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In Vitro Model of Penetration and Intracellular Growth of *Listeria monocytogenes* in the Human Enterocyte-Like Cell Line Caco-2

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Penetration and replication of *Listeria monocytogenes* within intestinal epithelial cells were studied by infecting the human enterocyte-like cell line Caco-2. Entry was due to directed phagocytosis, as suggested by the inhibiting effect of cytochalasin D on bacterial entry and by electron microscopy showing bacteria inside membrane-limiting vacuoles at the early stage of infection. Only bacteria from pathogenic species (*L. monocytogenes* and *Listeria ivanovii*) were able to induce their own phagocytosis by Caco-2 cells, as opposed to *Listeria seeligeri*, *Listeria welshimeri*, and *Listeria innocua*. *L. monocytogenes* multiplied readily within Caco-2 cells, with an apparent generation time of about 90 min. Listeriolysin O was found to be a major factor promoting intracellular growth of *L. monocytogenes*. After being internalized at the same rate as that of its hemolytic revertant strain, a nonhemolytic mutant from *L. monocytogenes* failed to replicate significantly within Caco-2 cells. Electron microscopic study demonstrated that bacteria from the nonhemolytic mutant remained inside phagosomes during cellular infection, whereas hemolytic bacteria from *L. monocytogenes* were released free within the cytoplasm. This indicates that disruption of vacuole membranes by listeriolysin O-producing strains of *L. monocytogenes* might be a key mechanism allowing bacteria to escape from phagosomes and to multiply unrestricted within cell cytoplasm.

Listeria monocytogenes is a gram-positive bacterium responsible for perinatal infections, septicemia, and meningoencephalitis in humans and many animal species (9). Although conjunctival, nasal, and respiratory routes have occasionally been mentioned as either natural or artificial means of exposure (1, 13, 17, 18), there is now evidence that the intestine is the usual site of entry of this microorganism. Gastrointestinal symptoms frequently occur at the onset of illness, and recent outbreaks of listeriosis were clearly shown to be associated with the ingestion of contaminated food, principally dairy produce (5, 14, 29). Experimental works also support this view, since animals inoculated orally with high doses of *L. monocytogenes* (10^8 to 10^9 bacteria in conventional mice) regularly develop a systemic illness, with bacterial spreading to the spleen and liver (3, 15). However, there are conflicting data about the primary site of intestinal tissue invasion by *L. monocytogenes*. An electron microscopic study has shown that oral administration of *L. monocytogenes* in guinea pigs resulted in infection of epithelial cells, predominantly in the small intestine (23). On the other hand, it has been found that *L. monocytogenes* given orally to mice penetrated into the Peyer's patches and not into the intestinal villi (15). This discrepancy partially reflects the difficulty in investigating bacterial invasion of intestinal tissues during experimental infection. Moreover, these in vivo studies did not allow a precise analysis of bacterial virulence factors acting at the cellular level. The in vitro infection of intestinal epithelial cells is therefore an attractive approach in studying the interaction of *L. monocytogenes* with enterocytic cells.

It has been established by G. B. Mackaness that the virulence of *L. monocytogenes* is due to the capacity of this microorganism to grow within macrophages (16). After this finding, most studies devoted to the pathogenicity of *L. monocytogenes* have focused on the behavior of this bacterium within professional phagocytes, especially macrophages. However, it has been reported that *L. monocytogenes* invaded cells of corneal, conjunctival, pulmonary, urinary bladder, and intestinal epithelia during experimental infections (22–24), suggesting that *L. monocytogenes* enters and multiplies within nonprofessional phagocytes. This phenomenon could be of crucial importance in explaining some aspects of the invasion process of *L. monocytogenes*, including penetration into the host and infection of target tissues. Moreover, the elucidation of molecular mechanisms promoting intracellular replication of *L. monocytogenes* within nonprofessional phagocytes should provide some insight into the ability of this bacterium to grow within professional phagocytes, especially macrophages and related cells.

For all these reasons, an in vitro model of infection of intestinal epithelial cells by *L. monocytogenes* was developed in the present work. We used the human colon carcinoma cell line Caco-2, which displays the remarkable property of expressing typical enterocytic differentiation under standard culture conditions (27). It is shown in this study that, unlike nonpathogenic species of the genus *Listeria*, *L. monocytogenes* was able to enter enterocyte-like Caco-2 cells, by inducing its own phagocytosis, and to multiply within these cells. Bacterial growth within Caco-2 cells depended on the secretion of a hemolytic factor recently characterized and termed listeriolysin O (8). This opinion is supported by the finding that a nonhemolytic mutant obtained from *L. monocytogenes* by transposon mutagenesis (7) was unable to multiply significantly within Caco-2 cells. It is concluded that infection of enterocyte-like Caco-2 cells appears to be a reliable in vitro model of pathogenesis.

