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Effect of Cell Polarization and Differentiation on Entry of *Listeria monocytogenes* into the Enterocyte-Like Caco-2 Cell Line

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The entry of *Listeria monocytogenes* into the enterocyte-like Caco-2 cell line was studied as a function of cell polarization and differentiation. *L. monocytogenes* entered through the entire surface of nonpolarized cells and, predominantly, through the basolateral surface of polarized cells based on the following observations: (i) sites of *L. monocytogenes* invasion paralleled the distribution of the transferrin receptor, a well-known basolateral marker of polarization; (ii) numbers of internalized bacteria decreased dramatically when Caco-2 monolayers cultured beyond confluency were used (about 0.1% of the inoculated bacteria versus 1 to 2% with nonconfluent monolayers); and (iii) *L. monocytogenes* entry into postconfluent monolayers was greatly enhanced by treating cells with Ca^{2+}-free medium, a procedure that disrupts intercellular junctions and thus exposes the basolateral surface to bacteria. Ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) had contradictory effects on *L. monocytogenes* entry as this reagent opened intercellular junctions but inhibited binding and internalization of bacteria. Finally, the role of the *inlAB* locus in *L. monocytogenes* entry was confirmed because an *inlAB* mutant was 50- to 100-fold less invasive than the parental strain regardless of the monolayer's age. However, the *inlAB* mutant was still able to enter cells and to induce intracellular actin polymerization. Entry of *inlAB* bacteria into Caco-2 cells was not inhibited by EGTA.

Listeria monocytogenes is a facultative intracellular bacterium that has emerged as an important food-borne pathogen in developed countries in the last decade (8). It causes life-threatening infections, mainly in the settings of pregnancy, aging, and immunosuppression. Perinatal infections may result in abortion, stillbirth, and neonatal meningitis or sepsis. Meningitis, encephalitis, and septicemia account for most cases of infection in nonpregnant adults. Approximately 1,700 cases of listeriosis occur annually in the United States, resulting in 450 adult deaths and 100 fetal and postnatal deaths (16).

Outbreaks of listeriosis are related to the ingestion of contaminated food (8), strongly suggesting that the infection is initiated via the intestinal route (8, 13). *L. monocytogenes* presumably crosses the intestinal epithelial barrier prior to induction of systemic disease. Animal models of oral infection have been used to study the process of translocation. MacDonald and Carter demonstrated that the initial sites of *Listeria* invasion in mice are the ileal Peyer's patches (26). In an extensive electron microscopic study of guinea pigs, Racz et al. observed dividing listeriae in absorptive intestinal epithelial cells within 3 h of infection; at later stages of infection, listeriae were found in phagocytic cells present in the lamina propria (32).

Invasion of intestinal epithelial cells by *L. monocytogenes* has been studied in vitro using the human colon carcinoma cell line Caco-2 (12, 27). The entry of listeriae into Caco-2 cells is a phagocytic-like process and is blocked by treating monolayers with cytochalasin D, a drug which inhibits actin polymerization (12). Invasion requires the expression of a chromosomal locus comprising two bacterial genes, *inlA* and *inlB* (11). These genes are transcribed both individually and in an operon by PrfA-dependent and -independent mechanisms (7, 25). The *inlA* gene is essential for *L. monocytogenes* entry into Caco-2 cells (5, 11). This gene encodes an 800-amino-acid protein, internalin, which is structurally analogous to the M protein from *Streptococcus pyogenes* and to some other surface proteins from gram-positive organisms involved in mammalian cell contact and recognition (6, 11). The *inlB* gene is very similar to *inlA* and encodes a 630-amino-acid protein (5, 11). It plays only a minor role in the entry of *L. monocytogenes* into Caco-2 cells, whereas its expression is absolutely required for the entry of this pathogen into cultured hepatocytic cells of human and murine origin (5, 15).

Although they are derived from a colon adenocarcinoma, Caco-2 cells are similar to enterocytes from the small intestine (33). Grown under standard culture conditions, they undergo dramatic changes over time that mimic the maturation process of intestinal epithelial cells during crypt-to-villus migration (30, 38). Subconfluent Caco-2 cells form roughly circular islets that spread progressively. Peripheral cells are undifferentiated and proliferate, whereas central cells become polarized and differentiate. After confluency, i.e., typically after 5 to 6 days of culture, the process of polarization involves the whole monolayer. The process of differentiation requires about 10 days more to complete.

Polarization and differentiation of Caco-2 cells are related but distinct processes (30). Polarization is characterized by the presence of tight junctions that separate and define the apical and basolateral domains of the plasma membrane. Membranes of these two cell domains have different protein and lipid compositions. Surface molecules that are expressed uniformly in nonpolarized cells and asymmetrically in polarized cells constitute markers of polarization. These molecules are found in virtually all epithelial cell types. Examples of basolateral mark-

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ers of polarization are receptors for cell-cell (e.g., cadherins) and cell-substratum (e.g., integrins) adherence and for uptake of nutrients from the circulation (e.g., transferrin receptor) (35). Differentiation is a progressive maturation process resulting in dramatic morphological changes (e.g., formation of an apical brush border). At a molecular level, it is characterized by the appearance of membrane-associated components that are essential to enterocytic functions. These molecules constitute markers of differentiation. They may be apical (e.g., peptidases and dissacharidases [30] and blood group H substance [1]) or basolateral (e.g., vasoactive intestinal peptide receptor [24]).

A number of bacterial pathogens specifically interact with definite target domains of Caco-2 cells. They include enterotoxigenic *Escherichia coli* (ETEC) and *Salmonella*, *Shigella*, and *Yersinia* spp. ETEC organisms bind to the apex of cells carrying receptors for ETEC colonization factor antigens; the expression of these receptors is a differentiation-related event (21). Salmonellae enter differentiated Caco-2 cells through their apical surface; invasion is associated with a local degeneration of microvilli and the formation of membrane ruffles (9). *Shigella* invasion is basolateral, and the entry process occurs only after the basolateral surface of Caco-2 cells has been exposed by disruption of the intercellular junction by treatment of cell monolayers with a Ca^{2+} chelator (28). Finally, *Yersinia pseudotuberculosis* enters predominantly proliferating undifferentiated Caco-2 cells that express large amounts of the cell receptor ($\alpha_5\beta_1$ integrin) for invasin (4).

