence, but not the presence, of serum, and their invasion of endothelial cells was diminished under both conditions. Thus, endothelial cell infection by *L. monocytogenes* can occur by two distinct mechanisms: direct bacterial invasion of the endothelial cells in an internalin-mediated fashion or cell-to-cell spread from adherent, infected mononuclear phagocytes. These data support the idea that endothelial cell infection by *L. monocytogenes* is an important event in the pathogenesis of listeriosis.

Endothelial cells can be targets for bacterial infection and cellular refuges in which bacteria avoid host defenses (4). In response to bacterial infection, endothelial cells demonstrate profoundly altered functions such as leukocyte adhesion and cytokine secretion (22, 38, 44, 45). The cellular and molecular events by which bacteria infect endothelial cells bear similarity to those involved in microbial invasion of other cells such as epithelial cells and macrophages. In these situations, microbes first must bind the surface of the eukaryotic cell before cellular invasion can occur (28). Not surprisingly, specific molecules on both the endothelial cell and the microbe are necessary for these events to take place (10, 29, 52). For example, after entering endothelial cells *Staphylococcus aureus*, group B streptococci, and *Pseudomonas aeruginosa* persist intracellularly but do not replicate (24, 42, 53). Spirochete pathogens, on the other hand, traverse endothelial cell monolayers without disrupting them (11, 50), and *Rickettsia rickettsii* and perhaps *Bartonella bacilliformis* and *Rochalimaea* species can enter and replicate within endothelial cells (2, 21, 30, 48).

Direct invasion of the vascular endothelium by bacteria, however, may not be the only means of cellular infection. A second possible mechanism could involve parasitism of an intermediary blood cell of host origin, followed by endothelial cell invasion. Part of this scenario, blood cell parasitism and microvascular adhesion, is involved in adherence of *Plasmodium falciparum*-infected erythrocytes to cerebral vascular endothelial cells (3, 5, 35–37). Similarly, intracellular pathogens of monocytes may use infection of host phagocytes not merely as a primary means of immune avoidance, but also for dissemination throughout the host. This Trojan horse mechanism of trafficking to, and infection of, the central nervous system has been proposed for visna virus and human immunodeficiency virus (HIV) (39, 40).

Listeria monocytogenes is a facultative intracellular bacterial parasite of many eukaryotic cells including epithelial cells, fibroblasts, hepatocytes, and macrophages (19, 26, 27, 51). Recently, Gaillard et al. identified an 80-kDa surface protein on *L. monocytogenes* named internalin that enables these bacteria to bind and invade epithelial cells in vitro (18). Dramsi et al. (13a) showed that invasion of cultured human epithelial cells by *L. monocytogenes* requires the expression of *inlA* alone, while *inlB* expression is required for invasion of cultured mouse hepatocytes. After entering nonmicrobicidal cells, including professional and nonprofessional phagocytes, *L. monocytogenes* lyses the phagosome, replicates in the cytoplasm, and becomes coated with F-actin, which polarizes and propels the bacterium from the first infected cell into adjacent cells (13, 51).

L. monocytogenes is also a pathogen of immunocompromised humans and domestic animals (6, 23, 25, 34). Invasive *L. monocytogenes* infection in nonpregnant humans usually assumes one of two general forms, sepsis and/or invasive central nervous system disease (6, 23, 34). These clinical syndromes suggest that *L. monocytogenes* may interact with endothelial cells and infect them, but this notion has not yet been addressed experimentally. The present study tested the hypothesis that *L. monocytogenes* could invade human endothelial cells directly by a process dependent in part on the internalin molecule and indirectly by using a phagocyte as an intermediate host cell. Our findings support the concept that invasion of endothelial cells by *L. monocytogenes* is a central event in the pathogenesis of listeriosis.

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MATERIALS AND METHODS

Bacteria. *L. monocytogenes* EGD was originally obtained from G. B. Mackaness. Bug 8, a mutant of *L. monocytogenes* with a transposon inserted in the *inlAB* gene, does not express either the internalin A or internalin B protein (18). This mutant was the generous gift of J.-L. Gaillard. The nonhemolytic, avirulent *L. monocytogenes* strain 43250 was purchased from the American Type Culture Collection (Rockville, Md.) (41). The nonhemolytic, avirulent transposon mutant of *L. monocytogenes*, CNL 85/162, and a hemolytic revertant, CNL 85/163, were made by Gaillard et al. (20) and kindly provided by K. Ziegler. Bacteria were stored at −70°C in tryptose phosphate broth containing 15% glycerol. For each experiment, a log-phase culture of bacteria was prepared by inoculating 0.5 to 1.0 ml of an overnight culture into 4 ml of fresh tryptose phosphate broth. The new culture was incubated for 3 to 5 h at 37°C with agitation to allow bacterial growth. Bacteria were washed twice by centrifugation at 12,000 × *g* for 3 min and then resuspended and vortex mixed in balanced salt solution (BSS) (33).

Cells. Human umbilical vein endothelial cells (HUVEC) were purchased from the American Type Culture Collection. The cells were cultured in F-12K medium (Gibco BRL, Grand Island, N.Y.) containing 10% fetal calf serum (FCS) (HyClone Laboratories, Logan, Utah) and 50 μg of endothelial cell growth supplement per ml (Collaborative Biomedical Products, Bedford, Mass., and Sigma Chemical Co., St. Louis, Mo.). The lyophilized growth supplement contained streptomycin which was at a final concentration of 19.5 μg/ml in the complete medium. No additional antibiotics were used. The human dermal microvascular cell line CDC.HMEC-1 (HMEC) was obtained from Edwin Ades at the Centers for Disease Control and Prevention, Atlanta, Ga. (1). These cells were cultured in MCDB 131 medium (Gibco) with 10% FCS, epidermal growth factor at 10 ng/ml (Gibco), and hydrocortisone at 1 μg/ml (Sigma). No antibiotics were added to the HMEC culture medium. HUVEC were used between passages 15 and 30 and were maintained in 100-mm dishes, fed twice weekly, and split 1:1 weekly or every other week into 24- or 6-well plates as needed for experiments. HMEC cells were used between passages 19 and 24, were maintained in 100-mm petri dishes, and were split 1:5 every 5 to 6 days. All experiments were performed with cells in complete monolayers that were cultured for no less than 72 h after the most recent split.

