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Interaction of *Listeria monocytogenes* with Mouse Dendritic Cells

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In this study, the interaction of murine dendritic cells with *Listeria monocytogenes* was investigated. Dendritic cells are efficient antigen-presenting cells, play a key role in the immune response, and are capable of migrating over substantial distances between sites of infection and lymphoid tissues. *L. monocytogenes* EGD invaded dendritic cells, escaped from phagosomes into the cytoplasm, and there directed actin nucleation, polymerization, and polarization in a typical fashion, thereby achieving intracellular movement and cell-to-cell spread. The internalization process appears to be independent of the *inl* locus. Interestingly, an intact microtubular function was essential for efficient uptake, whereas in a previous report, microtubule disruption did not affect bacterial spread in Caco-2 cells. The results obtained also suggest that *L. monocytogenes* binds to glycosylated receptors of dendritic cells. Uptake of *Listeria* cells was mediated by a protein kinase-dependent transducing phosphorylation signal that induces the actin polymerization-dependent phagocytic process. To achieve efficient uptake, de novo protein synthesis of eukaryotic and prokaryotic cells is also required. Despite the killing of dendritic cells, wild-type bacteria were found to persist in small numbers in some cells for at least 24 h. When different isogenic mutants of the EGD strain were analyzed for their capability to interact with dendritic cells, it was observed that some virulence-attenuated mutants (i.e., *prfA* and Δ*hly*) persisted in large numbers for even longer times. Invasion of dendritic cells by *L. monocytogenes*, which in turn could result in either cell death or persistent infection, might have an important role in the pathogenesis of listeriosis, leading to impaired immune responses with inefficient bacterial clearance and/or promoting bacterial spread.

Listeria monocytogenes is an important pathogen of humans and animals, and epidemic listeriosis has been reported after consumption of contaminated food (9, 14). While severe clinical infections can result in septicemia, meningoencephalitis, and death, an acute sublethal infection leads to long-lasting immunity to an otherwise fatal infection (14). This invasive bacterium exhibits a peculiar mode of cell-to-cell spread that enables the microorganisms to evade host defences. *L. monocytogenes* is actively internalized by host cells; the bacteria then lyse the phagosomal membrane, escaping into the cytoplasm where they orchestrate reorganization of host cell cytoskeletal proteins (32). This results in actin polymerization at one pole of the bacterium, which propels the bacterium within the infected cell and mediates invasion of new cells through the formation of a protrusion which enters a neighboring cell (32).

Dendritic cells (DCs) are the most efficient antigen-presenting cells and play a central role in immune responses (30). These highly specialized cells are present in the intestine-associated lymphoid tissues (23, 30), which constitute the portal of entry for listerial infections (9, 14). Infected DCs may serve as a reservoir for *L. monocytogenes* spread in human infections as a result of their specific homing in on lymphoid tissues after contact with antigens in the periphery. Moreover, their damage may lead to an impaired immune response with reduced bacterial clearance. For these reasons, interactions of *L. monocytogenes* with DCs are pertinent to the pathogenesis of listerial infections.

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In the present work, the infection of DCs by *L. monocytogenes* was analyzed by the use of a fully functional murine spleen DC line (22). *L. monocytogenes* cells were able to infect DCs, efficiently achieving intracellular and cell-to-cell spread, and persisted for at least 24 h. The influence of virulence-attenuating mutations and various metabolic inhibitors on bacterial uptake by and survival within DCs has provided interesting insights into the interaction of *Listeria* cells with these important cells.

MATERIALS AND METHODS

Bacterial strains and media. The bacteria employed in this study were the *L. monocytogenes* wild-type strain, EGD (serotype 1/2a), and its isogenic *prfA*1 (8), Δ*actA*2 (1), Δ*mpl*2, Δ*plcB*2, Δhly2, ΔinlA2, and Δ*inlB*2 mutants (described below), which were routinely grown at 37°C in brain heart infusion (BHI) broth or BHI agar (Difco Laboratories, Detroit, Mich.), supplemented where appropriate with 5 μg of erythromycin per ml. Liquid cultures were aerated by shaking at 300 rpm in a New Brunswick environmental incubator shaker.

Construction of the chromosomal deletion mutants Δ*mpl*, Δ*hly*2, Δ*plcB*2, Δ*inlA*2, and Δ*inlB*2. The plasmids pLM47, pLM47-300, and pLM54 served as templates for construction of chromosomal deletions in the genes *mpl*, *hly*, and *plcB* in *L. monocytogenes* (8). To generate a deletion within the *mpl* gene, plasmid pLM47-300 (6) was digested with the restriction endonucleases *Bal*I and *Nru*I, and the plasmid was religated to generate plasmid pLM47-Δ*mpl*. The 225-bp *Bal*I-*Nru*I restriction fragment encodes a region harboring the active site of the metalloprotease. Plasmid pLM47-Δ*mpl* was digested with restriction endonuclease *Ssp*I to generate a 1.85-kb *Ssp*I restriction fragment harboring the Δ*mpl* gene, which was then cloned into the *Sma*I restriction site of the temperature-sensitive suicide vector pAUL-A to generate plasmid pAUL-Δ*mpl*, which served for the generation of *L. monocytogenes* mutant strain Δ*mpl*2. To generate an in-frame deletion in the listeriolysin gene, an internal 1,080-bp *Cla*I fragment from pLM47 was removed by digestion with the restriction endonuclease *Cla*I and the religated to create pLM47-Δ*hly*. The resulting DNA fragment harboring the Δ*hly* gene was cloned into the *Kpn*I-*Bam*HI sites of pAUL-A, generating pAUL-Δ*hly*, which served for the generation of *L. monocytogenes* mutant strain Δ*hly*2. To create a Δ*plcB* mutant, an internal *Nde*I fragment harboring one-third

of the *plcB* gene was removed by digestion of plasmid pLM54 with *Nde*I followed by religation to generate pLM54-Δ*plcB*. The *Eco*RI-*Bam*HI DNA fragment of pLM54-Δ*plcB* harboring Δ*plcB* was cloned into pAUL-A to generate pAUL-Δ*plcB*, which served for the generation of *L. monocytogenes* mutant strain Δ*plcB2*.