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TABLE 1. Bacterial strains

Strain	Serovar	Hemolysin production[a]	Source or reference
L. monocytogenes			
EGD	1/2a	+	Trudeau Institute
CNL 85/162 (Hly$^-$)[b]	1/2a	−	Obtained by insertion of the transposon Tn*1545* into the genome of a spontaneous streptomycin-resistant mutant from EGD (7)
CNL 85/163 (Hly$^+$)[b]	1/2a	+	Spontaneous revertant from mutant Hly$^-$ by loss of Tn*1545* (7)
CNL 86/087[b]	1/2a	+	Clinical isolate from Hôpital Necker-Enfants Malades, Paris
CNL 85/08[b]	1/2a	+	Clinical isolate from Hôpital Necker-Enfants Malades, Paris
SLCC 5156	1/2a	+	Special *Listeria* Culture Collection, Würzburg, Federal Republic of Germany (19)
SLCC 3551	4b	+	Special *Listeria* Culture Collection, Würzburg, Federal Republic of Germany (19)
L. ivanovii CIP 7842	5	+	Collection de l'Institut Pasteur, Paris
L. seeligeri CIP 100100	1/2a	+	Collection de l'Institut Pasteur, Paris
L. welshimeri CIP 8149	6a	−	Collection de l'Institut Pasteur, Paris
L. innocua CIP 8011	6a	−	Collection de l'Institut Pasteur, Paris

[a] On 5% horse blood–tryptic soy agar.
[b] Strain deposited in the collection of the Centre National des *Listeria*, Nantes, France.

MATERIALS AND METHODS

Bacteria. The bacterial strains used in this study are shown in Table 1. The nonhemolytic (Hly$^-$) mutant (CNL 85/162) from *L. monocytogenes* differed from its hemolytic (Hly$^+$) revertant (CNL 85/163) only with respect to the production of an inactive, 52-kilodalton truncated listeriolysin O (C. Geoffroy and J. L. Gaillard, unpublished data). All strains demonstrated similar growth rates in tryptic soy broth (Diagnostics Pasteur, Marnes La Coquette, France), and their susceptibilities to gentamicin were comparable (MICs ranging from 0.12 to 1 mg/liter). Hemolysin production was tested on 5% horse blood–tryptic soy agar (Diagnostics Pasteur). Bacteria were grown in tryptic soy broth, harvested in log-phase growth (~10^8 bacteria per ml), and stored in 1-ml aliquots at −70°C until required. For each experiment, 1 ml of the frozen stock was rapidly thawed and inoculated in 10 ml of tryptic soy broth. After 4 h of incubation at 37°C, bacteria were washed once in phosphate-buffered saline and then used at the appropriate dilution in Dulbecco modified Eagle minimum essential medium (DMEM) (GIBCO Laboratories, Inc., Grand Island, N.Y.) to infect Caco-2 cells. Viable bacteria were determined by plating 0.1-ml quantities of serial dilutions on tryptic soy agar (Diagnostics Pasteur). CFUs were counted after 24 h of incubation at 37°C.

Culture of Caco-2 cells. The human colon carcinoma cell line Caco-2, established by J. Fogh in 1974 (6), was kindly provided by A. Zweibaum and M. Rousset (Institut National de la Santé et de la Recherche Médicale U178, Villejuif, France). The cell line, used between passages 76 and 90, was cultured as described previously (20). The culture medium was DMEM supplemented with 20% fetal bovine serum (GIBCO) and 1% nonessential amino acids (Flow Laboratories, Inc., McLean, Va.). Caco-2 cells were routinely cultured in 25-cm^2 plastic tissue culture flasks (Corning Glass Works, Corning, N.Y.) at 37°C in a humidified atmosphere of 5% CO_2 in air. Confluent cell monolayers were trypsinized and adjusted to a concentration of 2.5×10^5 cells per ml in culture medium. One milliliter of the cell suspension was dispensed into each 35-mm plastic tissue culture dish (Corning) and then incubated for 48 to 72 h to obtain a semiconfluent monolayer (~5×10^5 cells per culture dish). Cell monolayers were washed once with nonsupplemented DMEM before infection.

Infection of Caco-2 cells. The semiconfluent monolayers of Caco-2 cells were routinely inoculated with bacterial suspensions adjusted to obtain a multiplicity of infection (MOI) of 100 bacteria per cell. Penetration was allowed to proceed for 1 h at 37°C. Infected cells were then washed three times with Earle balanced salt solution (EBSS) (GIBCO) and covered with 2 ml of DMEM containing gentamicin at bactericidal concentration (5 mg/liter) to kill extracellular bacteria. Cells were incubated for an additional period of 18 h, the starting point of which was defined as time zero. At various times during this 18-h period, cells were washed three times with EBSS and lysed by cold distilled water before viable intracellular bacteria were counted. Experiments were carried out in duplicate and repeated three times for each bacterial strain tested. Results were expressed as the mean $\log_{10}$ viable bacteria per well. In some experiments, semiconfluent monolayers were infected at a lower MOI (1 and 10 bacteria per cell). The procedure was similar to that described above, and the course of infection was followed over an 8-h period.

Assay for detachment of infected Caco-2 cells. Caco-2 cells were labeled for 18 h before infection in a culture medium containing 0.5 μCi of [^{3}H]uridine (Amersham Corp., Buckinghamshire, England) per ml. Radiolabeled cells were then washed three times with EBSS, and infection was performed as described above. At intervals during the 18-h period of infection, monolayers were washed three times with EBSS, and adherent Caco-2 cells were lysed with 1 ml of cold distilled water. A 100-μl portion of lysates was then precipitated into 900 μl of 5% trichloroacetic acid and passed through a GF/C filter (Whatman Ltd., Maidston, England). Filters were then washed three times with 5% trichloroacetic acid and baked at 80°C for 30 min before being soaked for counting in an NCS/OCS (1:9) preparation. The percentage of adherent cells during the infection was determined by calculating the ratio of the residual radioactivity of infected cells to the residual radioactivity of noninfected cells throughout the experiment. Experiments were carried out in triplicate and repeated at least two times for each bacterial strain. Results were expressed as the mean percentage of adherent Caco-2 cells per well.