Data concerning the Caco-2 cell surfaces invaded by *L. monocytogenes* are contradictory. It has been reported that only nonconfluent Caco-2 monolayers (i.e., typically after 2 to 3 days of culture) are permissive for this pathogen (12, 27). It has been also noted that only the periphery of the cell islets that are formed at this stage are invaded (27). Taken together, these findings suggest that the entry of *L. monocytogenes* into Caco-2 cells may occur through the basolateral surface. However, using scanning and transmission electron microscopy, Karunasagar et al. recently found that *L. monocytogenes* attaches to and penetrates the apex of Caco-2 cells (20). In addition, these investigators found that Ca^{2+} chelators greatly reduced the uptake of listeriae in spite of their ability to open the intercellular space and to render the basolateral surface accessible to the bacteria. Whether or not the *inlAB* locus was involved in the process of apical entry was not investigated.

We demonstrate that *L. monocytogenes* binds to and enters through the entire surface of nonpolarized Caco-2 cells and, predominantly, the basolateral surface of differentiated and nondifferentiated polarized Caco-2 cells, using both *inlAB*-dependent and *inlAB*-independent mechanisms. We also show that the processes of binding and entry mediated by *inlAB* products are inhibited by Ca^{2+} chelators.

MATERIALS AND METHODS

Bacterial strains and growth media. *L. monocytogenes* EGD-SmR, a streptomycin-resistant derivative of strain EGD (14), has previously been shown to be invasive for Caco-2 cells (11). BUG8 is a Tn*1545* transposon mutant from EGD-SmR, which is about 50-fold less invasive for nonconfluent Caco-2 monolayers than its parent. The transposon insertion, 417 bp upstream of *inlA* (6), prevents the transcription of *inlA* and *inlB* (11). The BUG8-derivative strains JLG101, JLG102 and JLG103 have been described elsewhere (15). JLG101 harbors the vector pAT28; JLG102 harbors the pAT28 derivative pGM4, which also encodes *inlAB*; JLG103 harbors the pAT28 derivative pGM2, which encodes *inlA*. *Listeria innocua* CLIP11254, which is not invasive for Caco-2 cells (11), was used as negative control. *Listeria* strains were grown in tryptic soy broth (Diagnostics Pasteur, Marnes-la-Coquette, France) at 37°C without shaking. Spectinomycin (60 mg/liter) was added to cultures of strains harboring plasmid pAT28 derivatives.

Cyto- and immunochemicals. Fluorescein isothiocyanate (FITC)-conjugated *Ulex europaeus* agglutinin I (UEA-I) was purchased from Sigma, St. Louis, Mo. FITC-phalloidin and rhodamine-phalloidin were supplied by Molecular Probes Inc. (Eugene, Oreg.). Polyclonal rabbit anti-*L. monocytogenes* EGD serum and mouse monoclonal OKT9 anti-human transferrin-receptor antibody were gifts from Patrick Berche (INSERM U411, Faculté Necker-Enfants Malades, Paris, France) and from Wilfred A. Jefferies (University of British Columbia, Vancouver, Canada), respectively. Polyclonal rabbit anti-sucrase-isomaltase serum was kindly provided by M. Rousset and A. Zweibaum (INSERM U178, Villejuif, France). Secondary antibodies were all supplied by Jackson ImmunoResearch Laboratories Inc. (Bio/Can Scientific, Mississagua, Ontario, Canada) and included FITC- or rhodamine-goat anti-mouse and FITC- or rhodamine-goat anti-rabbit antibodies.

Cell culture. The human colon carcinoma cell line Caco-2 (ATCC HTB37) was used between passages 25 and 40 as older passages are less permissive to infection by *L. monocytogenes* (29). Cells were grown in Dulbecco's modified Eagle minimum essential medium (DMEM) (25 mM glucose) (GIBCO Laboratories, Grand Island, N.Y.), supplemented with 20% inactivated fetal calf serum (GIBCO) and 1% nonessential amino acids (Flow Laboratories, Inc., McLean, Va.), in a 10% CO_2 atmosphere at 37°C. Cell monolayers were prepared for the assays as follows. Propagated cells were seeded at 2×10^5 cells per cm^2 into 75-cm^2 flasks (Falcon; Becton Dickinson and Company, Paramus, N.J.). After 3 days of culture, cells were harvested by trypsinization. Cells used for the quantitative invasion assay were seeded at 6×10^4 cells per cm^2 in 24-well tissue culture plates (Falcon), whereas those to be used for fluorescence studies were seeded at 8×10^4 cells per cm^2 onto 12-mm-diameter glass coverslips in 24-well plates. The medium was changed daily until confluency and thereafter every second day. Confluency occurred after 5 to 6 days of incubation and was concomitant with the appearance of monolayer domes, which indicate cell polarization (30). Monolayers were used 1 to 21 days after seeding for the assays. The state of cell differentiation was monitored by detecting the brush border-associated markers sucrase-isomaltase (30) and substance H (1). Some experiments were performed using Caco-2 cells grown on Transwell tissue culture-treated polycarbonate filters (filter diameter, 6.5 mm; pore size, 3.0 μm; Costar Corp., Cambridge, Mass.). Monolayers were used 12 days after seeding and had electrical resistances between 350 and 400 Ωcm^2.

Disruption of intercellular junctions. Caco-2 monolayers, grown either on coverslips or on Transwell filters, were washed three times with Ca^{2+}-free DMEM (GIBCO) and treated with the same medium, with or without 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) (Sigma) 1 h prior to infection.

Quantitative invasion assay. Bacterial inocula were prepared from 18-h cultures in tryptic soy broth, containing antibiotics when required. Bacteria were pelleted by centrifugation, washed once, and diluted 1:100 ($\sim 2 \times 10^7$ bacteria per ml) in DMEM or in Ca^{2+}-free DMEM with or without EGTA; 500 or 150 μl of this bacterial suspension was added to wells of 24-well tissue culture plates or to Transwell filters, respectively ($\sim 10^7$ bacteria per cm^2). After 1 h of incubation at 37°C to allow bacterial entry, cells were washed twice and overlaid with fresh DMEM containing gentamicin (Sigma) (10 mg/liter) to kill extracellular bacteria. The cells were incubated at 37°C for 2 additional hours. They were then washed twice and lysed by adding 1 ml (coverslips) or 0.5 ml (Transwell filters) of cold distilled water. The titers of viable bacteria released from the cells were determined on agar plates. Each experiment was carried out in triplicate and repeated three times. Cells from noninfected monolayers were collected by trypsinization and enumerated in parallel.