The human histiocytic lymphoma cell line U937 (49) was obtained from the American Type Culture Collection and was cultured in RPMI medium (Gibco) with 10% FCS and 2 mM L-glutamine with no added antibiotics. Human peripheral blood mononuclear cells (PBMC) were obtained by venipuncture from healthy, HIV-negative donors following informed consent. Mononuclear cells were separated from whole blood by centrifugation through neutrophil isolation medium (Cardinal Associates, Santa Fe, N. Mex.). The bands containing mononuclear cells were collected and washed twice with BSS before use. Cytocentrifuge preparations of the PBMC were made and stained with Diff-Quik (Baxter Healthcare Corporation, McGaw Park, Ill.), and the purity of the preparation and percentage of monocytes were checked by light microscopy.

Serum. Normal human serum (NHS) was obtained following informed consent by venipuncture from a single, normal HIV-negative donor. Freshly clotted whole blood was centrifuged to separate serum from the cellular components. The serum was stored at −70°C, and a fresh sample was thawed immediately before use. Where indicated, bacteria were incubated in serum by mixing 10^5 to 10^6 *L. monocytogenes* per ml with 3, 10, or 30% NHS for 60 min at 37°C before adding them to wells containing cells.

Production of anti-internalin antibodies. *L. monocytogenes* genomic DNA was prepared by the method described by Flamm et al. (17). The *inlA* gene was cloned from genomic DNA by PCR. An oligonucleotide with a *Bam*HI site was used in the sense direction (5′-ATGGATCCTGAAGACGGTCTTAGGAAA), and an *Eco*RI oligonucleotide was used in the antisense direction (5′-ATGAAT TCCTATTTACTA). This *inlA* fragment was ligated into the pGEX3X expression vector (Pharmacia, Piscataway, N.J.) and transformed into *Escherichia coli* DH5α. Recombinant internalin A was isolated from inclusion bodies, bound to glutathione-agarose, and cleaved with factor Xa. Factor Xa-cleaved internalin A was separated on sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis minigels and visualized by staining with Coomassie blue dye and then was excised from the gel, emulsified with a Dounce homogenizer, and used to immunize mice. Spleen cells from the recombinant internalin A-immunized mice were fused with SP2/O myeloma cells to obtain monoclonal antibodies. The immunoglobulin G (IgG) fraction of ascitic fluid from mice immunized with the hybridomas was isolated from ascitic fluid by chromatography on Affi-Gel–protein A (Sigma) and adjusted to a final concentration of 500 μg/ml in sterile phosphate-buffered saline (PBS).

Direct infection of endothelial cells by *L. monocytogenes*. Bacteria were diluted to the indicated concentrations in F-12K medium, and then 0.25 ml of the bacterial suspension was added to triplicate wells of endothelial cells in 24-well plates. In experiments using six-well plates, 1.0 ml of the bacterial suspension was added to each of duplicate wells. In experiments in which the cells were not incubated in a CO_2 incubator, 15 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) was added to the medium to stabilize the pH. Control experiments showed that HEPES did not affect binding or invasion of bacteria. Binding experiments were performed in two ways with similar results. The plates containing cells and the indicated concentration of bacteria were incubated at 37°C for 60 min on a rotary platform without centrifugation. Alternatively, bacteria were centrifuged at 1,000 × *g* onto the cell monolayers and then incubated for 60 min at 37°C in a CO_2 incubator as previously described (18).

Bacteria associated with the monolayer were collected by washing each well twice with 0.5 ml of warmed BSS, and then 1.0 ml of sterile dH_2O containing 0.5% saponin was added and the cell lysates were collected with a Pasteur pipette. Bacterial CFU were quantified by serial dilution in sterile dH_2O and plating on Trypticase soy agar (Remel, Lenexa, Kans.). The percentage of bacteria bound to the monolayers was determined by averaging the CFU of bacteria recovered per well in triplicate wells and dividing that number by the CFU of bacteria inoculated per well. Experiments that compared the abilities of internalin-negative mutants and wild-type listeriae to bind and invade HUVEC were performed in the same plate on the same cells split from the same culture dish on the same day. In some experiments, monoclonal antibody (MAb) anti-internalin A 292 (mouse IgG1) or control MAb B344.1, an anti-staphylococcal enterotoxin B antibody (mouse IgG1) kindly provided by P. Marrack, was tested for its ability to inhibit binding of bacteria to HUVEC. In these experiments, 10^5 bacteria were incubated with the indicated concentration of MAb for 60 min at 37°C and then added to cell monolayers as described above. Control experiments showed that the MAbs used did not inhibit or promote bacterial growth.

Intracellular growth of *L. monocytogenes* in HUVEC was measured by a standard gentamicin protection assay as described by Portnoy et al. (43). Bacteria were centrifuged onto HUVEC monolayers and incubated for 60 min as described above. The cells were washed, and one set of triplicate wells was lysed to determine the number of bound bacteria. The remaining wells were incubated another 1 to 4 h at 37°C in medium containing 10 μg of gentamicin per ml to kill extracellular bacteria, and then CFU of listeriae were measured as above. Control experiments were performed to test the ability of gentamicin to kill extracellular listeriae. For this, 10^6 listeriae were incubated in 24-well plates in the absence of HUVEC in medium with 10 μg of gentamicin per ml. In these experiments, all bacteria were killed by 4 h.

Indirect infection of endothelial cells by *L. monocytogenes* within mononuclear phagocytes. Phagocytes were infected with *L. monocytogenes* by coincubation of cells and bacteria as previously described (14, 15). To induce phagocytosis of listeriae, U937 cells were differentiated by culturing in medium with 25 ng of phorbol myristate acetate per ml for 3 to 5 days (32). Then approximately 2×10^6 phorbol myristate acetate-differentiated U937 cells per ml were mixed 1:3 with *L. monocytogenes* in BSS containing 10% NHS. For PBMC, approximately 5×10^6 total PBMC per ml were mixed 1:10 in BSS containing 10% NHS. Cells and bacteria were rotated for 2 h at 37°C to allow phagocytosis and phagosomal escape of listeriae. Extracellular bacteria were removed carefully by washing the cells three times with 2 ml of BSS, and then they were centrifuged through a 1-ml layer of 30% sucrose-PBS as described previously (16). Cytocentrifuge preparations stained with Diff-Quik showed that 15 to 30% of the U937 cells and >75% of the monocytes were infected.