Treatment by cytochalasin D. Monolayers of Caco-2 cells were incubated before infection for 1 h with various concentrations of cytochalasin D (Sigma Chemical Co., St. Louis, Mo.). Routine infection procedure was then performed as

described, except that bacterial suspensions contained the original concentration of cytochalasin D. Viable bacteria were determined at 0 and 2 h postinoculation.

Electron microscopy. Semiconfluent cell monolayers were infected at an MOI ranging from 20 to 40 bacteria per cell according to the procedure described above. Samples were taken up at 0 and 4 h postinoculation. Infected cells were fixed for 1 h at 4°C with 2.5% glutaraldehyde in phosphate buffer (0.1 M, pH 7.4). They were then scraped off the surface of culture dishes, washed, and rinsed overnight at 4°C in the same buffer. Postfixation was performed in 2% osmium tetroxide for 1 h. Cells were then dehydrated and embedded in Epon. Serial ultrathin sections were collected on 150-mesh grids and then counterstained with uranyl acetate and lead citrate. Sections were observed on a Philips EM 300 transmission electron microscope at 80 kV. Most of the morphological studies were qualitative, but the capacity of hemolytic and nonhemolytic bacteria from *L. monocytogenes* to induce lysis of phagocytic vacuole membranes was compared quantitatively. For each strain, 100 to 300 bacteria localized within 100 to 300 distinct cells were examined at 0 and 4 h postinoculation for their location within the cell cytoplasm or inside phagosomes.

RESULTS

Entry of *L. monocytogenes* into Caco-2 cells and intracellular growth. The capacity of *L. monocytogenes* to enter the intestinal epithelial cells and to grow intracellularly was evaluated by using ar in vitro model of infection of the enterocyte-like Caco-2 cell line. Cell monolayers were infected for 1 h at 37°C with five strains of *L. monocytogenes* shown in Table 1 (EGD, CNL 86/087, CNL 85/08, SLCC 5156, and SLCC 3551), at an initial MOI of 100 bacteria per cell. After being washed extensively (time zero), infected cells were incubated for 18 h in culture medium supplemented with gentamicin at a bactericidal concentration (5 mg/liter), and the number of viable bacteria was determined at intervals. The kinetics of Caco-2 cell infection were very similar for the five strains tested (Fig. 1A). Initial bacterial counts (time zero) ranged from 2 to 4% of the inoculum. An early 2-h period of decrease in bacterial number was then observed, probably corresponding to the killing of extracellular cell-associated bacteria by gentamicin. This phase was followed by a 6-h period of rapid bacterial growth due to intracellular multiplication, preceding a slow decline in bacterial number during the remaining 10 h.

In fact, the kinetics of bacterial growth must be interpreted in view of the results of cellular survival during infection, estimated from the detachment of Caco-2 cells (Fig. 1B). Caco-2 cells were radiolabeled before inoculation, and the amount of radioactivity bound to the dish and the number of intracellular bacteria were assessed simultaneously over the 18-h period of infection. Cellular radiolabeling did not alter the kinetics of bacterial infection, which was similar to that illustrated on Fig. 1A (results not shown). The percentage of adherent cells progressively declined to 65 to 80% (depending on the strain tested) at 8 h and to 30 to 50% at the end of the experiment (Fig. 1B). This suggested that the rate of intracellular replication of *L. monocytogenes* was significantly underestimated during infection at an MOI of 100 bacteria per cell, since bacterial multiplication progressively induced detachment or lysis of Caco-2 cells.

The rate of intracellular growth of *L. monocytogenes* was accurately evaluated with strain EGD in Caco-2 cells infected at a lower MOI (1 and 10 bacteria per cell) over a limited period (8 h). In these conditions, no significant detachment of radiolabeled cells was observed during infection (data not shown). *L. monocytogenes* EGD multiplied readily within Caco-2 cells, with an 18- and 14-fold increase in bacterial number from 2 to 8 h at an MOI of 1 and 10 bacteria per cell, respectively (Fig. 2), as opposed to a fivefold increase found with the same strain at an MOI of 100 bacteria per cell (Fig. 1A). Therefore, the apparent generation time of *L. monocytogenes* within Caco-2 cells was estimated to be about 90 min.