Fluorescence microscopy of adherent bacteria. Caco-2 monolayers grown on coverslips were washed twice, overlaid with 0.5 ml of DMEM, and incubated on ice for 1 h. Five microliters of a bacterial suspension containing $\sim 2 \times 10^9$ bacteria per ml was added to the wells. Monolayers were incubated for 90 min on ice. Cells were then washed three times with phosphate-buffered saline (PBS), fixed with 3% paraformaldehyde (wt/vol in PBS) for 30 min at room temperature, and washed three times again with PBS before being processed for fluorescence labeling. Adhesion was analyzed both qualitatively and quantitatively. The quantitative adhesion assay was carried out in triplicate. Adherent bacteria were counted by examining 500 (3- to 4-day-old monolayers) or 2,000 (14-day-old monolayers) Caco-2 cells in randomly picked microscopic areas.

Fluorescence microscopy of internalized bacteria. Cells grown on coverslips were infected as above. Two or four hours after gentamicin addition, cells were washed twice with PBS, fixed with 3% paraformaldehyde (wt/vol in PBS) for 30 min at room temperature, and permeabilized for 5 min in 0.1% Triton X-100 (Sigma) in PBS. Cells were then washed three times with PBS before being processed for fluorescence labeling. In some experiments, the invasion assay was initiated by centrifugation of the bacteria onto cells at $1,000 \times g$ for 10 min.

Fluorescence microscopy. Permeabilized (invasion assay) or nonpermeabilized (adhesion assay) fixed cells were washed three times with PBS and processed further. For detection of substance H, cells were incubated with FITC–UEA-I (10 μg/ml) for 30 min at room temperature. For F-actin staining, FITC- or rhodamine-phalloidin was used as described previously (27). For immunolabeling, cells were incubated sequentially with appropriate dilutions of primary and secondary antibodies in 1% bovine serum albumin–PBS; incubations were carried out for 30 min at room temperature and were followed by three washings in PBS. Coverslips were mounted on slides and examined by fluorescence microscopy with a Zeiss Axioskop microscope.

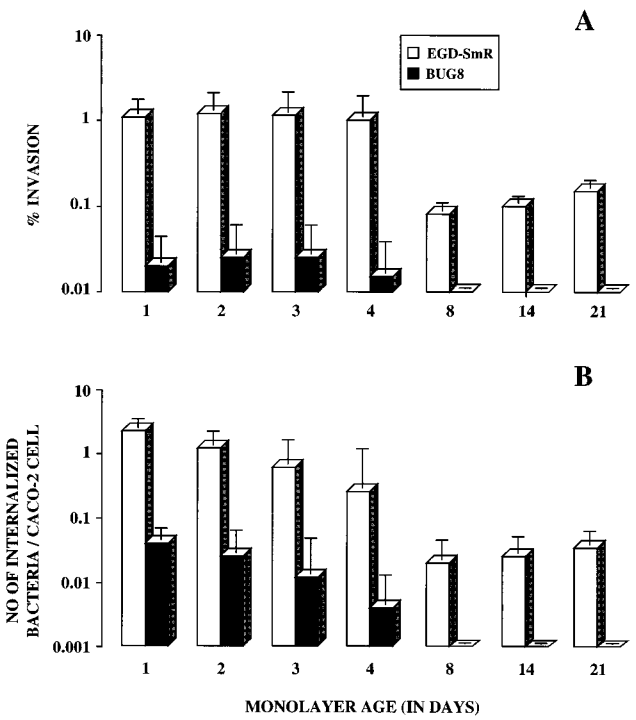

FIG. 1. *L. monocytogenes* invasion as a function of Caco-2 monolayer age. Caco-2 monolayers of different ages were infected with EGD-SmR and BUG8 for 1 h and further incubated for 2 h with gentamicin. Caco-2 cells in monolayers were enumerated in parallel. Each experiment was performed in triplicate and repeated three times. The results of one series of experiments are shown (means and standard deviations). Results are expressed as the number of bacteria surviving gentamicin treatment as a proportion of inoculated bacteria (A) or per Caco-2 cell (B).

RESULTS

Invasion of Caco-2 cells by *L. monocytogenes* as a function of monolayer age. One- to 21-day-old Caco-2 monolayers were infected with the invasive *L. monocytogenes* strain EGD-SmR and its *inlAB* derivative BUG8, with or without *inlA* or *inlAB* in *trans*. After a 1-h incubation with bacteria, cells were incubated for 2 h with gentamicin to kill extracellular bacteria and lysed for bacterial counting.

The results of these experiments are expressed as the number of bacteria surviving incubation with gentamicin as a proportion of inoculated bacteria (percent invasion) (Fig. 1A). The proportion of EGD-SmR bacteria recovered after gentamicin treatment was about 1% for monolayers grown 1 to 4 days and dropped to about 0.1% for monolayers grown 8 days or more. Decreased invasion was also observed with BUG8 when the assay was performed using postconfluent monolayers: entry was about 0.02% of the inoculum for monolayers grown 1 to 4 days and dropped to about 0.002% for monolayers grown 8 days or more (Fig. 1A). The inoculated BUG8 bacteria surviving in the presence of gentamicin were not wild-type revertants. Independent colonies obtained after infection of representative monolayers were randomly selected (20 colonies for each monolayer) and assayed for entry, using 2-day-old monolayers. In each case, the bacteria exhibited the same entry-defective phenotype as BUG8. The BUG8-derivative strains harboring either *inlA* or *inlAB* invaded 1- to 21-day-old monolayers similarly to invasive EGD-SmR (results not shown), suggesting that *inlA* is essential to *Listeria* entry irrespective of monolayer age. It has been reported that a small proportion of extracellular bacteria survive after 2 h of exposure to gentamicin (11). To determine the background levels resulting from this phenomenon, Caco-2 monolayers were infected with noninvasive *L. innocua* CLIP11254. The proportion of inoculated bacteria recovered after gentamicin treatment was 0.001% or less regardless of monolayer age (results not shown).

The number of cells in Caco-2 monolayers approximately doubled daily from seeding to confluency. To account for the increase in Caco-2 cells, the results of bacterial invasion obtained with EGD-SmR and BUG8 are also expressed as the number of bacteria surviving gentamicin treatment per Caco-2 cell (Fig. 1B). The number of EGD-SmR bacteria per cell was greatest for 1-day-old monolayers, reaching two bacteria per cell, decreased progressively as a function of the monolayer's age for nonconfluent monolayers, and dropped to 0.025 bacterium per cell for postconfluent monolayers. The number of internalized BUG8 bacteria per cell also decreased with the monolayer's age and was about 50-fold less than that found with EGD-SmR for any given monolayer age (Fig. 1B).