Deletion mutants in the internalin locus were generated with a PCR employing specific primers with incorporated restriction sites to introduce an in-frame deletion in the *inlA* and *inlB* genes. To create the *inlA*2 mutant, the oligonucleotide pair A (5′-ACTTCATCTGCTGCAGGCTTAAAAGCA-3′) and B (5′-AACTTGGTCTGGATCCGTTTGCGAGAC-3′), with a *Bam*HI restriction site, were used to amplify a 1,245-bp DNA fragment (positions 520 to 1766 of the published sequence [10]) at the 5′ region of *inlA* encoding the first 78 N-terminal amino acid residues. The oligonucleotide pair C (5′-ATGAACGCTTAGGATC CTTATAATTCA-3′), with a *Bam*HI restriction site, and D (5′-TACTTTAC CACGCATGCTAAATTGATA-3′) served to amplify a 1,835-bp DNA fragment at the 3′ region encoding the last 47 C-terminal amino acids of InlA (positions 3792 to 5627). The two PCR products were digested with *Bam*HI and used in a ligation. The ligation product harboring the deletion was selectively amplified with the oligonucleotide pair A and D. To create the *inlB*2 mutant, the oligonucleotide pair E (5′-CTAAAAGAACCAAAGGTACCAACGAAAGCCGG A-3′), with a *Kpn*I restriction site, and F (5′-CACGGTGATAGGATCCGCT TGTACTTTCGC-3′), with a *Bam*HI restriction site, were used to amplify a 632-bp DNA fragment at the 5′ region of *inlB* (positions 3507 to 4139) encoding the first 40 N-terminal amino acid residues, and the oligonucleotide pair G (5′-GCAGCTAATTTAAGGGATCCGAAATAACTGAAAAAGACC T-3′), with a *Bam*HI restriction site, and H (5′-GTCATTAAATCTAGAC GATTCCATACA-3′), with an *Xba*I restriction site, served to amplify a 733-bp DNA fragment at the 3′ region (positions 5866 to 6618) encoding the last 8 C-terminal amino acids of InlB. The two PCR products were cut with *Bam*HI and used in a ligation. The ligation product harboring the deletion was selectively amplified with the oligonucleotide pair E and H. The resulting PCR products were cut with *Kpn*I and *Xba*I and cloned into the shuttle vector pAUL-A.

Plasmids pAUL-Δ*mpl*, -Δ*hly*, -Δ*plcB*, -Δ*inlA*, and -Δ*inlB* were transformed into *L. monocytogenes* EGD via electroporation, and recombinants were isolated at a growth temperature of 28°C from BHI agar plates supplemented with 5 μg of erythromycin ml^{-1} 2 to 4 days later. The recombinant clones were screened by plasmid DNA extraction from a pure colony, followed by digestion with appropriate restriction enzymes. For integration events, a single colony of the respective *L. monocytogenes* recombinant was incubated on BHI agar plates with 5 μg of erythromycin ml^{-1} at 42°C for 2 days. This procedure was repeated three times, and then the presence of the integrated plasmid was confirmed by Southern blot analysis of chromosomal DNA. The merodiploid intermediates contained the wild-type allele as well as the deleted alleles. To obtain spontaneous excision of the integrated plasmids through intramolecular homologous recombination, the strains were incubated twice at 28°C without erythromycin in BHI broth for 24 h, and then they were grown at 42°C in BHI broth overnight, diluted in saline, and plated on BHI agar plates. The bacterial colonies were transferred via a replica technique to BHI agar plates containing 5 μg of erythromycin ml^{-1}; the erythromycin-sensitive colonies, which were recovered at a frequency of about 17%, were screened for the presence of the deletion by PCR with specific oligonucleotides flanking the respective gene. A shorter PCR product was amplified from those strains with an allelic exchange of the deleted version of the wild-type allele on the chromosome. The appropriate gene deletions were confirmed by PCR sequencing of chromosomal DNA from mutants and further verified by immunoblotting with the appropriate monoclonal antibody (MAb [data not shown]). All DNA manipulations were performed as described by Sambrook et al. (29).

Tissue culture methods and invasion assays. The spleen DC line CB1, obtained from DBA/2 mice (22), was maintained in Iscove's modified Dulbecco's medium (Sigma Chemie GmbH, Deisenhofen, Germany) supplemented with 5% fetal calf serum and 5 mM glutamine (GIBCO Laboratories, Eggenstein, Germany). Cells were seeded at a concentration of approximately 5×10^4 per well in 24-well Nunclon Delta tissue culture plates (Inter Med Nunc, Roskilde, Denmark). Bacteria were grown for 16 h in BHI broth, recovered by centrifugation ($3,000 \times g$ for 10 min), and suspended in complete Iscove's medium. Infection assays were performed as previously described (15) at a cell/bacterium ratio of 1:100. After 1 h of coincubation, the medium was replaced with complete Iscove's medium supplemented with 50 μg of gentamicin ml^{-1} (Sigma Chemie GmbH) and incubated at 37°C for 1 h to kill the remaining extracellular bacteria (time zero). Thereafter, the supernatant fluids were discarded, and the cells were washed twice with phosphate-buffered saline (PBS) and lysed at different intervals. The number of CFU recovered from each well was determined by plating 10-fold dilutions on BHI agar with a Spiral Plater model C (Spiral Biotech, Inc., Bethesda, Md.).