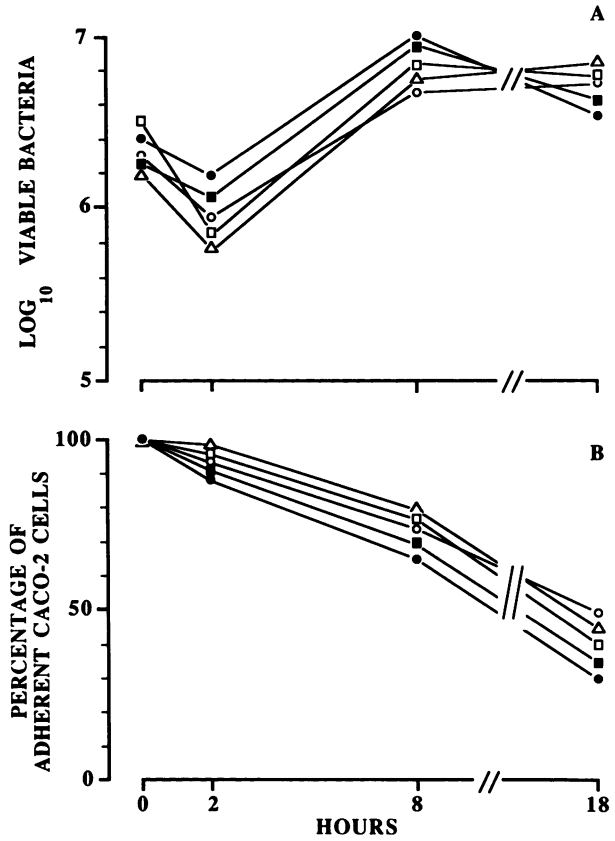

FIG. 1. Infection of Caco-2 cells with various strains of *L. monocytogenes*. The *L. monocytogenes* strains were EGD (●), CNL186/087 (■), CNL185/08 (○), SLCC15 56 (△), and SLCC13551 (□). The cells were infected with an MOI of 100 bacteria per cell. For experimental conditions, see Materials and Methods. (A) Bacterial growth within Caco-2 cells. Results are expressed as the mean $\log_{10}$ viable bacteria per well (mean of three determinations; standard deviation of ≤0.30). (B) Simultaneous assessment of detachment of radiolabeled Caco-2 cells. Results are expressed as the mean percentage of adherent Caco-2 cells per well (mean of two determinations; standard deviation of ≤0.15). The five strains of *L. monocytogenes* were able to enter Caco-2 cells and to replicate intracellularly. Significant cellular detachment occurred during infection, resulting in the underestimate of bacterial growth.

Directed phagocytosis of *L. monocytogenes* by Caco-2 cells. The next step was to study whether penetration of *L. monocytogenes* was due to phagocytosis by Caco-2 cells. Such a demonstration would imply that entry of bacteria requires the endocytic activity of Caco-2 cells, as well as a specific interaction between invasive bacteria and phagocytic cells. The first point was investigated by pretreating Caco-2 cells with cytochalasin D (0.1, 0.5, 1, and 5 μg/ml) before infection by *L. monocytogenes* (strain EGD) at an MOI of 100 bacteria per cell. Bacterial survival was then

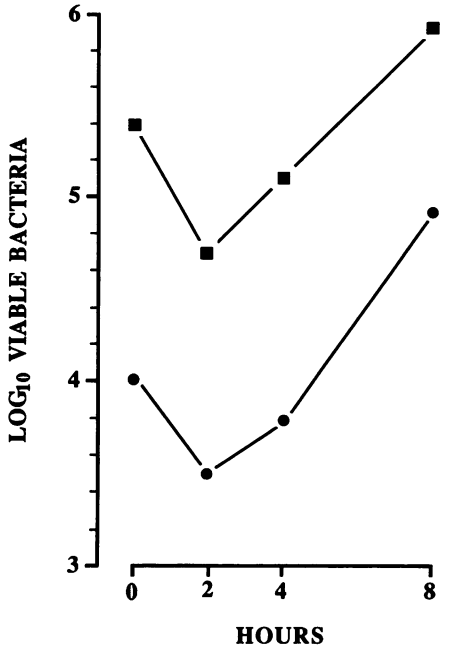

FIG. 2. Kinetics of intracellular growth of *L. monocytogenes* (strain EGD) in conditions avoiding detachment of Caco-2 cells, with an MOI of 1 (●) and 10 (■) bacteria per cell. For experimental conditions, see Materials and Methods. Results are expressed as the mean $\log_{10}$ viable bacteria per well (mean of three determinations; standard deviation of ≤0.30). *L. monocytogenes* multiplied readily within Caco-2 cells, with an apparent generation time of about 90 min.

followed for the next 2 h in the presence of gentamicin at a bactericidal concentration. Entry of bacteria was markedly reduced by increasing doses of cytochalasin D (Fig. 3). After a 2-h incubation, when most extracellular bacteria have been eliminated by gentamicin, the number of cell-associated bacteria per well dropped to about 10^4 with the highest concentrations of cytochalasin D (1 and 5 μg/ml), compared with about 10^6 with nontreated Caco-2 cells.

The existence of a specific interaction between invasive bacteria and Caco-2 cells was investigated by infecting cell monolayers with pathogenic and nonpathogenic *Listeria* species at an MOI of 100 bacteria per cell. Kinetics of infection and detachment of radiolabeled Caco-2 cells were followed over an 18-h period (Fig. 4). *Listeria ivanovii*, a pathogenic species producing a streptolysin O-related hemolysin (19), displayed a pattern of infection very similar to that of *L. monocytogenes* (Fig. 4A), although cellular detachment was induced more slowly than during infection with *L. monocytogenes* (Fig. 4B). In contrast, it was found that the nonpathogenic *Listeria* species (*L. seeligeri*, *L. welshimeri*, and *L. innocua*) totally failed to invade Caco-2 cells. This was evidenced by very low counts of residual bacteria observed after 2 h of infection, corresponding to about 0.01% of the initial inoculum (Fig. 4A). These bacteria did not induce cellular detachment during the 18-h period of infection (Fig. 4B). Interestingly, only with the hemolytic nonpathogenic species (*L. seeligeri*) was it found that the rarely observed intracellular bacteria were capable of rapid multiplication from 8 to 18 h, with a 100-fold increase in bacterial number (Fig. 4A). These bacteria remained sensitive to gentamicin.

Restriction of intracellular growth of a nonhemolytic mutant from *L. monocytogenes*. It has recently been suggested that hemolysin (listeriolysin O) might be an important factor promoting intracellular growth of *L. monocytogenes*, as indicated by loss of virulence of nonhemolytic mutants from *L. monocytogenes* obtained by transposon mutagenesis (7, 11). This view is in agreement with the above results showing that only the hemolytic *Listeria* species (*L. monocytogenes*, *L. ivanovii*, and *L. seeligeri*) could replicate within Caco-2 cells. The role of listeriolysin O in intracellular multiplication was further studied in this work by infecting Caco-2 cells with a nonhemolytic mutant from *L. monocytogenes* or with its hemolytic revertant strain.