Next, 0.5 ml of medium containing 2.5×10^4 monocytes per ml or 2×10^4 to 4×10^4 U937 cells per ml with 10% FCS and 10 μg of gentamicin per ml was incubated at 37°C with endothelial cells. A separate aliquot of infected phagocytes not mixed with gentamicin was used to measure CFU of *L. monocytogenes* added to the endothelial cells. Bacterial CFU associated with the monolayer were measured as described above at the indicated time points. Control experiments demonstrated that cell-free *L. monocytogenes* which were inoculated directly into medium containing gentamicin did not infect the endothelial cell monolayers. Other experiments estimated CFU of *L. monocytogenes* present in the phagocyte population during incubation with endothelial cells. For this, infected phagocytes were cultured in wells without endothelial cells, and CFU of *L. monocytogenes* were determined in parallel with wells containing endothelial cells.

Microscopy of endothelial cells infected with *L. monocytogenes*. Endothelial cells were cultured on glass coverslips (22 by 22 mm) in six-well plates and then were infected with approximately 2.5×10^5 *L. monocytogenes* or were incubated with infected phagocytes. The cells were cultured in the presence of 10 μg of gentamicin per ml and then were stained with Diff-Quik and evaluated by light microscopy. To detect *L. monocytogenes* coated with host cell F-actin, infected monolayers were fixed with 4% paraformaldehyde, quenched with 100 mM glycine, and permeabilized with 0.2% Triton X-100 (Sigma). The cells were then incubated in 10 U of Bodipy phallicidin per ml (Molecular Probes, Eugene, Oreg.) to detect F-actin, with or without staining of listeriae with rabbit antilisteria antiserum followed by CY3-labeled donkey anti-rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, Pa.) as previously described (15). These cells were evaluated by fluorescence microscopy on a Leitz Diaplan microscope equipped with epifluorescence or by confocal laser scanning microscopy with a Bio-Rad MRC 500 system. For confocal microscopy, laser stimulation was at 488 nm with two-channel detection on green (Bodipy–F-actin) and red (CY3-listeria) channels. Laser power and gain were set so that there was negligible crossover of red to green or green to red.

Discrimination between intracellular and extracellular bacteria was performed by double immunofluorescence staining of HUVEC after incubation with listeriae (54). Wild-type and internalin-negative mutants of *L. monocytogenes* were diluted to 10^6 CFU/ml in F-12K medium with and without 10% NHS and were incubated for 60 min at 37°C. Then 0.25 ml was added to the endothelial cells, and the bacteria were centrifuged onto HUVEC monolayers that had been cultured on coverslips. The cells and bacteria were incubated for 90 min at 37°C

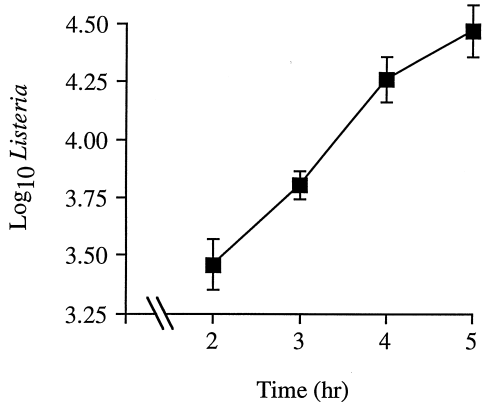

FIG. 1. Intracellular growth of *L. monocytogenes* in endothelial cells. HUVEC were infected by centrifuging 2.5×10^5 bacteria onto confluent cell monolayers in 24-well plates. The cells were incubated for 60 min and then washed and incubated for another 1 to 4 h in medium containing 10 μg of gentamicin per ml. At the indicated times, the cells were washed again, and then listeriae and cells were collected by Pasteur pipetting with dH_2O containing 0.5% saponin. CFU of listeriae were determined by serial dilution of cell lysates and plating on agar. Results are the mean ± SEM of CFU of *L. monocytogenes* per well from four separate experiments; each time point-experiment was performed in triplicate.

in a humidified incubator with 5% CO_2 and then were washed twice, fixed with 2 to 4% paraformaldehyde, and stained with antilisteria antiserum followed by CY3 (red)-labeled secondary antibody. Because the cells had not been permeabilized, only extracellular bacteria were stained at this point. Next, the same cells were permeabilized with 0.2% Triton X-100, reincubated with antilisteria antiserum, and then stained with a fluorescein isothiocyanate (green)-labeled secondary antibody. Thus, extracellular bacteria fluoresced both green and red whereas intracellular bacteria fluoresced green only. The cells on coverslips were mounted, and then 10 consecutive high-power fields of cells were examined for red and green bacteria by fluorescence microscopy with fluorescein isothiocyanate-specific band-pass (green) and 580-nm long-pass (red) filters.

RESULTS

Intracellular growth of *L. monocytogenes* in HUVEC. The first series of experiments tested whether *L. monocytogenes* could invade and grow within HUVEC. For this, a standard gentamicin protection assay was used. Approximately 2.5×10^5 *L. monocytogenes* were centrifuged onto HUVEC monolayers, which then were incubated for 60 min, washed, and incubated another 1 to 4 h in medium containing 10 μg of gentamicin per ml to kill extracellular bacteria. As shown in Fig. 1, there was a steady increase in bacterial CFU associated with the cells despite the presence of the bactericidal antibiotic. Control experiments showed that no bacteria could be recovered from wells without HUVEC by the 4-h time point. Bacteria were seen easily in infected HUVEC by light microscopy (Fig. 2), and some of the bacteria had tails similar in appearance to the actin associated with *L. monocytogenes* in the cytoplasm of other host cells (51). Fluorescence staining for F-actin (Fig. 2) showed numerous F-actin-coated bacteria, and confocal microscopy of infected cells stained for both F-actin and listeriae demonstrated colocalization of bacteria and F-actin (not shown). These experiments demonstrate that *L. monocytogenes* invaded HUVEC and that once inside these cells the bacteria completed their parasitic life cycle of phagosomal escape, cytoplasmic replication, and F-actin-based motility.