Immunofluorescence study of invasion of Caco-2 monolayers by *L. monocytogenes*. Bacterial invasion of Caco-2 monolayers grown for 1 to 21 days was also monitored by immunofluorescence. Infected cells were fixed and permeabilized for immunolabeling of bacteria. Double-fluorescence labeling of bacteria and the transferrin receptor was also performed to determine which cell surfaces were invaded. The transferrin receptor is expressed on the entire surface of nonpolarized enterocytic cells and on the basolateral surface of polarized cells (17).

Different patterns of invasion were observed when Caco-2 monolayers of different ages were infected with EGD-SmR. These patterns were also seen with BUG8-derivative strains harboring *inlA* or *inlAB* in *trans*, which behaved similarly to EGD-SmR (results not shown). Monolayers grown for 1 to 2 days (Fig. 2) consisted mainly of individual cells and small islets containing less than 10 cells. Nearly all individual cells and small islets were invaded by large numbers of bacteria. Bacteria infecting islets were seen in peripheral as well as central cells. In contrast, large islets were invaded by small numbers of bacteria and invasion appeared to be restricted to the outer edge of the islets. Double-labeling experiments showed that the bacteria were located in areas where the transferrin receptor was detected. The transferrin receptor was uniformly expressed on the cell surface of individual cells and on cells forming small islets, whereas it was expressed only at the outer edge of the peripheral cells in large islets. Very few BUG8 bacteria were seen under these experimental conditions (results not shown). Some were visualized after F-actin staining when infection was extended over 4 h (results not shown), demonstrating their intracellular location.

Monolayers grown for 3 to 4 days consisted of large islets containing 25 to 100 cells (Fig. 3). Some central cells expressed sucrase-isomaltase and substance H (results not shown) and were thus already differentiated. EGD-SmR bacteria were seen almost exclusively at the outer edge of the islets (Fig. 3a). As previously found for the largest islets formed after 1 to 2 days of culture, bacteria colocalized with the transferrin receptor (results not shown). BUG8 bacteria were infrequently found in monolayers (Fig. 3b); some, which were always seen inside peripheral cells, appeared coated with F-actin after 4 h of infection (results not shown). Centrifugation of the EGD-SmR bacteria onto cells greatly enhanced infection of peripheral cells but did not promote entry of bacteria into central cells (Fig. 3c), demonstrating that the restriction of the invasion process to the outer edge of the islets was not due to mechanical reasons. Only a few BUG8 bacteria entered cells

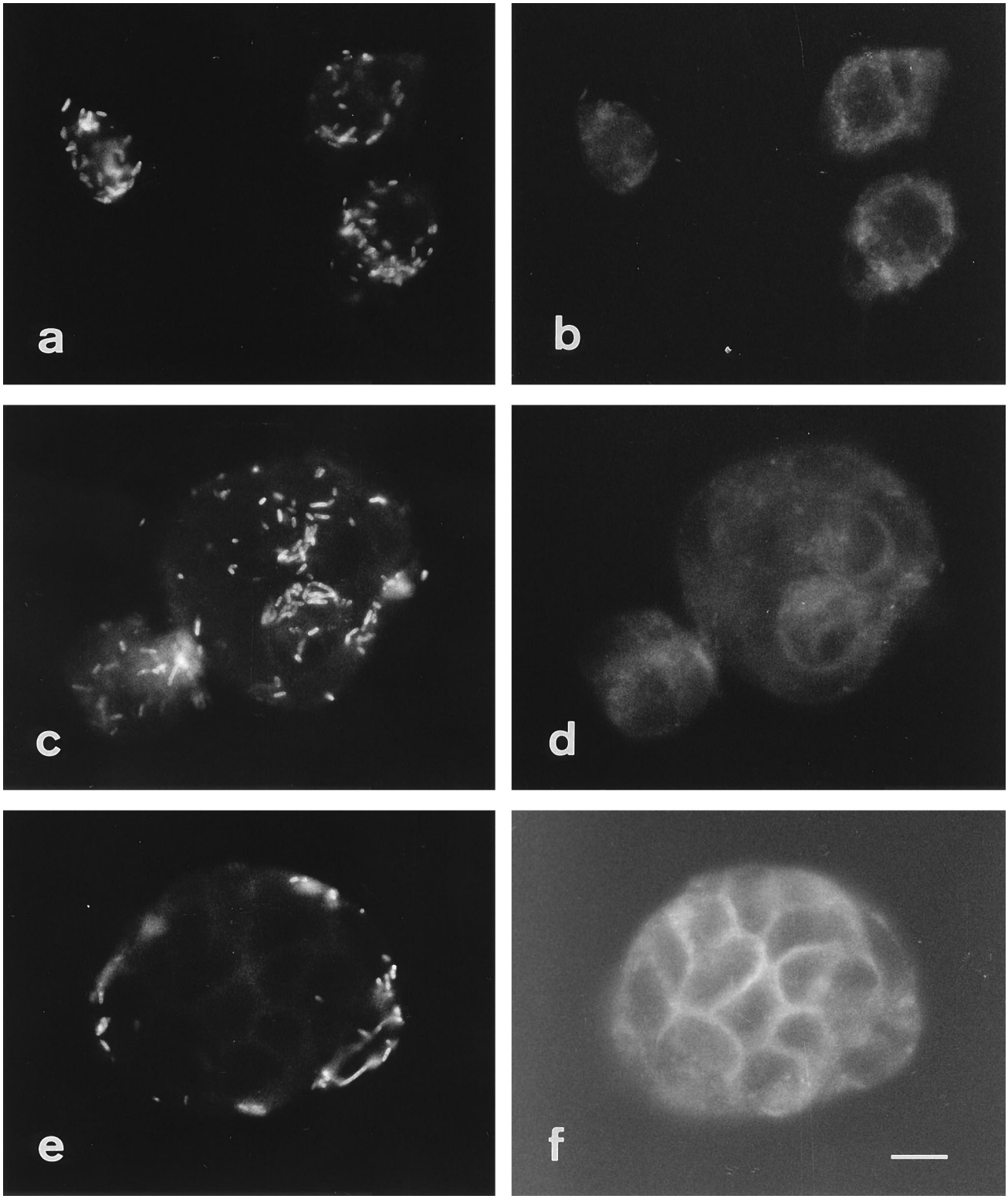

FIG. 2. Sites of *L. monocytogenes* entry into 1- to 2-day-old Caco-2 monolayers and cell distribution of the transferrin receptor. One- to two-day-old Caco-2 monolayers were infected with EGD-SmR for 1 h and further incubated for 2 h with gentamicin. Cells were then fixed, permeabilized, and processed for double-fluorescence labeling of bacteria (a, c, and e) and the transferrin receptor (b, d, and f). *Listeria* invasion of individual Caco-2 cells (a and b), of a small islet (c and d), and of a large islet (e and f) is shown. Sites of entry parallel the distribution of the transferrin receptor on cells. Bar = 5 μm.