In the first series of experiments, the two bacterial strains were tested separately. Caco-2 cells were infected with microorganisms at an initial MOI of 10 bacteria per cell for 1 h, and viable intracellular bacteria were then counted at regular intervals during an 8-h period. The kinetics of bacterial growth are illustrated in Fig. 5A. The hemolytic revertant strain multiplied intracellularly at the same rate as strain EGD, with a 13-fold increase in bacterial number from 2 to 8 h. Conversely, the nonhemolytic mutant almost completely lost its ability to grow within Caco-2 cells, since only a twofold increase in bacterial count was found during the same period. The number of hemolytic bacteria at 0 h was already higher than that of nonhemolytic bacteria, probably because intracellular hemolytic bacteria already initiated their replication during the 1-h incubation period. In the second series of experiments, Caco-2 cells were infected by a mixture of the two strains at an MOI of 20 bacteria (12

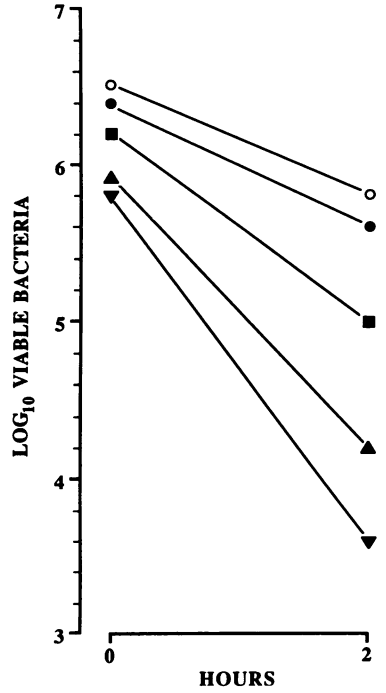

FIG. 3. Penetration of *L. monocytogenes* (strain EGD) into Caco-2 cells treated with cytochalasin D. The cells were treated with 0.1 (●), 0.5 (■), 1(▲), or 5 (▼) μg of cytochalasin D per ml; the controls (○) were not treated. The MOI was 100 bacteria per cell. For experimental conditions, see Materials and Methods. Results are expressed as the mean $\log_{10}$ viable bacteria per well (mean of three determinations; standard deviation of ≤0.25). Entry of *L. monocytogenes* was inhibited by treatment of Caco-2 cells with cytochalasin D at concentrations of ≥0.5 μg/ml.

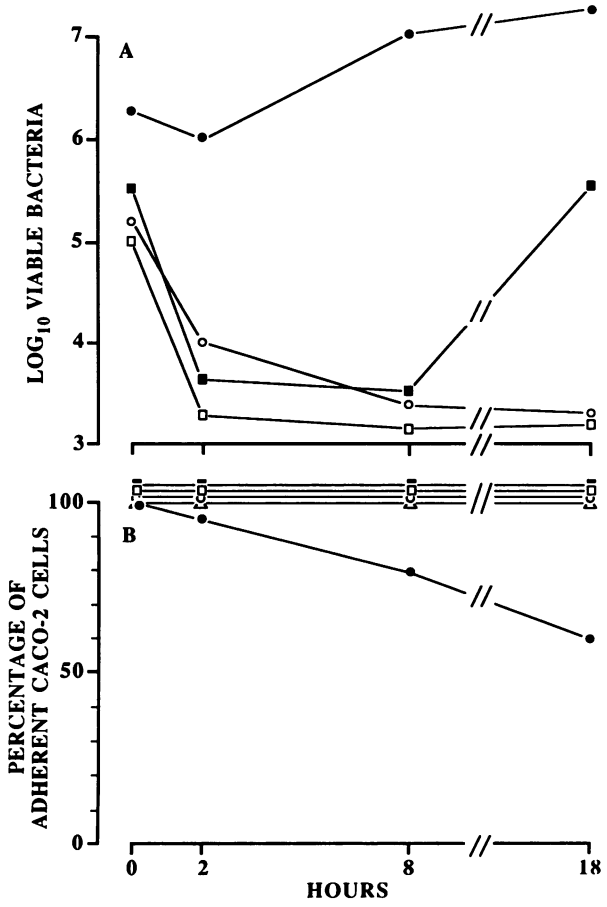

FIG. 4. Infection of Caco-2 cells with various species belonging to the genus *Listeria*. *L. ivanovii* CIP 7842 (●), *L. seeligeri* CIP 100100 (■), *L. welshimeri* CIP 8149 (□), and *L. innocua* CIP 8011 (○) with an MOI of 100 bacteria per cell were used. For experimental conditions, see Materials and Methods. (A) and (B) See the legend to Fig. 1. The various species of *Listeria* exhibited quite different patterns of infection.

Hly^-/8 Hly^+) per cell, allowing us to obtain the same number of both hemolytic and nonhemolytic bacteria within monolayers after 1 h of incubation (time zero). Again it was found that nonhemolytic bacteria failed to grow within Caco-2 cells despite the coinfection (Fig. 5B), suggesting that listeriolysin O could act as a promoting factor of intracellular multiplication within a restricted cellular compartment, probably remaining confined around the ingested bacteria.