Fluorescence staining of actin and bacteria and confocal laser scanning microscopy. Cells were grown on round 13-mm-diameter Thermanox coverslips (Inter Med Nunc) in 24-well Nunclon Delta tissue culture plates. After infection with *L. monocytogenes*, the coverslips were rinsed with PBS, and the cells were fixed in 3.7% formaldehyde in PBS for 30 min and permeabilized by a 5-min treatment with 0.2% (vol/vol) Triton X-100 in PBS to allow entry of antibodies. Coverslips were processed for double fluorescence microscopy with rabbit polyclonal antibodies raised against *L. monocytogenes* cells, together with tetramethyl-rhodamine-isothiocyanate-labelled phalloidin for F-actin labelling (Sigma Chemie GmbH). Coverslips were then carefully rinsed with PBS, treated with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulins (Dianova, Hamburg, Germany), washed three times, and then mounted for microscopy with a Zeiss inverted microscope (Carl Zeiss, Oberkochen, Germany) attached to the Bio-Rad MRC600 confocal system (Bio-Rad Laboratories GmbH, München, Germany), combined with an argon-krypton laser with the K1 and K2 filter set at 488 nm (green channel) and 568 nm (red channel). Z series with an optical section thickness of 0.5 μm were obtained (14 sections per sample), and the collected Z series were combined into one picture.

Transmission electron microscopy. Infected cells were fixed and processed as previously described (15). After embedding, ultrathin sections were cut with a glass knife and counterstained with 4% aqueous uranyl acetate (pH 4.5) and lead citrate. Sections were examined with a Zeiss EM 910 transmission electron microscope at an acceleration voltage of 80 kV at calibrated magnifications.

Pretreatment of CB1 cells or bacteria with inhibitors or blocking compounds. In several experiments, cells or bacteria were pretreated with cytochalasin D (0.5 μg ml^{-1}), nocodazole (3 μg ml^{-1}), tunicamycin (1 μg ml^{-1}), actinomycin D (1 μg ml^{-1}), chloramphenicol (50 μg ml^{-1}), staurosporine (1 μM), genistein (200 μM), and galactose (50 mM). The results were expressed as percentages of the untreated control CFU recovered per well.

Inhibition experiments were also performed with the following MAbs specific for surface proteins and receptors of CB1 cells: for MAC-2, clone ATCC TIB166; for CD11a, clone ATCC TIB213; for FcγRII, clone ATCC HB197; for F4/80, clone ATCC HB198; for CD11b, clone ATCC TIB128; for ICAM-1, clone ATCC CRL1878; and for CD11c, clone ATCC HB224. As a negative control (100% value), cells were treated with a MAb specific for the marker Thy-1 (clone ATCC HB23), which is absent in DCs.

Cells were pretreated for 1 h before addition of bacteria and throughout the invasion assays. The hybridoma supernatants containing MAbs specific for surface proteins were used at a 1:4 dilution to pretreat CB1 cells for 2 h. All compounds for which there is no specific information were provided by Sigma Chemie GmbH. The concentration of the inhibitor used was selected as previously described (15); treatments of bacteria and cells with the different compounds did not significantly reduce cell viability (data not shown).

Statistical calculations. The results obtained after pretreatment with inhibitors were analyzed for significance by analysis of variance and Student's *t* test. Differences were considered significant at $P \leq 0.05$.

RESULTS

Morphological follow-up of *L. monocytogenes* during infection of CB1 cells. The invasion process of CB1 cells by *L. monocytogenes* was investigated by immunofluorescence staining followed by confocal laser scanning microscopy. CB1 cells were demonstrated to be highly susceptible to *L. monocytogenes* infection. Almost all of the cells observed contained large numbers of intracellular bacteria, which were spread diffusely within the cytoplasm and which were surrounded by polymerized actin (Fig. 1A and B). Analysis of *L. monocytogenes*-infected cells by scanning electron microscopy revealed the typical *Listeria*-induced cellular protrusions involved in cell-to-cell spread, which result from bacterial recruitment of host cell cytoskeletal proteins (data not shown). Transmission electron microscopy analysis confirmed that at 4 h postinfection, most of the bacteria had escaped from the phagosome into the cytoplasm (Fig. 2A) and were surrounded by electroluminescent sponge-like material, which correlates with F-actin staining by immunofluorescence.

Uptake and intracellular survival of wild-type and attenuated *L. monocytogenes* cells within CB1 cells. When CB1 cells were infected with the wild-type strain, EGD, approximately 17% were taken up at time zero, and the number of viable intracellular bacteria recovered per well remained constant for approximately 4 h. Thereafter, the number of bacteria decreased by 50% 8 h and by 4 orders of magnitude 24 h after infection (Fig. 3). This reduction was due to cellular damage, which causes release of bacteria into the antibiotic-containing medium. In fact, 7 h after infection, 60% of the cellular lactate dehydrogenase activity was detected in the medium (data not shown), and confocal laser scanning microscopy performed 24 h after infection showed bacteria surrounded by cellular debris (Fig. 4A and B). However, some viable bacteria were still