Role of internalin in binding of *L. monocytogenes* to HUVEC. Next, we tested whether internalin, a surface protein on *L. monocytogenes* that mediates binding and invasion of epithelial cells (18), was involved in *L. monocytogenes* binding to HUVEC. For this, we compared the abilities of wild-type *L. monocytogenes* and an internalin-negative mutant of *L. monocytogenes* to bind HUVEC. This mutant, Bug 8, contains a transposon insertion which prevents transcription of both InlA and InlB, here collectively called internalin. As shown in Fig. 3, over a broad range of bacterial concentrations, wild-type bacteria bound to HUVEC about 10 to 20 times more efficiently than did the internalin-negative mutant. Similar results were obtained when bacteria were centrifuged onto the monolayers of HUVEC and incubated for 60 min (data not shown). Control experiments showed that wild-type and mutant bacteria bound equally well to tissue culture plastic, with a mean CFU ± standard error of the mean (SEM) percent inoculum bound of (15.2 ± 1.8)% for wild type and (17.7 ± 3.3)% for the internalin-negative mutant ($n = 9$). Moreover, an anti-internalin A MAb significantly inhibited binding of wild-type bacteria to the cells, whereas control MAb had little effect (Fig. 4). Anti-internalin A MAb did not alter binding of the internalin-negative mutant to HUVEC (data not shown). These data indicate that internalin, probably internalin A, mediates the invasion of cultured endothelial cells, but it is possible that the

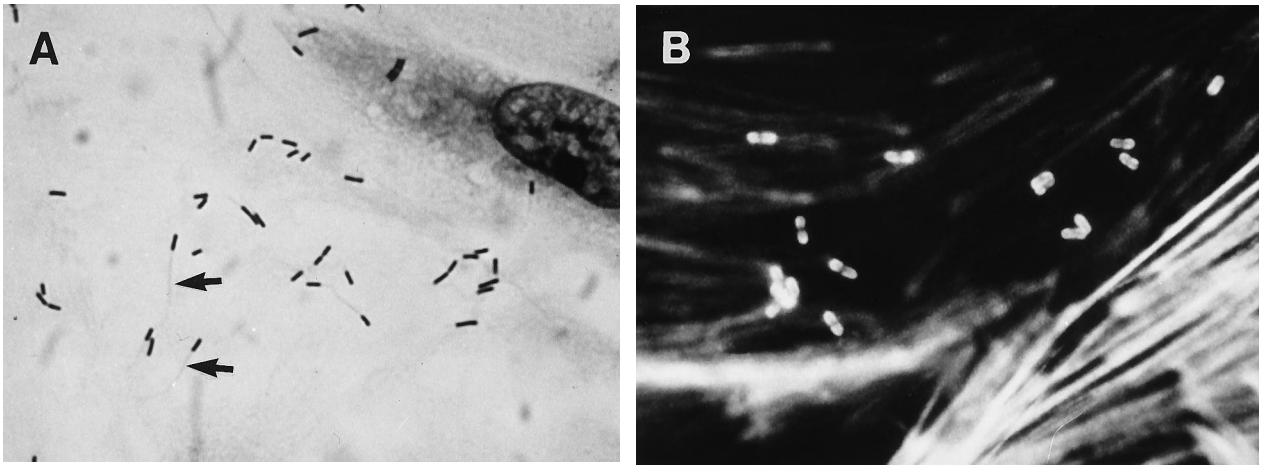

FIG. 2. Microscopy of endothelial cells infected with *L. monocytogenes*. HUVEC on coverslips were infected as for Fig. 1. The cells were incubated for 60 min and then washed and incubated for another 4 to 6 h. The cells were stained with Diff-Quik for light microscopy (A) or with a fluorescent phallicidin to detect F-actin-coated listeriae by fluorescence microscopy (B). Arrows in panel A are directed at tails. Final magnification, approximately ×1,500.

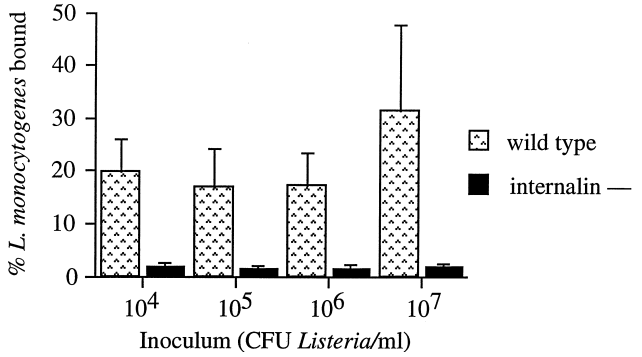

FIG. 3. Binding of wild-type and internalin-negative mutants of *L. monocytogenes* to endothelial cells. HUVEC cultured in 24-well plates were incubated by orbital rotation at 37°C with the indicated concentration of wild type and internalin-negative mutants in medium without serum. CFU of listeriae per well were determined as before. Results from four experiments are presented as the mean ± SEM percent bacteria bound to cells, with each point-experiment performed in triplicate.

internalin B gene product is also involved in binding and invasion of endothelial cells by *L. monocytogenes*.

Other experiments tested the effect of serum on binding of listeriae to HUVEC. Incubation in 10% NHS did not affect binding of wild-type *L. monocytogenes* to HUVEC (not shown), but binding of the internalin-negative *L. monocytogenes* mutant increased from (0.45 ± .07)% to (18.9 ± 7.6)% (mean ± SEM; $n = 3$). Increasing the concentration of NHS to 30% did not increase or decrease binding of either bacterium over that seen with 10% NHS (not shown). Similarly, anti-internalin MAb did not inhibit binding of wild-type bacteria to HUVEC in the presence of 10% NHS (not shown). Thus, these data indicate that internalin mediates binding of *L. monocytogenes* to HUVEC in the absence of serum. However, opsonins in serum which associate with *L. monocytogenes* enable internalin-negative mutants to bind to HUVEC as well as do wild-type bacteria, suggesting that the role of internalin as a binding protein may be diminished in the presence of serum.

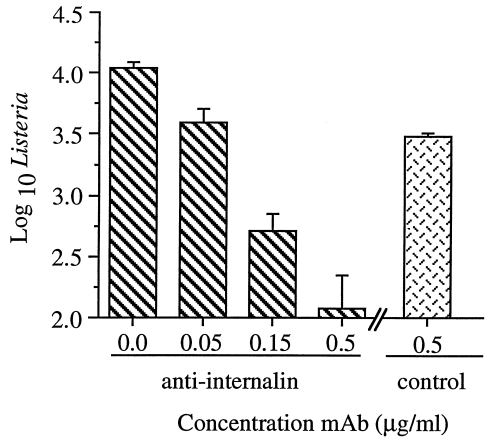

FIG. 4. Anti-internalin A MAb inhibits binding of *L. monocytogenes* to HUVEC. The indicated concentrations of anti-internalin A MAb or control MAb were incubated with 10^5 listeriae for 60 min, and then the bacteria were centrifuged onto HUVEC monolayers. Cells and bacteria were incubated for 60 min at 37°C, and CFU of listeriae per well were determined as before. The results are presented as mean ± standard deviation CFU per triplicate wells from one of four experiments with similar results.