Electron microscopic study of *L. monocytogenes*-infected Caco-2 cells. *L. monocytogenes* EGD was incubated with Caco-2 cells for 1 h at MOIs ranging from 20 to 40 bacteria per cell. Samples were taken after extensive washes at the end of this inoculation period (time zero) and 4 h later. At 0 h, rare bacteria could be seen in loose association with the cell membrane (Fig. 6A) or in the process of ingestion (Fig. 6B). During the process of entry, the cytoplasmic membrane in contact with bacteria appeared unchanged and bridging points were sometimes observed (Fig. 6A). Most cell-associated bacteria were intact and located within cell phagosomes (Fig. 6C), as verified by serial sections showing the endocytic nature of vacuoles. The ultrastructural aspect of infected Caco-2 cells was preserved at these early stages. After 4 h of infection, most bacteria were free within the cytoplasm (Fig. 6D), thus demonstrating the ability of *L. monocytogenes* to lyse the phagocytic vacuole membranes during the infectious process. Intracytoplasmic bacteria appeared undamaged. They were often dividing and surrounded by dense material (Fig. 6D). On the contrary, rare microorganisms still enclosed inside vacuoles were being destroyed (Fig. 5E), suggesting that Caco-2 cells were capable of bactericidal activity in the phagosomal compartment. The structure of bacterium-containing cells was generally preserved, but heavily infected cells were lysed and microorganisms were released into the extracellular environment (Fig. 6F). Similar results were found by infecting Caco-2 cells with *L. ivanovii* in the same conditions. Bacteria were seen inside phagosomes at the early phases of infection (time zero) but were outside phagosomes and replicating within the cytoplasm after 4 h of cellular infection (results not shown). Finally, when Caco-2 cells were infected with bacteria from nonpathogenic *Listeria* species (*L. innocua*, *L. seeligeri*, and *L. welshimeri*) at an MOI of 100 bacteria per cell, no intracellular microorganisms could be observed at 0 h postinoculation.

To visualize the mechanism(s) whereby listeriolysin O acts, electron microscopic study was further performed on Caco-2 cells infected with the nonhemolytic mutant from *L. monocytogenes* or with its hemolytic revertant strain at an

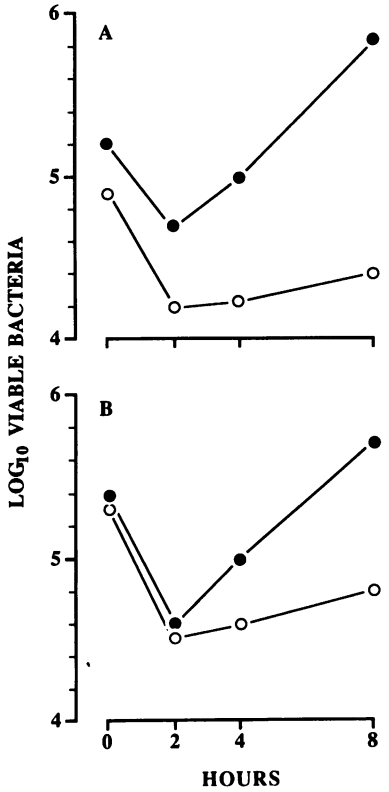

FIG. 5. Kinetics of intracellular growth of a nonhemolytic mutant from *L. monocytogenes* (○) and of its hemolytic revertant (●). For experimental conditions, see Materials and Methods. Results are expressed as the mean $\log_{10}$ viable bacteria per well (mean of three determinations; standard deviation of ≤0.25). (A) Separate infections (MOI, 10 bacteria per cell); (B) coinfection (MOI, 20 bacteria per cell at a 12:8 ratio of Hly^-/Hly^+). The nonhemolytic mutant from *L. monocytogenes* almost completely lost the ability to replicate within Caco-2 cells.