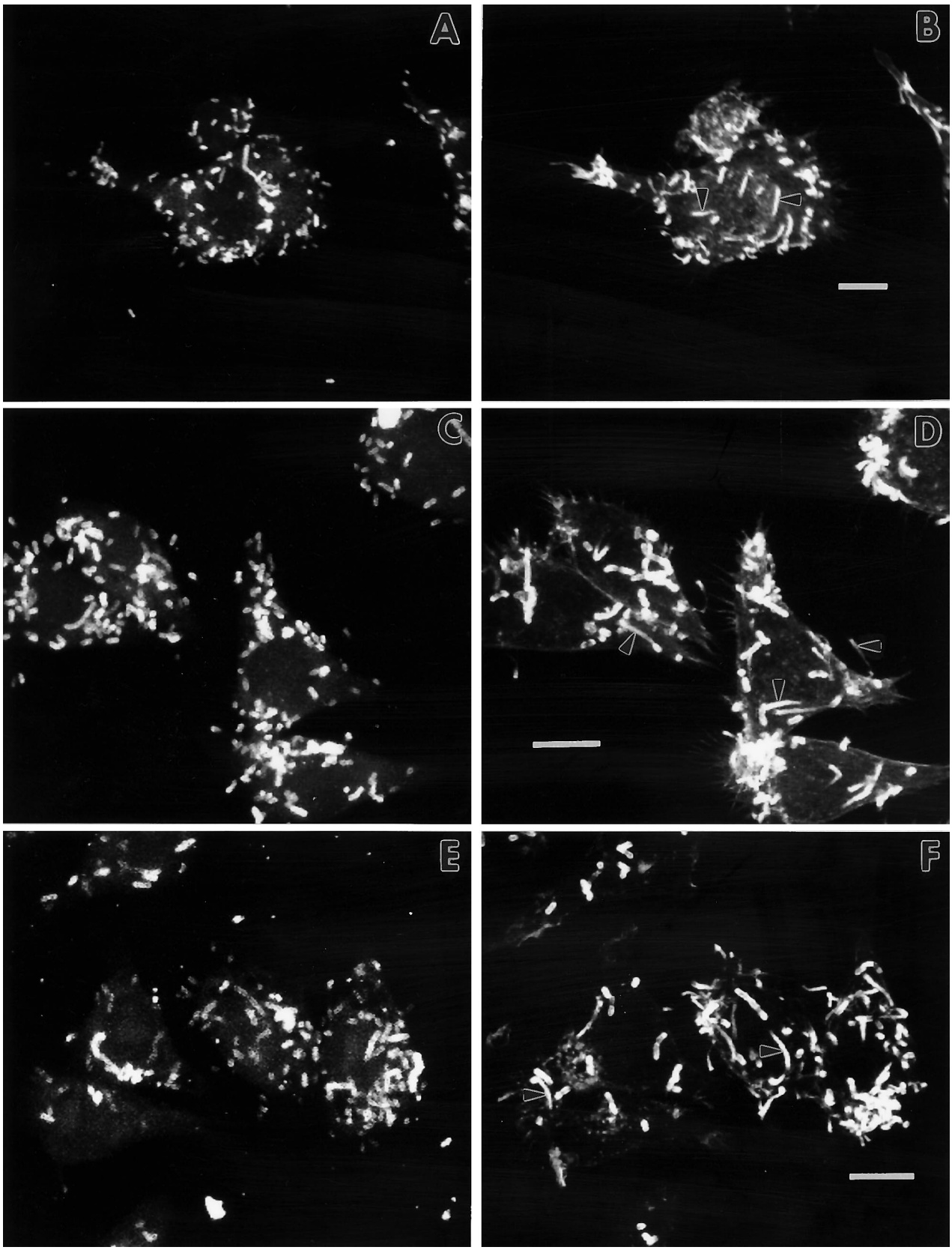

FIG. 1. Confocal laser scanning microscopy of *L. monocytogenes*-infected cells. CB1 cells were infected with *L. monocytogenes* EGD (A and B) and its isogenic Δ*plcB*2 (C and D) and *inlA*2 (E and F) mutants. The samples were stained as described in Materials and Methods for bacteria (green channel [A, C, and E]) or actin (red channel [B, D, and F]). Samples were processed after 4 h of infection, and Z series with an optical section thickness of 0.5 μm (14 sections) were collected and combined into one picture. Actin tails are indicated by arrowheads. Bars, 10 μm.