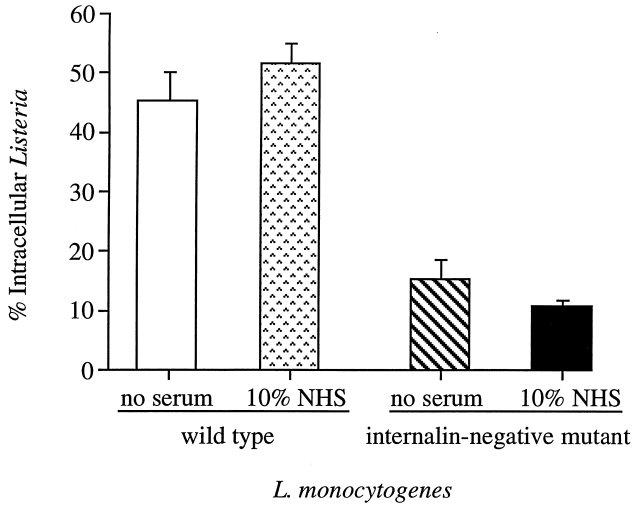

FIG. 5. Invasion of endothelial cells by *L. monocytogenes* requires internalin. Approximately 2.5×10^5 wild-type or internalin-negative *L. monocytogenes* mutant organisms, with and without prior incubation in 10% NHS, were centrifuged onto monolayers of HUVEC on coverslips and then incubated for 90 min at 37°C. The cells were washed, fixed, and then stained with antilisteria antiserum and CY3-labeled secondary antibody to label extracellular bacteria red. Then the cells were permeabilized and stained with antilisteria antiserum and fluorescein isothiocyanate-labeled secondary antibody to label intracellular and extracellular bacteria green. The cells on triplicate coverslips were examined by fluorescence microscopy with band-pass green and long-pass red filters. Red and green bacteria associated with cells in 10 to 15 random high-power fields were counted, and percent intracellular bacteria was calculated as 1 − (red bacteria/green bacteria). Results are presented as the mean ± SEM percent intracellular bacteria from three or four identical experiments.

Role of internalin in invasion of HUVEC by *L. monocytogenes*. Next, we used double immunofluorescence to determine the ability of wild-type and internalin-negative *L. monocytogenes* to invade HUVEC in the presence and absence of serum. As shown in Fig. 5, 45.3% of wild-type listeriae were intracellular when incu-bated with HUVEC in the absence of serum. In contrast, not only did fewer internalin-negative mutants bind to HUVEC, but of the bound bacteria, only 15.3% were able to invade them. In the presence of 10% NHS, the percentage of intracellular wild-type *L. monocytogenes* increased to 51.6%, but the percentage of intracellular internalin-negative mutants decreased slightly to 10.9%. A control experiment in which bacteria were incubated with HUVEC for 120 min with orbital rotation instead of centrifugation demonstrated similar results (not shown). These experiments show that *L. monocytogenes* can invade endothelial cells rapidly and that the presence of internalin is required for optimal invasion. Furthermore, these experiments demonstrate that the importance of internalin as an invasion protein is not diminished by serum and that the serum components which enable internalin-negative mutants to bind HUVEC do not enhance their invasiveness.

Infection of endothelial cells by infected phagocytes. The next series of experiments tested whether endothelial cells could be infected by *L. monocytogenes* within infected phagocytes. For this, we infected phorbol myristate acetate-stimulated U937 cells with listeriae and then incubated the infected U937 cells with endothelial cells. After 3 h of cocultivation, some of the U937 cells were adherent to the endothelial cell monolayer and many listeriae could be seen extending outwards from the phagocytes in filopodia (Fig. 6). Fluorescence staining for F-actin demonstrated that these were the typical F-actin comet tails associated with *L. monocytogenes* when in the cytoplasm of nonlistericidal cells (not shown) (51). When

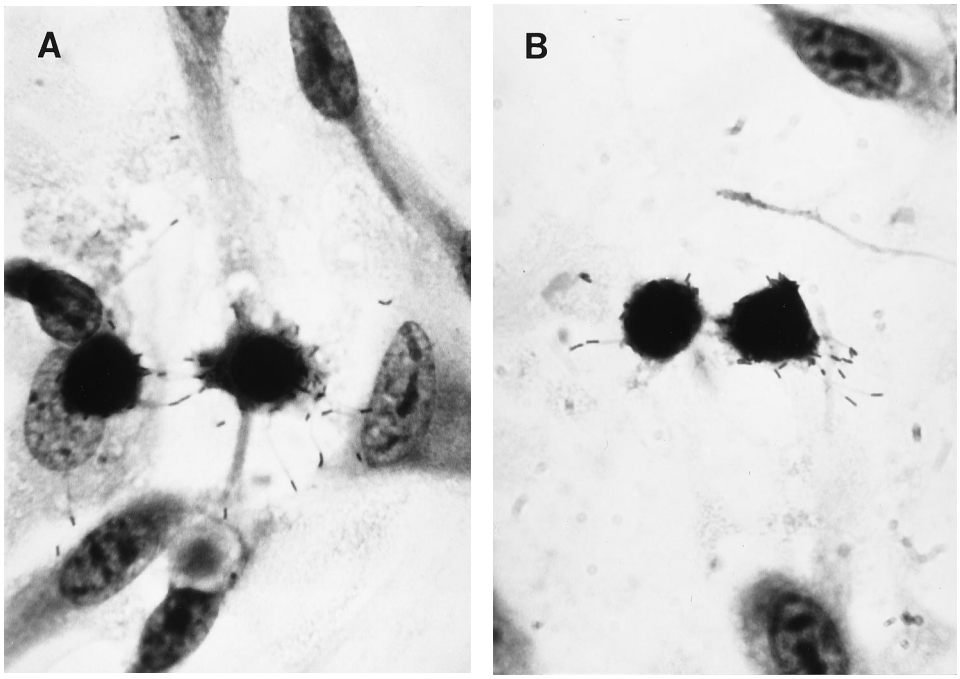

FIG. 6. Transfer of *L. monocytogenes* from mononuclear phagocytes to endothelial cells. U937 cells were incubated with *L. monocytogenes* for 2 h, and then unbound bacteria were washed away and the cells were resuspended in medium containing 10 μg of gentamicin per ml. The phagocytes were cultured on HUVEC monolayers for 3 h and then stained with Diff-Quik. Panels A and B show two different fields in which listeriae are clearly seen projecting away from the U937 phagocytes. Final magnification, approximately ×800.