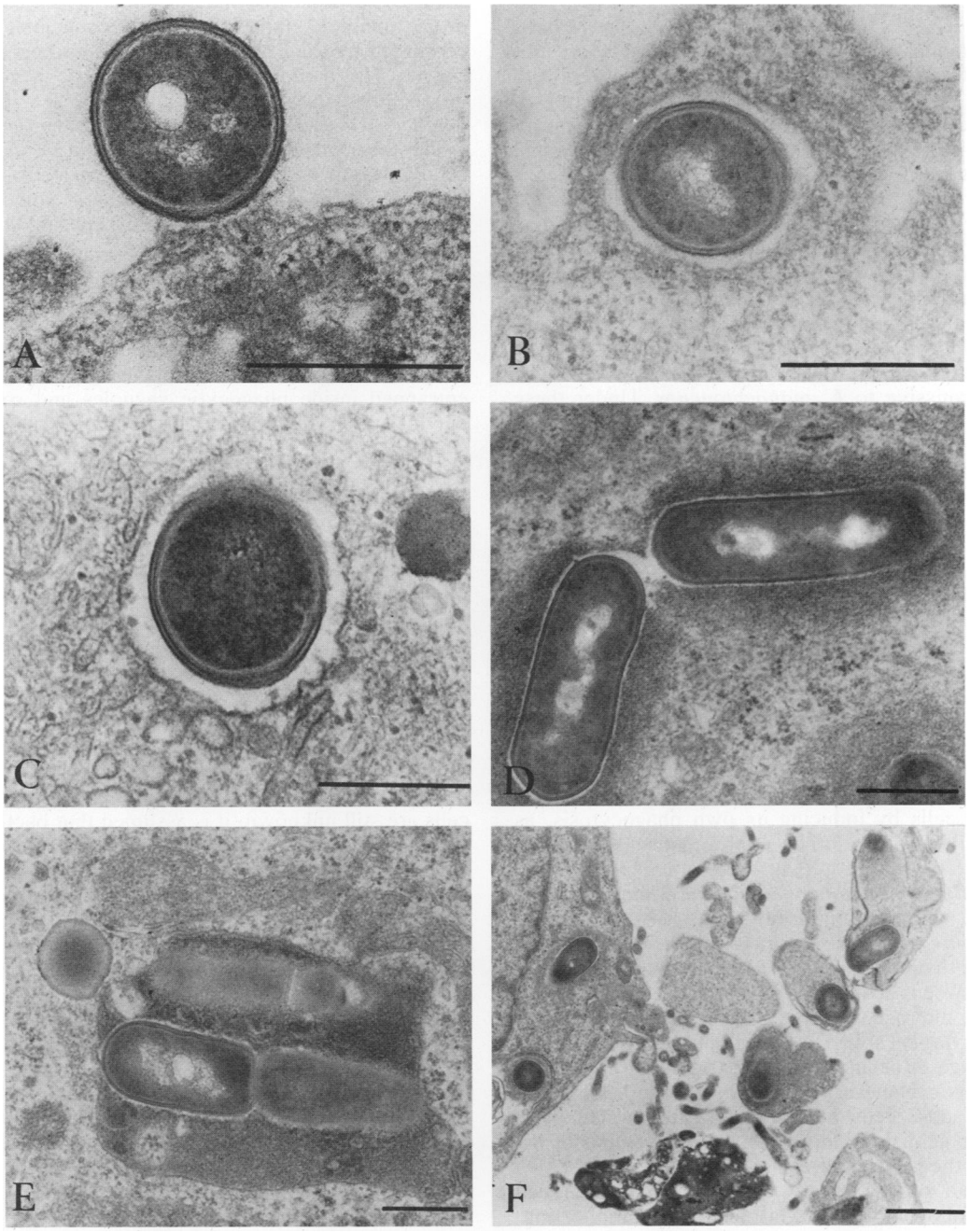

FIG. 6. Electron micrographs of Caco-2 cells infected with *L. monocytogenes* (strain EGD) at time zero (A, B, and C) and 4 h postinoculation (D, E, and F). (A) Cell-associated *Listeria* cells; (B) bacterium being internalized by induced phagocytosis; (C) bacterium inside phagocytic vacuole (membrane surrounding the phagocytic vacuole appears intact); (D) divided bacteria in the cytoplasm (note the dense material surrounding the microorganisms); (E) rarely observed, degenerating bacteria inside a phagolysosome; (F) lysis of a heavily infected cell with bacterial release. Bars, 0.5 (A through E) and 1.0 μm (F).

MOI of 20 to 40 bacteria per cell. For each strain, 100 to 300 bacteria were observed in 100 to 300 different Caco-2 cells during infection. At 4 h, 100% of bacteria from the nonhemolytic mutant appeared as single organisms located inside phagosomes. In contrast, as previously illustrated with strain EGD (Fig. 6), 90% of hemolytic bacteria from the revertant strain were free within the cytoplasm and 5% of dividing microorganisms were observed. On the other hand, it was confirmed by electron microscopy that the nonhemolytic mutant penetrated Caco-2 cells, as well as its hemolytic revertant strain (0.3 nonhemolytic bacterium per cell versus 0.4 hemolytic bacterium per cell at 0 h), but failed to multiply intracellularly (0.3 nonhemolytic bacterium per cell versus 3 hemolytic bacteria per cell at 4 h). The results of this quantitative electron microscopic study strongly suggest that listeriolysin O produced by internalized bacteria promotes intracellular growth by disrupting phagocytic vacuole membranes during the infectious process.

DISCUSSION

An in vitro model of infection of the human colon carcinoma cell line Caco-2 was developed in the present report to investigate the ability of *L. monocytogenes* to enter intestinal epithelial cells and to replicate intracellularly. As opposed to cell lines which originated from normal intestinal epithelium that fail to express any differentiation characteristics of mature enterocytes (21), these malignant cells are able to display many features of differentiated enterocytes, including a typical brush border containing enzymes observed in the small intestine (20, 27). For these reasons, the Caco-2 cell line appears suitable for investigating in vitro interaction of *L. monocytogenes*, as well as many other procaryotic cells, with enterocytes. We used in this study 48 to 72-h cultures of Caco-2 cells, which were revealed to be exquisitely permissive to *L. monocytogenes*. Under these conditions, it is generally assumed that Caco-2 cells mimic the dividing crypt enterocytes of the small intestine (27).

L. monocytogenes was capable of entering Caco-2 cells and of multiplying within these cells. The penetration rate of *L. monocytogenes* into Caco-2 cells could not be precisely estimated, since intracellular growth of early internalized bacteria did occur during the 1-h period of inoculation. On the other hand, bacteria found at 0 h postinoculation were both intra- and extracellular. It is, however, likely that penetration of *L. monocytogenes* into Caco-2 cells was moderate. Indeed, with an MOI of 20 to 40 bacteria per cell, it was found by quantitative electron microscopic study that about 1% of the infecting bacteria (0.3 to 0.4 bacterium per cell) were inside the cells at 0 h. Bacterial entry was greatly reduced when Caco-2 cells were treated with cytochalasin D (0.5, 1, and 5 μg/ml) (Fig. 3), suggesting that *L. monocytogenes* invaded cells by inducing its own phagocytosis, as previously evidenced for other microorganisms infecting cultured cells (10, 31). Moreover, nonpathogenic *Listeria* species (*L. seeligeri*, *L. innocua*, and *L. welshimeri*) failed to invade Caco-2 cells, as opposed to the two pathogenic species (*L. monocytogenes* and *L. ivanovii*). These results clearly indicate that entry of *Listeria* cells requires a specific interaction between invasive bacteria and enterocyte-like cells. Electron microscopic study confirmed that bacteria from *L. monocytogenes* were all inside phagocytic vacuoles at the early stage of cellular infection (time zero). The fact that nonhemolytic bacteria from *L. monocytogenes* could easily enter Caco-2 cells at the same rate as hemolytic bacteria unambiguously eliminates any involvement of listeriolysin O in the process of cellular invasion.