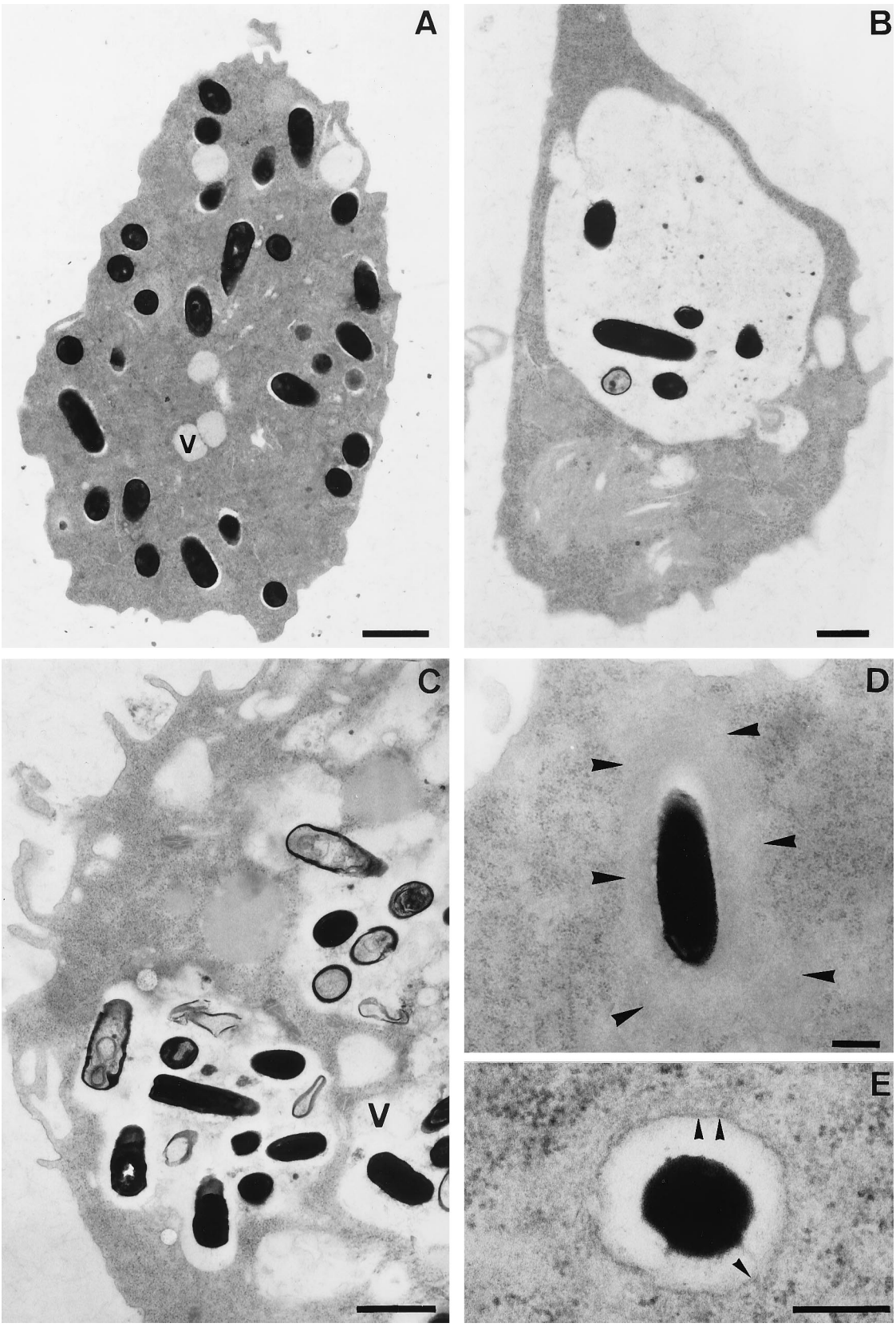

FIG. 2. Transmission electron microscopic analysis of CB1 cells infected with *L. monocytogenes*. Cells after 4 (A) and 24 (B to E) h of infection with *L. monocytogenes* EGD (A) and its isogenic Δ*hly*2 (B) and Δ*plcB*2 (C to E) mutants are shown. Actin accumulation (D) and the vacuole membrane (E) are indicated by arrowheads. Bars, 1 μm (A to C) and 0.25 μm (D and E). v, vacuole.

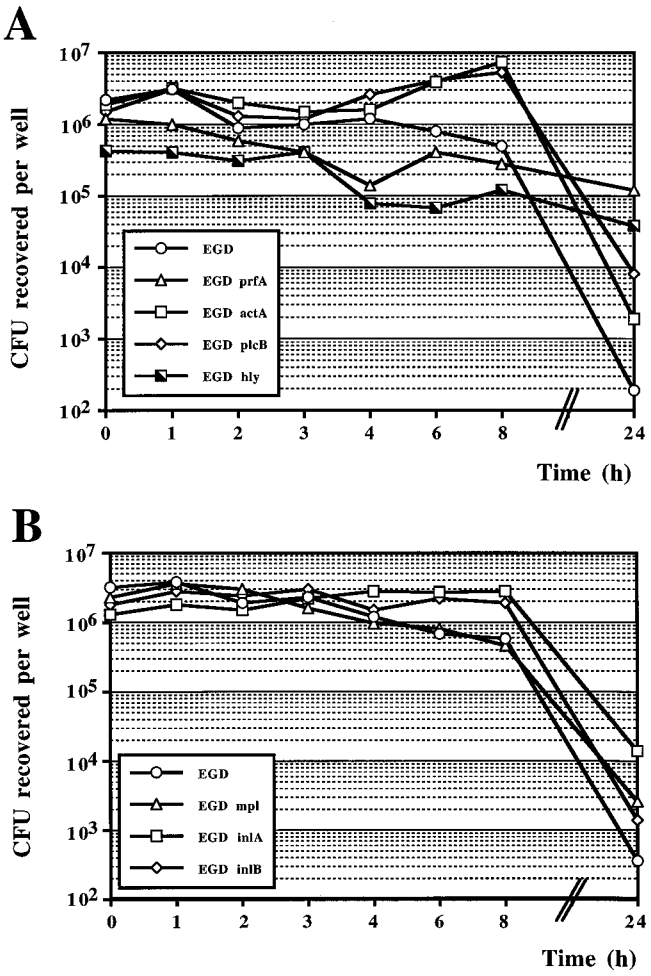

FIG. 3. Intracellular survival of *L. monocytogenes*. CB1 cells were infected with *L. monocytogenes* EGD and its isogenic *prfA*1, ΔactA2, Δ*plcB*2, Δ*mpl*2, Δmpl2, Δ*hly*2, *inlA*1, and *inlB*2 mutants. After 1 h of coincubation of *L. monocytogenes* cells with CB1 cells, gentamicin-containing medium was added, and the mixtures were further incubated for 1 h (time zero). Thereafter, cells were washed and lysed at different intervals, and the number of CFU recovered per well was determined. Results are presented as mean values of three independent experiments; in all cases, the standard deviation was lower than 15%.

present after 24 h, suggesting the possibility of persistent infection.

The *prfA* gene encodes a positive regulator of several *L. monocytogenes* virulence genes (2, 17, 25). The *L. monocytogenes prfA* mutant (Fig. 3A) was taken up by CB1 cells with a slightly lower level of efficiency than that of the EGD parental strain: 11% of the inoculum was recovered at time zero, after which the number of viable bacteria decreased over the next few hours at the same rate as that of the wild-type strain. Therefore, bacterial entry in CB1 cells appears to be largely independent of *prfA* regulation. However, while the number of viable bacteria recovered from cells infected with EGD fell dramatically between 8 and 24 h, the number of bacteria isolated from *L. monocytogenes prfA*1-infected cells was only slightly reduced over the same period (1 order of magnitude).

During the *L. monocytogenes* infection cycle, bacteria invade and then escape from phagosomes, spread intracellularly, and infect surrounding cells. Listeriolysin mediates lysis of the phagocytic vacuole and liberation of bacteria into the cytoplasm and is one of the main virulence factors which allows tissue colonization (11, 25, 26). The kinetics of DC infection by the *L. monocytogenes* listeriolysin-defective Δ*hly*2 mutant were similar to those of the *prfA*1 mutant, except that only 2% of the inoculum was taken up by DCs at time zero (Fig. 3A).