these cells were cultured for an additional 12 to 18 h (Fig. 7), listeriae were found in HUVEC distant from U937 cells and had infected the entire monolayer, presumably by cell-to-cell spread between endothelial cells. Staining for F-actin showed numerous listeriae within the endothelial cells (Fig. 8), demonstrating the intracellular location of bacteria. Similar results were obtained when the human microvascular endothelial cell line HMEC-1 was used in place of HUVEC, or when infected PBMC were used in place of U937 cells to donate organisms to endothelial cells. These data indicate that *L. monocytogenes* which had been phagocytosed by U937 cells and PBMC invaded the endothelial cells.

The preceding experiments suggested that the *L. monocytogenes* which were transferred from phagocytes to the endothelial cell monolayer were viable and replicating. To confirm this, we measured bacterial CFU during incubation of infected phagocytes with endothelial cells. To account for bacterial replication in the phagocytes themselves, CFU of listeriae were measured in infected U937 cells and PBMC added to wells without endothelial cells. Figure 9 shows the results of two of six separate experiments in which infected U937 cells or PBMC were incubated with HUVEC, with HMEC, or on tissue culture plastic only. When infected phagocytes were incubated with endothelial cells, CFU of listeriae increased by 10- to 50-fold. By comparison, when infected phagocytes were cultured in the absence of endothelial cells there was net bacterial death as shown by declining CFU. This bactericidal activity probably was the result of lysis of infected cells and then

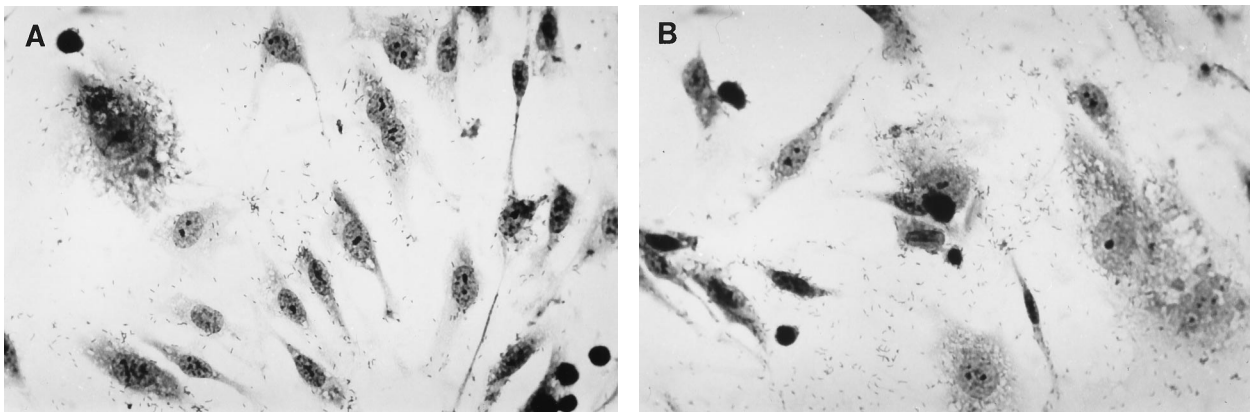

FIG. 7. Parasitism of HUVEC monolayers by *L. monocytogenes* from infected phagocytes. U937 cells were infected with listeriae as for Fig. 6, cultured with HUVEC monolayers for 19 h, and then stained with Diff-Quik. Panels A and B are from two different fields showing dissemination of bacteria throughout the endothelial cell monolayer. The small dark round cells are adherent U937 cells. Final magnification, approximately ×300.

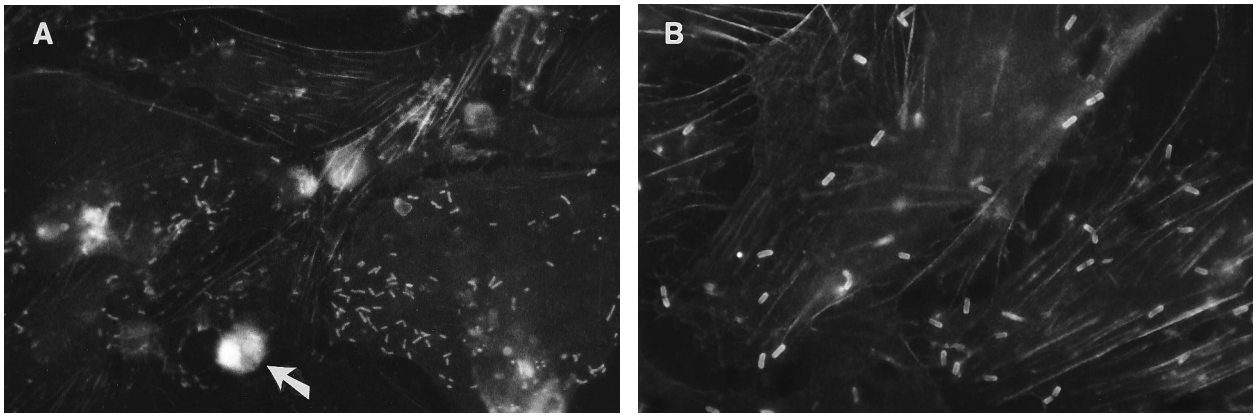

FIG. 8. F-actin-coated *L. monocytogenes* within HUVEC after transfer from U937 cells. Infected U937 cells were cultured on HUVEC monolayers for 24 h as for Fig. 7. The cells were fixed and stained for F-actin and then evaluated with fluorescence microscopy. Numerous F-actin-coated listeriae are seen throughout the HUVEC monolayer. U937 cells are present out of the plane of focus (arrow). Final magnification, approximately ×300 (A) and ×700 (B).

gentamicin killing of the listeriae released into the medium. With infected PBMC, there was sterilization of the culture in the absence of endothelial cells. This probably reflects the combined effects of gentamicin and monocyte bactericidal activity (12). Thus, the increase in CFU of *L. monocytogenes* in cultures of phagocytes and endothelial cells was not due to bacterial replication in the phagocytes. Rather, these data confirm the results of the microscopy experiments indicating that the *L. monocytogenes* transferred to endothelial cells were viable and grew within the endothelial cell monolayer.