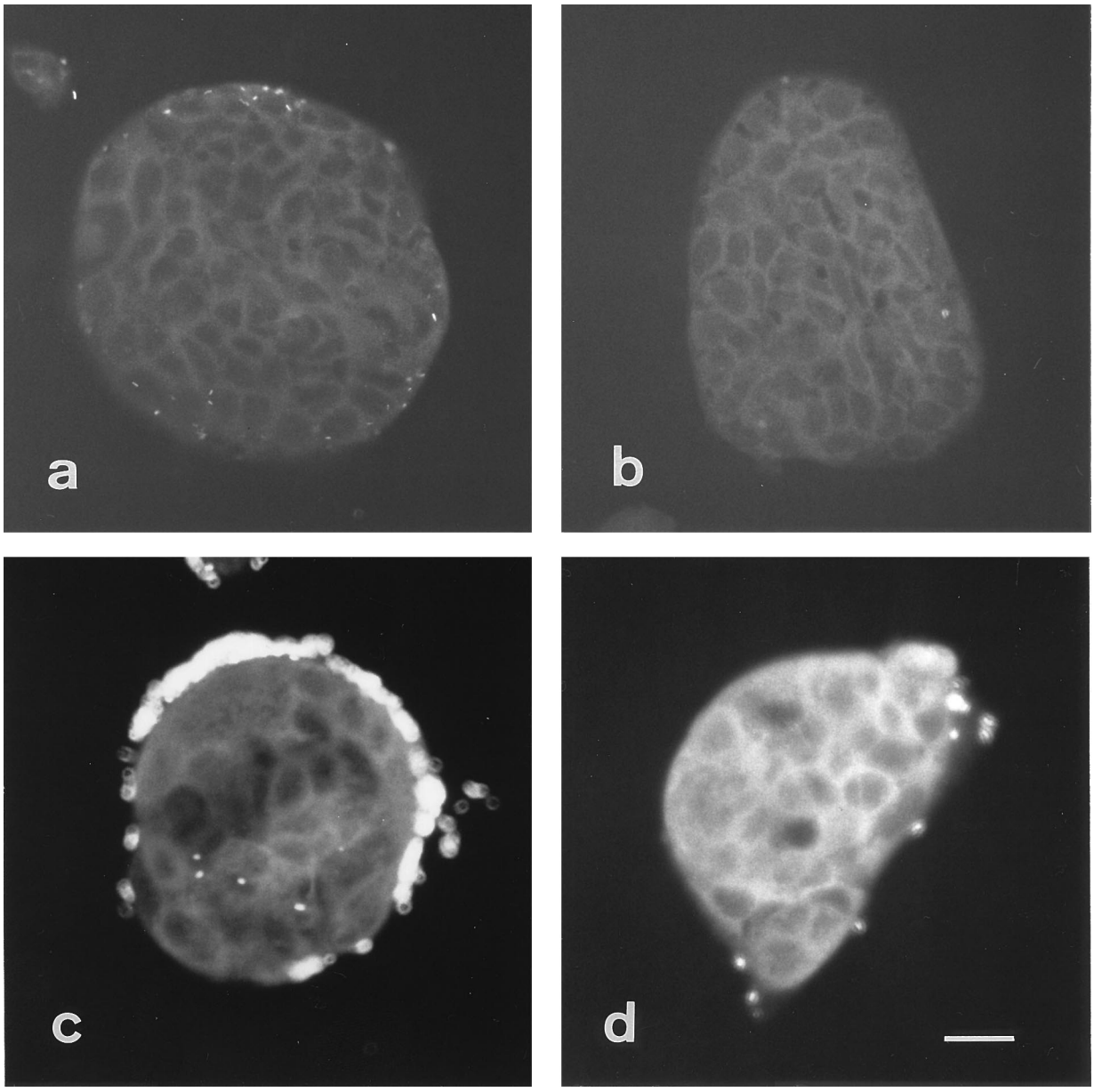

FIG. 3. Peripheral invasion of 3- to 4-day-old Caco-2 islets by *L. monocytogenes*. Three- to four-day-old monolayers were infected for 1 h with EGD-SmR (a and c) and BUG8 (b and d), with (c and d) or without (a and b) previous centrifugation of bacteria onto cells. Monolayers were then incubated for a further 2 h with gentamicin. At the end of the gentamicin treatment, cells were fixed, permeabilized, and processed for immunolabeling of bacteria. Note that bacteria invade almost exclusively the outer edges of the islets, even with centrifugation. Staining of Caco-2 cell junctions is probably due to the presence of intestine-specific antigens reacting with normal rabbit serum, as reported with other human adenocarcinoma cells (19). Bar = 10 μm.

after centrifugation. As observed with EGD-SmR, cell invasion was almost exclusively peripheral (Fig. 3d).

The observations made for infection of monolayers grown for 8 to 21 days were consistent with the results of the gentamicin survival assay (results not shown). Regardless of the monolayer's age, first, only a small number of internalized EGD-SmR bacteria could be seen (~20 bacteria per field at a magnification of 400), and second, few BUG8 bacteria were visible (one or two bacteria per field). F-actin staining was performed to determine if bacteria were truly intracellular (results not shown). After 4 h of infection, EGD-SmR as well as BUG8 bacteria coated with F-actin could be seen in some individual cells. About one cell per 10 fields (magnification of 400) contained F-actin-stained bacteria. No difference was observed whether monolayers were 8, 14, or 21 days old.