Bacterial phospholipase C is also involved in *L. monocytogenes* release from the phagosome and seems particularly important in the lysis of the restraining host double membrane formed during cell-to-cell spread (33). On the other hand, the ActA protein is involved in actin recruitment and polymerization around the bacterial cell (25) and thus plays a pivotal role in bacterial movement within infected cells and in cell-to-cell spread. The *L. monocytogenes* phospholipase C-deficient Δ*plcB*2 mutant and the ActA-deficient Δ*actA2* mutant exhibited comparable infection kinetics (Fig. 3A). They showed levels of entry similar to that of the wild-type strain: approximately 14 and 17%, respectively, of the inoculum were recovered at time zero. The number of viable microorganisms increased steadily with time, such that after 8 h, it was 1 order of magnitude higher than at time zero. This could be due to either improved intracellular growth or impaired cytotoxicity of these bacteria. After 24 h of infection, the number of viable bacteria recovered from both mutant strains was 1 order of magnitude higher than that of the parental strain but lower than that at time zero. Analysis by confocal laser scanning microscopy showed that both mutant bacteria grew as localized microcolonies within CB1 cells (particularly evident 24 h after infection [Fig. 1C and Fig. 4C and E]). Interestingly, while impairment of the actin polymerization process was not observed 4 h after infection of CB1 cells with the Δ*plcB*2 mutant (Fig. 1D), it was seen 24 h after cell infection with both Δ*plcB*2 and Δ*actA*2 mutants (Fig. 4D and F). Analysis by transmission electron microscopy demonstrated that after 24 h of infection, between 95 and 99% of the intracellular Δ*plcB*2 bacteria were surrounded by membranes (Fig. 2C and E) similar to that observed with the Δ*hly*2 mutant (Fig. 2B). Only a few *Listeria* cells were in the cytoplasm surrounded by polymerized actin (Fig. 2D). Hence, the inability of these bacteria to breach surrounding membranes resulted in them being trapped within vacuoles after cell-to-cell spread. Almost all of the vacuoles observed appeared to be surrounded by only a single membrane (Fig. 2E).

The metalloprotease of *L. monocytogenes* has been shown to be required for the production of biologically active PlcB (27). However, the initial course of infection of CB1 cells with the Δ*mpl22* metalloprotease-deficient mutant was not superimposable over that of the Δ*plcB* mutant (Fig. 3), suggesting that host cell proteases may compensate for the lack of metalloprotease during cell infection. Later in infections (24 h), 3 orders of magnitude fewer viable bacteria were recovered, which suggests that metalloprotease is also involved in the processing of other proteins involved in intracellular survival.

The *L. monocytogenes* locus *inl* is required by bacteria to invade epithelial cells (10). However, the Δ*inlA*2 and Δ*inlB*2 mutant bacteria were taken up by cells almost as efficiently as wild-type bacteria (12 and 15%, respectively [Fig. 1, compare A and B with E and F, and see Fig. 3B]). The *inlA*2 and *inlB*2 mutants did, however, exhibit intracellular growth kinetics different from those of the wild-type strain. A 20 to 60% increase in the number of CFU after 4 to 8 h of infection was observed, and 100 times more bacteria were recovered 24 h after infection with respect to the parental strain (Fig. 3B).

The uptake of wild-type *L. monocytogenes* requires intact microfilament and microtubule machineries. The mechanisms involved in the uptake of *L. monocytogenes* by CB1 cells were investigated with selected inhibitors. It has been previously shown that *L. monocytogenes* enters different eukaryotic cells by microfilament-dependent endocytosis (4, 11, 16, 20, 26, 32).