Internalization of *L. monocytogenes* into Caco-2 cell vacuoles was followed by rapid intracellular multiplication, resulting in cellular detachment or lysis in heavily infected cells (Fig. 1). When using low infecting doses of *L. monocytogenes*, which were unable to produce significant cellular detachment during the first 8 h of infection, the apparent generation time was estimated as about 90 min. By comparing this value with the 1-h generation time observed in vitro (16) and the 5-h generation time reported in mouse tissues during the early phase of infection (16), we found bacterial replication proceeds almost unrestricted in enterocyte-like cells. The role of listeriolysin O in the process of intracellular multiplication was clearly established by showing that a nonhemolytic mutant from *L. monocytogenes* failed to multiply significantly within Caco-2 cells, as opposed to its hemolytic revertant strain. Since these mutants only differed with respect to the secretion of a truncated, inactive listeriolysin O by the nonhemolytic mutant, this observation strongly suggests that listeriolysin O is an essential factor promoting intracellular growth. This conclusion was further confirmed by the observation that other hemolytic *Listeria* species (*L. ivanovii* and *L. seeligeri*) were capable of intracellular growth, although the exact nature of the hemolysin(s) secreted by *L. seeligeri* remains unknown.

The mechanism whereby listeriolysin O might act at the cellular level was visualized by electron microscopy. After 4 h of Caco-2 cell infection, most bacteria from *L. monocytogenes* EGD were free within the cytoplasm, suggesting that hemolytic bacteria lysed the phagosome membranes during the process of intracellular replication. This was also found with a strain of *L. ivanovii*, a species known to produce a streptolysin O-like hemolysin (19). Instead, nonhemolytic bacteria from *L. monocytogenes* remained confined in the phagosomal compartment, appearing as single organisms entrapped inside phagocytic vacuoles and sometimes displaying ultrastructural alterations after 4 h of infection. A similar strategy implying phagosome membrane lysis has been reported for other intracellular replicating pathogens. In *Shigella flexneri*, the membrane of the phagocytic vacuole is lysed within 1 h after entry, thus leading to rapid intracellular growth. A contact hemolytic activity has been proposed to account for such lysis (28). Molecular characterization of this contact hemolysin has not been presented as yet. In *Rickettsia tsutsugamushi* and *Rickettsia prowazekii*, similar lysis has been demonstrated (25, 31). Phospholipase A2 either from bacterial or host-cell origin may be involved (32). It would therefore appear that bacteria genera as different as *Listeria*, *Shigella*, and *Rickettsia* make use of different membrane-damaging systems to gain access to the cytoplasm.

Although the conditions inside phagosomes of Caco-2 cells are still unknown, it is assumed that bacterial multiplication of *L. monocytogenes* is restricted in these vacuoles by a hostile microenvironment, including low pH and presumably other microbicidal mechanisms. For instance, it is known that *L. monocytogenes* cannot replicate in vitro under pH 5.6 (9). Escaping from phagosomes might allow bacteria to find favorable conditions for growth in the cytoplasm (especially adequate pH and available iron required for *L. monocytogenes* multiplication [30]). In this connection, it is noteworthy that there is an inverse relationship between iron concentration and hemolysin production (4). It was reported long ago that hemolysin produced by internalized *Listeria* would disrupt phagocytic vacuole membranes of macrophages and related cells (2, 12). We infer therefore that listeriolysin O might be also a major factor promoting intracellular growth in professional phagocytic cells, as suggested in a recent work (7).

Although our observations must be cautiously extended to invasion of enterocytes in vivo, they are in full agreement with the results that Racz and co-workers obtained in experimental *Listeria* enteritis (23). Our data suggest that the enterocyte might be a target cell for entry of *L. monocytogenes* into the host, and even a primary replication site, where bacteria temporarily escape host defenses. This phase of bacterial growth would therefore give an additional opportunity for virulent *Listeria* to overwhelm the bactericidal capacity of macrophages lying within the lamina propria. The assumption that enterocytes might be critically involved in the initiation of the infectious process was further supported by the observation that nonpathogenic species of the genus *Listeria* (*L. innocua*, *L. welshimeri*, and *L. seeligeri*) failed to enter enterocyte-like Caco-2 cells. This favors the view that these species are not responsible for human

infections (26), primarily because they fail to invade host tissues. Although isolation of *L. ivanovii* in human listeriosis is unusual (26), this species, surprisingly, interacted with Caco-2 cells as well as *L. monocytogenes*. If infection of Caco-2 cells is really relevant to the events occurring in natural infections, one can postulate that *L. ivanovii* is either rarely transmitted by the oral route or readily eliminated in the human host at a step after the enterocytic phase. In conclusion, our results suggest that intracellular multiplication in nonprofessional phagocytes during the initiation of the infectious process might be an important phase of bacterial amplification preceding the macrophagic phase.

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