Immunofluorescence study of *L. monocytogenes* adherence to Caco-2 monolayers. The binding of EGD-SmR and BUG8, complemented or not with *inlA* or *inlAB*, to Caco-2 cells was studied as a function of monolayer age by immunofluorescence. Patterns of adhesion observed with EGD-SmR matched the patterns of invasion previously found. Bacteria adhered uniformly to 1-day-old and small 2-day-old cell islets (Fig. 4a) and to the outer edge of large 2-day-old and of 3- to 4-day-old cell islets (Fig. 4b); they all colocalized with the transferrin receptor (results not shown). Only a few bacteria bound to 8- to 21-day-old monolayers (Fig. 4c). The same observations

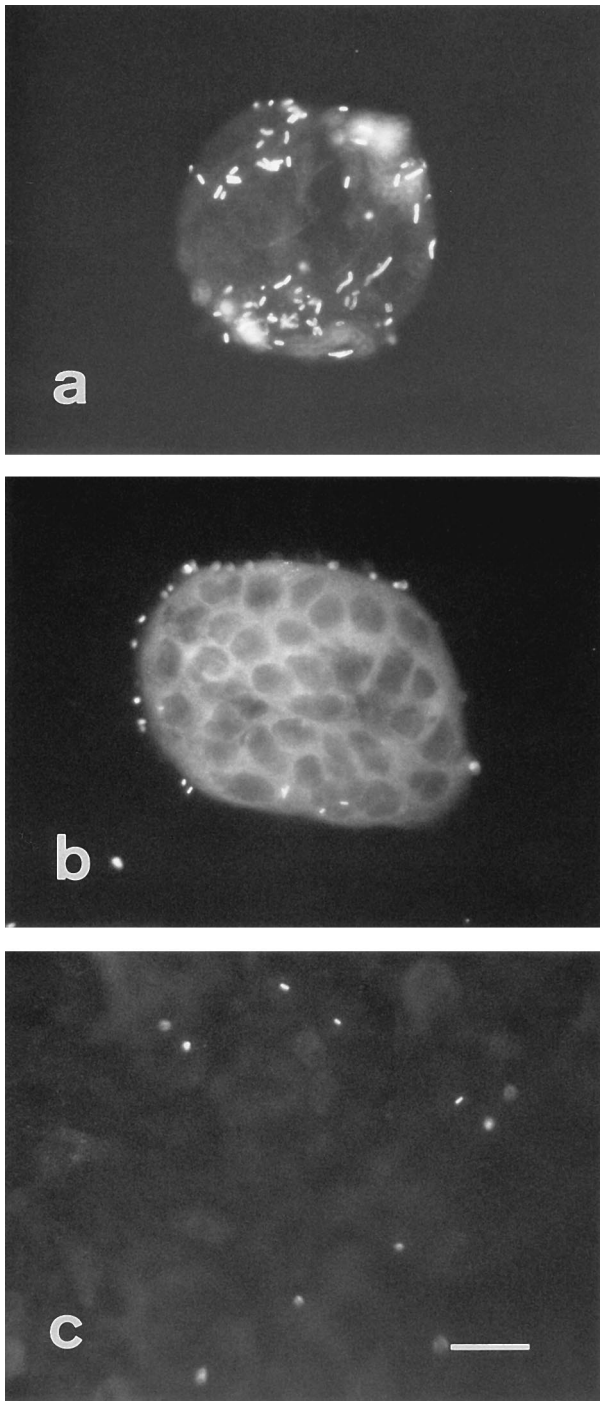

FIG. 4. Patterns of *L. monocytogenes* adhesion as a function of Caco-2 monolayer age. Monolayers were infected with EGD-SmR for 90 min at 4°C. Cells were then washed, fixed, and processed for immunolabeling of bacteria. (a) One-day-old monolayer; (b) 3-day-old monolayer; (c) 14-day-old monolayer. Bar = 10 μm.

were made with BUG8-derivative strains harboring either *inlA* or *inlAB* (results not shown). BUG8 bacteria adhered to cells similarly to EGD-SmR bacteria but were 20- to 30-fold less numerous regardless of the monolayer's age (results not shown).

Effects of intercellular junction disruption on *L. monocytogenes* entry. Restriction of the invasion process to the outer edges of 3- to 4-day-old islets (see above) suggested that *L. monocytogenes* invaded polarized Caco-2 cells basolaterally. We addressed this further by infecting cells whose intercellular junctions had been disrupted by Ca^{2+} depletion in the extracellular medium (31) in order to render the basolateral surface accessible to the bacteria (28). Ca^{2+} depletion was achieved by treating the monolayers with Ca^{2+}-free medium (17) or with EGTA (31). Both procedures were used because Ca^{2+} depletion is more severe with EGTA; however, this agent may reduce bacterial uptake by cells (3). Also, cell polarity is substantially affected by the support upon which cells are grown (nonpermeable versus permeable supports) (10), and therefore we used both plastic- and filter-grown monolayers.

The effects of disrupting cell-cell junctions on EGD-SmR and BUG8 entry were studied using gentamicin assays (Fig. 5). The entry of EGD-SmR bacteria into 3- to 4-day-old monolayers grown on plastic dishes was enhanced by a factor of 2 by treatment with Ca^{2+}-free medium without EGTA, whereas it was reduced by a factor of 12 in the presence of EGTA. The inhibitory effect of EGTA was reversible. When EGTA-pretreated cells were infected using medium with Ca^{2+}, invasion levels were comparable to those obtained with Ca^{2+}-free medium alone. For BUG8, treatments with Ca^{2+}-free medium with or without EGTA caused a two- to threefold increase in bacterial entry regardless of the medium used for infection. Cells of treated and untreated monolayers were enumerated by trypsinization. The treatment of monolayers with Ca^{2+}-free medium and Ca^{2+}-free medium with EGTA caused the detachment of 15 and 25%, respectively, of Caco-2 cells.

The effects of cell depolarization on EGD-SmR entry were more demonstrative with postconfluent plastic-grown monolayers because of the low invasion rates observed with untreated cells. Invasion levels were increased 20 times by treatment with Ca^{2+}-free medium and 3 times by treatment with EGTA. In the latter case, the effects of cell depolarization were probably overshadowed by the inhibitory effect of EGTA on bacterial uptake. Consistent with this view, invasion levels were enhanced 40 times when Caco-2 monolayers treated with EGTA were infected using medium with Ca^{2+}. In contrast to 3- to 4-day-old monolayers, no significant cell detachment occurred with any treatment. Invasion levels of BUG8 increased three- to fivefold following treatments with Ca^{2+}-free medium with or without EGTA regardless of the medium used for infection.