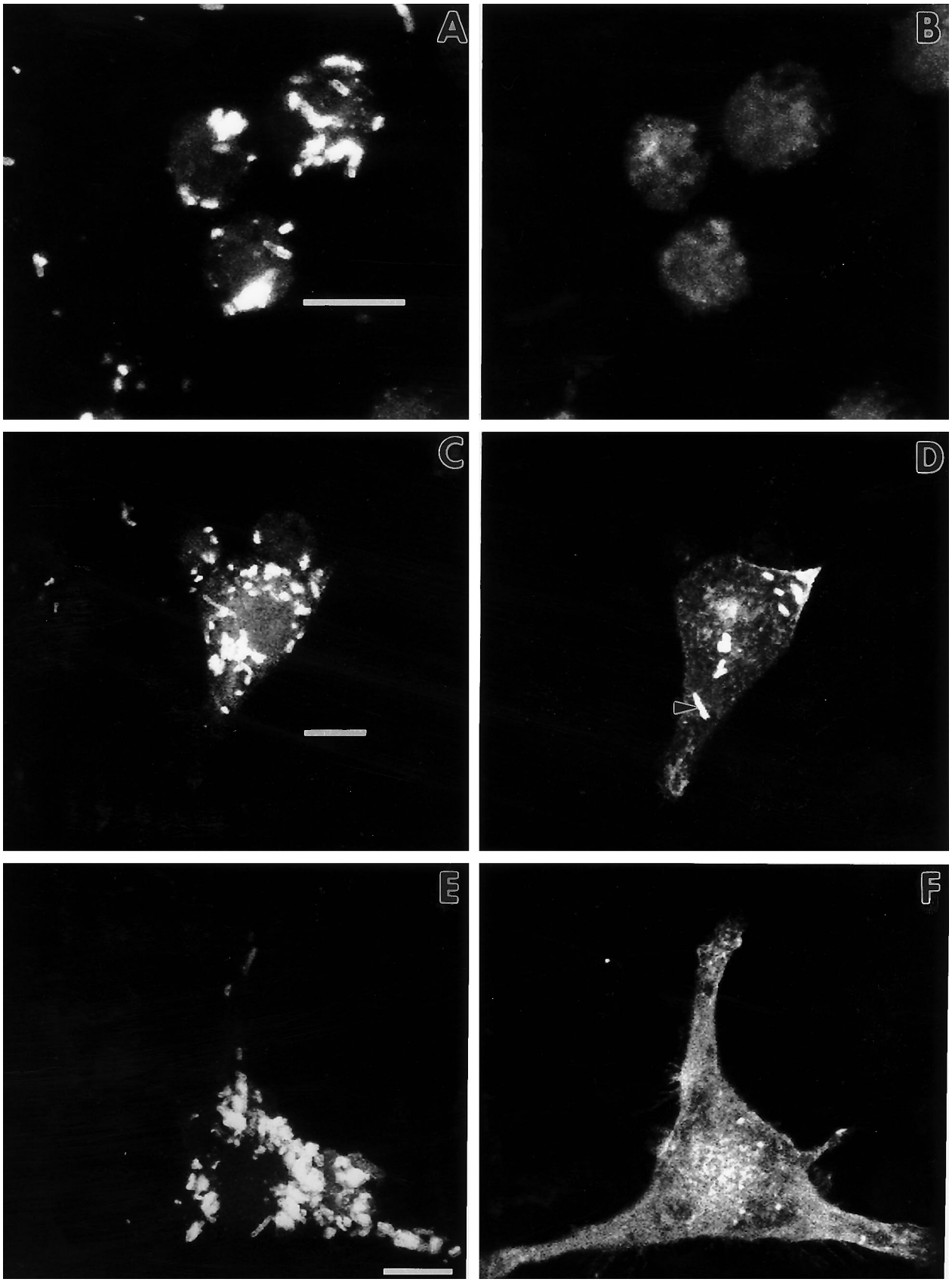

FIG. 4. Confocal laser scanning microscopy of CB1 cells infected with *L. monocytogenes*. Cells were infected with *L. monocytogenes* EGD (A and B) and its isogenic Δ*plcB*2 (C and D) and Δ*actA*2 (E and F) mutants. The samples were stained as described in Materials and Methods for bacteria (green channel [A, C, and E]) or actin (red channel [B, D, and F]). Samples were processed after 24 h of infection; Z series with an optical section thickness of 0.5 μm (14 sections) were collected and combined into one picture. Actin tails are indicated by arrowheads. Bars, 10 μm.

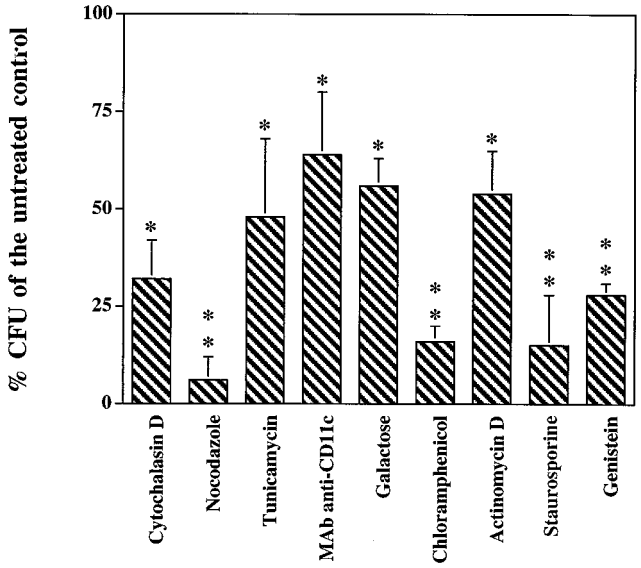

FIG. 5. Influence of various bioactive reagents on the uptake of *L. monocytogenes* bacteria by the CB1 DC line. Eukaryotic cells were pretreated with the indicated substances as described in Materials and Methods prior to addition of bacteria. After 1 h of coincubation of *L. monocytogenes* cells with CB1 cells, gentamicin-containing medium was added, and the mixtures of cells were further incubated for 1 h, washed, and lysed. Results are expressed as a percentage of the untreated control CFU recovered per well and represent mean values of three independent experiments ± standard deviations. The results are statistically significant compared with those of the untreated controls at $P \leq 0.05$ (*) and $P \leq 0.001$ (**).

The pretreatment of cells with the inhibitor of actin polymerization cytochalasin D (68% reduction) confirmed that the same mechanism is involved in the uptake of *L. monocytogenes* by DCs (Fig. 5).

The microtubule system plays an important role in intracellular trafficking (19) and has been implicated in the uptake of *Bordetella bronchiseptica* by CB1 cells (15). However, the intracellular spread and cell-to-cell spread of *L. monocytogenes* in Caco-2 cells have been reported to be microtubule independent (20). Nevertheless, pretreatment of CB1 cells with nocodazole, which inhibits microtubule polymerization, resulted in a 94% reduction in the number of bacteria taken up by CB1 cells (Fig. 5), suggesting involvement of microtubule machinery in the *Listeria* uptake pathway.

A 52% inhibition of uptake resulting from pretreatment of cells with the inhibitor of N glycosylation tunicamycin indicated that glycosylated receptors may have a role in the internalization of *L. monocytogenes* by CB1 cells (Fig. 5) and suggested an assessment of the effects of MAbs against well-characterized DC surface ligands on the uptake process. Pretreatment of CB1 cells with MAbs directed against the receptors MAC-2, CD11a, FcγRII, F4/80, CD11b, and ICAM-1 had no significant effect on internalization (127, 114, 115, 95, 86, and 106% of the level of the control, respectively), whereas the anti-CD11c MAb caused 36% inhibition (Fig. 5). This suggested that the CD11c receptor might be involved in the uptake process. Alternatively, the inhibition might result from a nonspecific masking of other surface ligands by the MAb directed against the highly expressed CD11c molecules (22, 30). It has been shown that attachment of *L. monocytogenes* to other eukaryotic cell lines is mediated in part by adhesins containing a galactose moiety (3). The 44% inhibition caused by pretreatment of CB1 cells with galactose (Fig. 5) suggests that similar listerial ligands may mediate interactions with DCs. When cells were treated with glucose to verify the specificity of the reduction, no inhibitory effect was observed (data not shown).

Bacterial binding to a cellular receptor is expected to generate an uptake signal which is transmitted through the membrane and which triggers the microfilament-dependent endocytic process. Protein kinases are involved in the regulation of cellular surface receptors and in the transmission of signals originating from them (24) and might be involved in the activation of proteins necessary for the uptake process of invasive pathogens (12, 15, 28, 31). The 85 and 72% reductions in the numbers of bacteria taken up by cells pretreated with the wide-range protein kinase inhibitor staurosporine and the tyrosine protein kinase inhibitor genistein, respectively, indicate that several protein kinases may be involved in signal transduction events associated with bacterial uptake by and initial intracellular survival in CB1 cells.

De novo bacterial protein synthesis has been demonstrated to be required for the interaction of *L. monocytogenes* with cytoskeletal proteins (4) and in the invasive processes of many pathogenic bacteria (15, 18, 21). The numbers of bacteria taken up by CB1 cells after pretreatment with chloramphenicol and actinomycin D, inhibitors of prokaryotic and eukaryotic protein synthesis, decreased by 84 and 46%, respectively, suggesting that this requirement is also characteristic of the *L. monocytogenes*-DC interaction.