The final series of experiments asked whether transfer of *L. monocytogenes* from infected phagocytes to endothelial cells required productive parasitism of the phagocyte by *L. monocytogenes*. For this, we infected U937 cells with wild-type *L. monocytogenes* or with two listeriolysin O-negative *L. monocytogenes* mutants, ATCC 43250 and CNL 85/162, which cannot escape phagosomes (20, 43). Figure 10 shows that productive infection of the endothelial cells by the two hemolytic *Listeria* strains, EGD and CNL 85/163, was accompanied by a 100- to 200-fold increase in bacterial CFU. In contrast, there was no increase in CFU of bacteria with the nonhemolytic *L. monocytogenes* mutants, ATCC 43250 and CNL 85/162. This suggests that the nonhemolytic bacteria did not establish infection in the endothelial cells. Microscopy of endothelial cell monolayers after 19 h of incubation with U937 cells infected with nonhemolytic *L. monocytogenes* mutants showed that the bac-

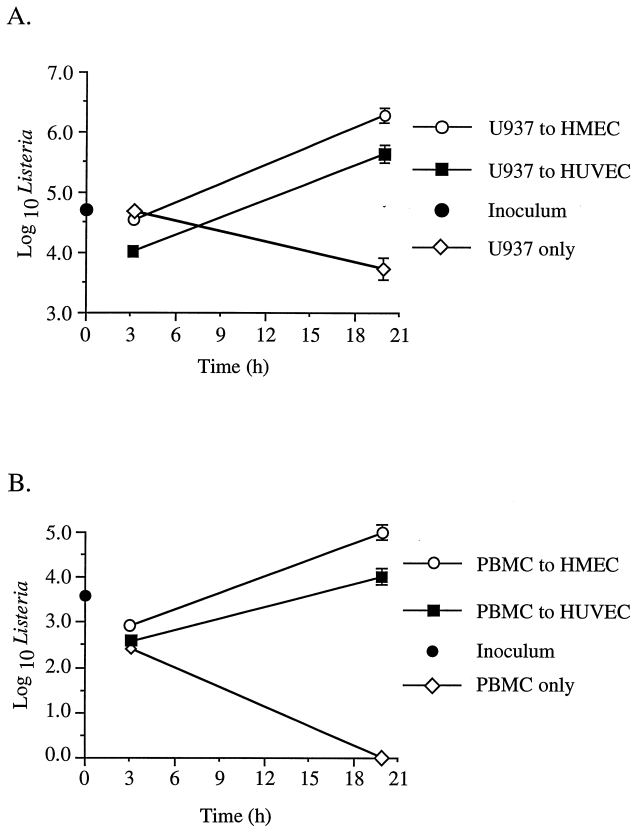

FIG. 9. Intracellular growth of *L. monocytogenes* in endothelial cells following transfer from infected mononuclear phagocytes. U937 cells (A) or peripheral blood monocytes (B) were infected with listeriae and then cultured on HUVEC or HMEC monolayers or on tissue culture plastic without endothelial cells. The inoculum represents CFU of listeriae per well present when the phagocytes were first cultured with the endothelial cells. At the indicated time, the monolayers in triplicate wells were washed, the cells were lysed, and bacterial CFU were determined by serial dilution and plating. The mean ± standard deviation CFU of listeriae from two of six similar experiments with identical results are shown.

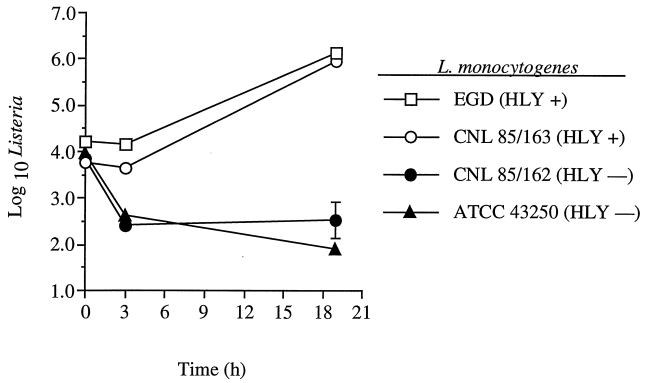

FIG. 10. Hemolytic but not nonhemolytic *L. monocytogenes* can invade endothelial cells from infected phagocytes. U937 cells were infected with the hemolytic *L. monocytogenes* strains EGD and CNL 85/163 or the nonhemolytic *L. monocytogenes* mutants ATCC 43250 and CNL 85/162 as for Fig. 9. They were then cultured on HMEC monolayers, and CFU of listeriae were determined in triplicate wells at the indicated time. The mean ± standard deviation CFU of listeriae from one of two similar experiments with identical results are shown.

teria did not spread within the endothelial cells and were rarely found (not shown). These findings support the idea that transfer of *L. monocytogenes* from phagocytes to endothelial cells required escape of the bacteria from the phagosome to the cytoplasm.

DISCUSSION

Endothelial cells are early and active participants in the nonspecific inflammatory response to blood-borne microorganisms. However, they are also targets for microbial invasion and parasitism. *L. monocytogenes* is a facultative intracellular bacterium that causes sepsis and invasive central nervous system disease in humans and in some domestic animals (6, 23, 25, 34). These observations indicate that blood-borne *L. monocytogenes* may target endothelial cells as a primary site for initial infection of the host and suggest that infection of these cells may be an important mechanism in the pathogenesis of central nervous system invasion by this bacterium. However, it is unknown whether *L. monocytogenes* can invade and parasitize endothelial cells, and the routes by which endothelial cell invasion can occur are also unknown. The purpose of the experiments described here was to test this and identify the infective routes.

The initial experiments demonstrated that *L. monocytogenes* grew within HUVEC. Evidence for this was obtained by measuring CFU of listeriae in the presence of a bactericidal concentration of gentamicin. There were nearly 3.3 bacterial doublings over 3 h, indicating a doubling time of approximately 55 min, similar to that reported for *L. monocytogenes* in other cells (19, 43). Moreover, the intracellular life cycle of *L. monocytogenes* included phagosomal escape, cytoplasmic replication, and F-actin-based intracellular motility and was the same in endothelial cells, including a mouse microvascular endothelial cell line (data not shown), as reported in other cell types.