The results obtained with filter-grown monolayers were different, probably due to the higher polarity of cells established on permeable supports (10). The uptake of EGD-SmR bacteria was enhanced by factors of 5 and 20, respectively, after treatment with Ca^{2+}-free medium and EGTA. However, when infection of monolayers pretreated with EGTA was performed in medium with Ca^{2+}, the uptake was enhanced by a factor of 50, confirming that EGTA was inhibitory. The uptake of BUG8 bacteria was enhanced by a factor of 4 to 5 regardless of the treatment used. Electrical resistance measurement showed that monolayers were depolarized much more efficiently with EGTA than with Ca^{2+}-free medium alone (80 versus 30% reduction in electrical resistance).

Immunofluorescence study of *L. monocytogenes*–Caco-2 cell interaction after disruption of intercellular junctions. The effects of intercellular junction disruption on EGD-SmR invasion were studied by immunofluorescence. In contrast to the observations made with untreated islets (Fig. 3a), bacteria were able to invade nonperipheral cells of 3- to 4-day-old Caco-2 islets treated with Ca^{2+}-free medium (Fig. 6a and b). Treatment of islets with EGTA also abolished the pattern of peripheral invasion found with untreated cells (Fig. 6c and d).

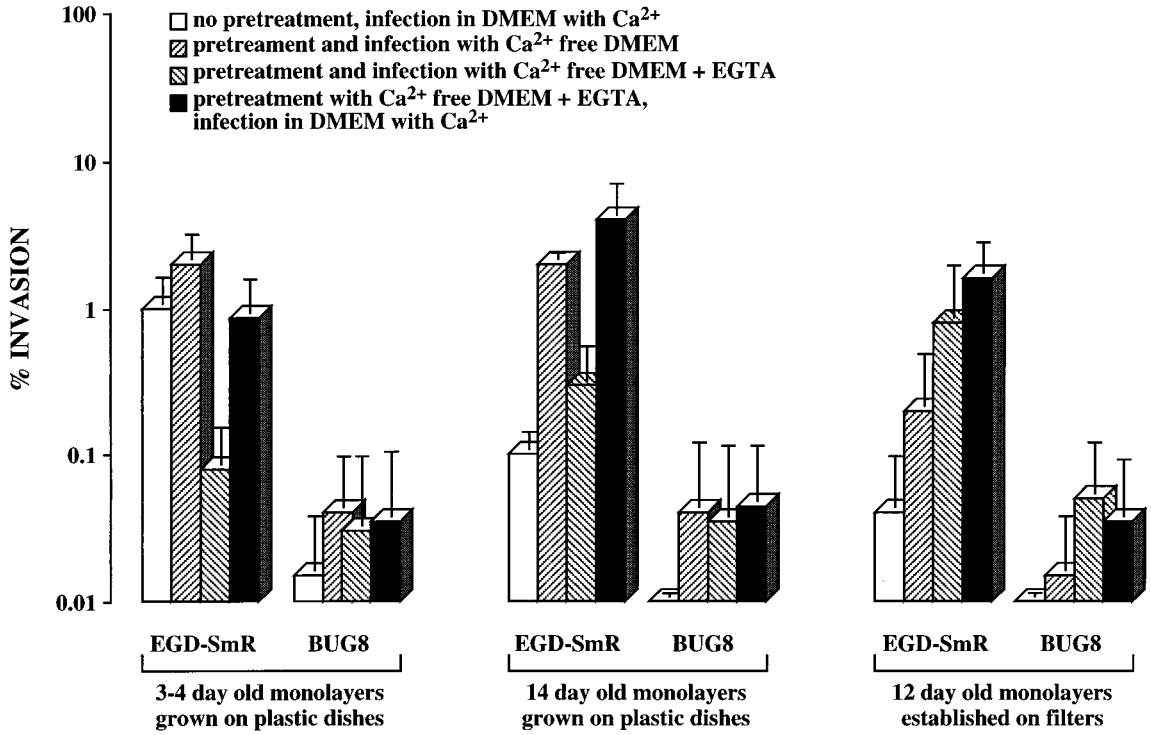

FIG. 5. Effects of disruption of intercellular junctions on *L. monocytogenes* entry. Three- to four-day-old plastic-grown, 14-day-old plastic-grown, and 12-day-old filter-grown Caco-2 monolayers were treated with Ca^{2+}-free medium or with Ca^{2+}-free medium plus EGTA and infected with EGD-SmR and BUG8 in the same medium or in medium with Ca^{2+}. Bacterial entry was assessed using the gentamicin invasion assay. Each experiment was performed in triplicate and repeated three times. The results of one series of experiments are shown, expressed as the mean number of bacteria (and standard deviation) surviving gentamicin treatment as a proportion of inoculated bacteria (percent invasion).

However, the number of bacteria interacting with EGTA-treated cells was smaller than after treatment with Ca^{2+}-free medium alone. Observations with postconfluent monolayers were in agreement with the results of quantitative invasion assays. Regardless of the monolayer's age, a much larger number of bacteria were seen after treatment with Ca^{2+}-free medium alone (Fig. 6e and f) than without treatment. Also, bacteria were rarely found in areas where treatment obviously failed to open intercellular junctions. The number of bacteria interacting with cells was dramatically reduced when monolayers were treated with EGTA (Fig. 6g and h). Consistent with the results of quantitative studies, the inhibitory effect of EGTA was suppressed when monolayers treated with this agent were infected using medium with Ca^{2+} (results not shown).

Finally, adherence of EGD-SmR to monolayers treated with Ca^{2+}-free medium with or without EGTA was studied by immunofluorescence. Treated cells were infected at 4°C for 90 min, fixed, and processed for immunolabeling with anti-*Listeria* antibody. Results were analyzed both qualitatively and quantitatively. Treatment of 3- to 4-day-old monolayers with Ca^{2+}-free medium resulted in the adherence of bacteria to central as well as peripheral cells (results not shown). The same pattern of adhesion was found in the presence of EGTA (results not shown), but the number of bacteria seen on cells was much smaller (Table 1). Similarly, treatment of postconfluent monolayers with Ca^{2+}-free medium alone promoted the adhesion of bacteria to cells (Table 1). In areas where phase-contrast examination suggested that the intercellular junctions were readily opened, bacteria were seen in large numbers in the intercellular spaces (results not shown). In contrast, although the addition of EGTA appeared to open the intercellular spaces more efficiently (results not shown), only a few bacteria interacted with cells (Table 1). As observed previously for invasion, the inhibitory effect of EGTA could be abrogated by performing the assay in medium with Ca^{2+} (results not shown).