DISCUSSION

DCs are the critical cells able to fully activate both naive CD4 and CD8 T lymphocytes, are essential for primary immune responses, and constitute the basis of efficient defense against infective agents (30). In addition, they are capable of migrating through lymphatic vessels between the periphery and the draining lymphoid tissues. Their interactions with microorganisms have not, however, been intensively studied thus far because of difficulties in purifying DCs and the lack of an established cell line that ensures reproducibility. The recent establishment of a murine spleen DC line (22) has now opened up the possibility of carrying out such studies. *L. monocytogenes* is a paradigm of an invasive bacterial pathogen. This and its ability to cause both acute nonlethal infections that result in long-lived protective immunity and severe often fatal infections have prompted us to analyze its interactions with DCs.

In this study, the interaction of *L. monocytogenes* with a murine DC line was investigated. We show that DCs are permissive hosts for *L. monocytogenes*; the bacteria invade CB1 cells and persist intracellularly for at least 24 h. However, the level of uptake efficiency remains low (17%), suggesting a decreased antigen-scavenging function compared with professional phagocytes. Investigation of the requirements for bacterial uptake by and propagation within CB1 cells by means of bacterial isogenic mutants and selected cell inhibitors showed that the interaction of *Listeria* cells with CB1 cells is largely similar to interactions of *Listeria* cells with other cell lines, either professional or nonprofessional phagocytes (4, 11, 16, 20, 26), and interactions of CB1 cells with *B. bronchiseptica* (15). *Listeria* cells are internalized by microfilament-dependent endocytosis and escape rapidly from the vacuolar compartment, whereupon free intracytoplasmic bacteria direct actin nucleation through a polarized process leading to intracellular movement and cell-to-cell spread. It is, however, worth emphasizing that some aspects appear to be unique for CB1-*Listeria* interaction. For example, the internalization process of *L. monocytogenes* appeared to be independent of regulation by

the *prfA* gene product and of the products of the *inl* locus. Therefore, other, presently unknown, *prfA*-independent invasins are involved in the internalization process. Moreover, unlike previous findings with Caco-2 cells (20), in which bacterial spread was not modified by nocodazole treatment, microtubules appear to be critical for initial interactions of *Listeria* cells with CB1 cells. This would be consistent with the inhibition of *B. bronchiseptica* uptake by CB1 cells after nocodazole treatment (15) and recent reports demonstrating a role for microtubules in the uptake and intracellular replication processes of several pathogenic microorganisms in other cellular systems (13, 21).

Uptake of *L. monocytogenes* into CB1 cells appeared to be mediated by glycosylated receptors. A MAb to the CD11c receptor also partially blocked uptake of *L. monocytogenes*. However, these latter results have to be interpreted with caution, since this protein is highly expressed by DCs (22, 30) and could mask other less-abundant receptors on the cell surface. The involvement of protein kinases in the uptake of pathogenic *Listeria* cells by eukaryotic cells has not been extensively studied. We show here that the tyrosine kinase inhibitor genistein inhibits uptake of *L. monocytogenes* into the CB1 DC line, a result which concurs with those previously obtained with kidney and epithelial cell lines derived from different hosts (34). In contrast to those studies, strong inhibition of uptake into CB1 cells was observed with the broad-range protein kinase inhibitor staurosporine, suggesting that distinct pathways of uptake may be operating in different cell lines.

An interesting phenotype that emerged in these studies was the ability of attenuated mutants to survive for long periods within CB1 cells. Unlike the wild-type strain, which causes cell death within 8 h of infection, the Δ*hly*2 and *prfA*1 mutants in particular were recovered in large numbers from intact CB1 cells even 24 h postinfection. Since these bacteria produce no or little listeriolysin, respectively, *L. monocytogenes* appears capable of survival in phagosomes of DCs (Fig. 2B). Extended survival was also observed, albeit to a lesser extent, with the Δ*actA*2 and Δ*plcB*2 mutants. These mutants produce wild-type levels of listeriolysin (data not shown). However, they either grow as localized microcolonies (Δ*actA*2 [Fig. 4E]) or are found trapped in vacuoles (Δ*plcB*2 [Fig. 2C and E]). From these observations, it could be extrapolated that the differential survival rates are manifestations of the distribution and amount of listeriolysin present in the cell.

Listeriolysin has been implicated as a key molecule in *L. monocytogenes* infection and is required for the lysis of phagosomal membranes (11, 16, 20, 25, 26). This protein also appears to have a major role in initial interactions of *L. monocytogenes* with CB1 cells. Although the reason for this is not immediately obvious, recent data have pointed to multiple effects of listeriolysin on eukaryotic cells. Listeriolysin appears to be responsible for induction of mitogen-activated protein kinase prior to bacterial entry (31), and expression of listeriolysin in eukaryotic cells leads to a strong mitogenic response (5). Hence, listeriolysin may be activating cellular pathways for uptake by modulating key enzymes in signal transduction. Since the *prfA*1 mutant, which produces only low levels of listeriolysin (7), enters cells at levels comparable to those of the wild type, sublethal levels of the toxin may be involved in inducing these subtle cellular changes. Interestingly, if persistence of infection is a measure of attenuation, then it appears that the *inl* mutants are also somehow enhanced for intracellular survival, suggesting an additional role for internalin in the intracellular stage of listerial infections.

The greater persistence in CB1 cells of some attenuated mutants and the persistence of wild-type *Listeria* cells for extended periods in a minority of invaded cells raise the intriguing possibility that in natural infections, modulation to an attenuated phenotype may occur in the intracellular microenvironment. Otherwise, actively virulent bacteria would lyse the carrier cell, rendering intracellular bacteria susceptible to specific and nonspecific clearance mechanisms. Persistently infected DCs could play an active role in listerial dissemination to different tissues or serve as sources of initiation of infection at different anatomical sites during the natural course of infection. Moreover, DCs have been shown to be essential for the development of an efficient immune response (30). Therefore, the colonization and/or damage of this key component of the immune system may lead to reduced bacterial clearance and subsequent progression of the infection with septicemia.

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