We investigated the role of the *L. monocytogenes* invasion protein, internalin, in binding and invasion of endothelial cells in the presence and absence of serum. An internalin AB-negative mutant of *L. monocytogenes* that was unable to bind or invade epithelial cells in vitro (18) and wild-type *L. monocytogenes* treated with anti-internalin A MAb had reduced binding to HUVEC in the absence of serum. Furthermore, we found that expression of internalin is required for efficient invasion of endothelial cells by *L. monocytogenes* and that this requirement is independent of serum. That internalin-negative mutants could invade endothelial cells at all is somewhat different from the results of Gaillard et al., who found them unable to invade the Caco-2 epithelial cell line (18). This difference may well reflect the fact that endothelial cells, in contrast to normally nonphagocytic epithelial cells, usually express some phagocytic activity (46). Alternatively, other internalin proteins encoded by the *inl* gene family (13a, 18) or noninternalin proteins such as the p60 protein (31) may mediate entry into endothelial cells. In the presence of serum, proteins may be acting as bridging molecules to promote binding of listeriae to endothelial cells, similar to the situation with *S. aureus* (8). Thus, there appears to be separation of binding and invasion mechanisms. It is possible that there are several receptors for the binding of *L. monocytogenes* to cells but that not all binding mechanisms lead to cell invasion.

Intracellular growth of bacteria in endothelial cells is not common, and growth of *L. monocytogenes* in endothelial cells was unknown. Previously, only *R. rickettsii* has been shown to replicate within endothelial cells in vitro (48). There is some evidence that *B. bacilliformis* and *Rochalimaea* species may inhabit endothelial cells in vivo (2, 21, 30), and the spirochetes *Treponema pallidum* and *Borrelia burgdorferi* appear to penetrate and pass through endothelial cells, but intracellular replication has not been demonstrated (11, 50). Other bacteria including *S. aureus*, group B streptococcus, and *P. aeruginosa* can enter endothelial cells, where they persist without demonstrable intracellular multiplication (24, 42, 53). Then, after a variable period of time, usually several hours, bacterial CFU begin to decrease. Whether declining intracellular bacterial viability is from intracellular starvation or bactericidal activity on the part of the endothelial cell or is a result of culturing the cells in antibiotics is not clear. However, the innate phagocytic activity of endothelial cells and the ability of certain bacteria to persist within them may be important components of the pathogenesis of intravascular infections.

Only a few bacterial proteins that are involved in binding and/or invasion of endothelial cells have been identified. The OspA protein of *B. burgdorferi* is the only bacterial protein previously shown to mediate binding directly to endothelial cells, as a MAb against OspA inhibits binding of *B. burgdorferi* to HUVEC (10). A series of studies with *S. aureus* by Cheung et al. (7–9) show that extracellular matrix protein-binding protein(s) encoded by the *sar* locus is important for *Staphylococcus* binding to endothelial cells. This is due to the ability of staphylococci to bind fibrinogen, which then acts as a bridging molecule between the bacteria and endothelial cells. Other work suggests that binding of group B streptococci and *Streptococcus pneumoniae* to HUVEC is mediated by components of the bacterial cell wall, but the specific molecules involved have not been identified (22, 24).

Infection of endothelial cells by pathogens such as bacteria, fungi, and viruses is thought to be an important aspect of their ability to cause disease (4). Bacterial pathogens usually infect endothelial cells directly by first binding to their surface and then invading them in a parasite-directed phagocytosis process. However, the ability of *L. monocytogenes* to grow within mononuclear phagocytes suggested the possibility of a second mechanism by which *L. monocytogenes* might infect vascular endothelial cells, specifically, cell-to-cell transfer of *L. monocytogenes* from infected phagocytes to endothelial cells. To test this hypothesis, we cocultured listeria-infected U937 cells or peripheral blood monocytes with endothelial cells. In these experiments, microscopy demonstrated infection of the endothelial cell monolayer, and CFU of listeriae increased more than 10-fold. By comparison, *L. monocytogenes* infection could not be sustained when infected phagocytes were cultured in the absence of endothelial cells, probably because listeriae released from lysed phagocytes were killed by the gentamicin in the medium. Furthermore, nonhemolytic *L. monocytogenes* mutants appeared to be unable to infect the endothelial cells, presumably because they could not establish productive infection in the phagocytes. These data support the conclusions that infected phagocytes can transport *L. monocytogenes* to endothelial cells and that these bacteria invaded endothelial cells via the phagocyte. In a host, monocytes that have phagocytosed blood-borne *L. monocytogenes* but which cannot kill them could play a role in bacterial dissemination. Along these lines, it is interesting to note that corticosteroids are a predisposing factor for human infection by *L. monocytogenes* and also inhibit monocyte listericidal activity (47). Moreover, studies by others have shown that listeriae infect hepatocytes (26). To arrive at this anatomical site, bacteria must enter the hepatocytes via invasion of the sinusoidal endothelium, which provides an anatomical barrier to the direct invasion of hepatocytes. Alternatively, as suggested by the present study, sinusoidal endothelium may be infected by *L. monocy-*

togenes from monocytes which subsequently infect hepatocytes. By extrapolation, based on data presented here, we hypothesize that the brain parenchyma or the meninges might also become infected via parasitized vascular endothelium.

There are other instances in which microbes use parasitized host cells to reach specific anatomic sites. For example, *P. falciparum*-infected erythrocytes localize to the cerebral vasculature by binding to several surface ligands including CD36, ICAM-1, VCAM-1, and ELAM-1 on the postcapillary endothelium (3, 5, 35–37). In a different model, visna virus harbored in peripheral blood monocytes crosses the blood-brain barrier during normal phagocyte migration through the central nervous system and then infects the brain parenchyma (39). A similar Trojan horse mechanism has been suggested for HIV infection of the central nervous system (39, 40).

In summary, we present evidence that *L. monocytogenes* can parasitize human endothelial cells in vitro by two different mechanisms: direct invasion or through an intermediary phagocyte. We show that the internalin protein of *L. monocytogenes* plays a major role in binding and invasion of endothelial cells. These data suggest that invasion of endothelial cells by *L. monocytogenes* could be an important event in the pathogenesis of invasive *Listeria* infections. Our data support the notion that infected mononuclear phagocytes can be vectors by which intracellular bacterial pathogens avoid host defenses and move from the bloodstream of a host into immunologically privileged compartments such as the central nervous system.

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