DISCUSSION

We demonstrated that *L. monocytogenes* enters through the entire surface of nonpolarized Caco-2 cells and the basolateral surface of differentiated and nondifferentiated polarized Caco-2 cells. First, double-labeling studies showed that the sites of *Listeria* invasion paralleled the distribution of the transferrin receptor on the cell surface. In young Caco-2 islets (1 to 2 days of culture), which were mostly composed of nonpolarized cells expressing the transferrin receptor on their entire surface, listeriae invaded uniformly. In older Caco-2 islets (3 to 4 days of culture), where the polarity of cells was established, the transferrin receptor was exclusively detected at the outer edge of peripheral cells. Similarly, the sites of *Listeria* entry were also restricted to the outer edges of cell islets. Secondly, after the monolayer reached confluency and no basolateral surfaces were accessible to bacteria, levels of invasion dramatically decreased compared with invasion of nonconfluent monolayers (~0.1 versus 1 to 2% of internalized bacteria). Thirdly, treatments that exposed the basolateral pole of cells by opening intercellular junctions generally enhanced *Listeria* entry.

The effects of treatments aimed at opening intercellular junctions varied widely according to the monolayer's age, the support upon which cells were grown (plastic dish or filter), and the treatment (Ca^{2+}-free medium alone or with EGTA). Entry into 3- to 4-day-old monolayers grown on plastic dishes was enhanced two times by treatment with Ca^{2+}-free medium

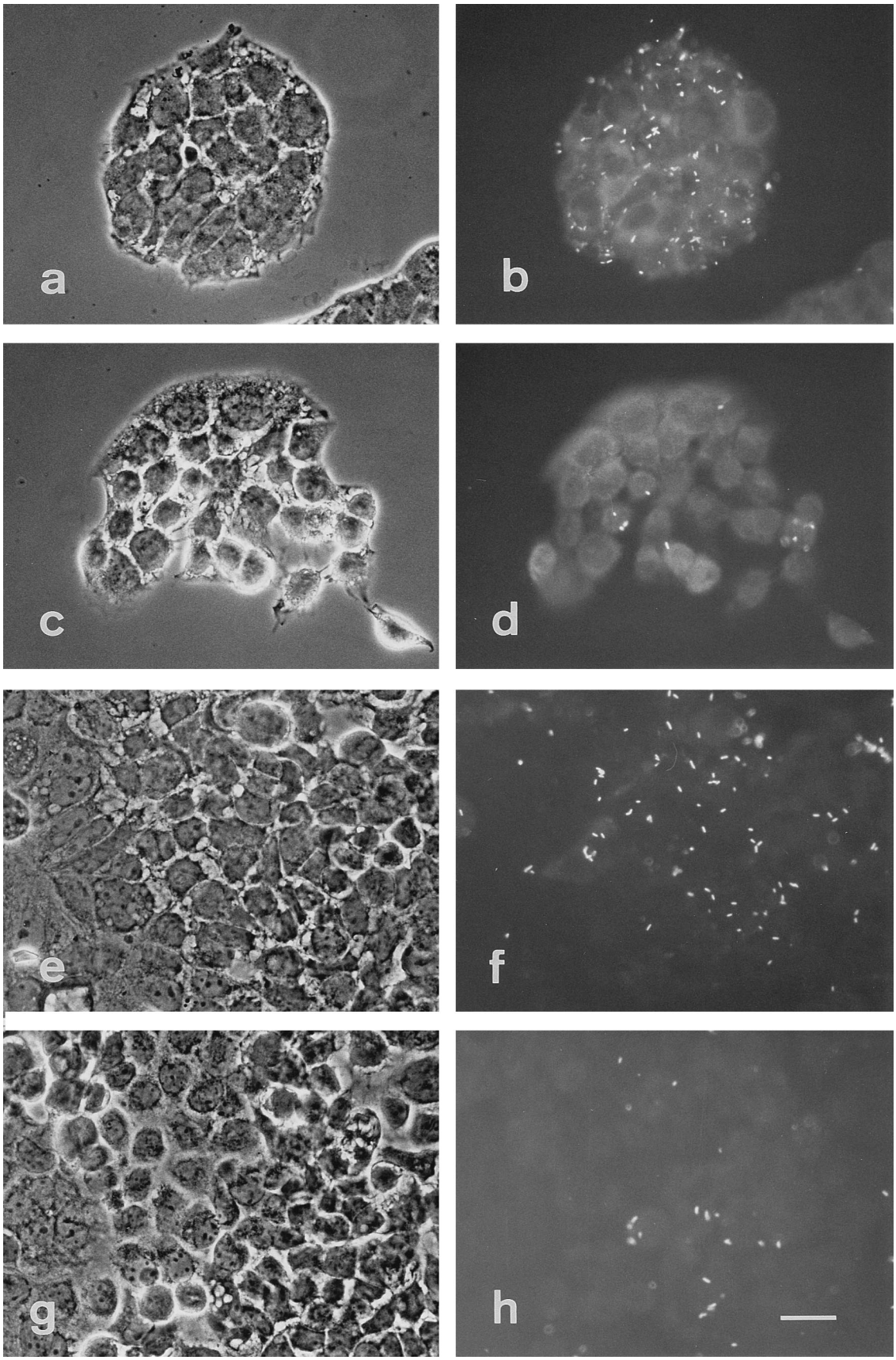
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FIG. 6. *L. monocytogenes* invasion of Caco-2 monolayers treated with Ca^{2+}-free medium with or without EGTA. Three- to four-day-old (a to d) and 14-day-old (e to h) monolayers pretreated with Ca^{2+}-free medium (a, b, e, and f) or Ca^{2+}-free medium plus EGTA (c, d, g, and h) were infected with EGD-SmR for 1 h and further incubated for 2 h with gentamicin. Cells were then fixed, permeabilized, and processed for immunolabeling of bacteria. Coverslips were examined by phase-contrast (a, c, e, and g) or fluorescence (b, d, f, and h) microscopy. Irrespective of the monolayer age, much more internalized bacteria were seen with treatment with Ca^{2+}-free medium than without treatment (see Fig. 3 and 5). Adding EGTA resulted in a dramatic decrease in the number of bacteria. Note in panels b and d that central as well as peripheral Caco-2 cells are invaded. Panels f and h show that only a few bacteria are found in areas where intercellular junctions are not well open (phase contrast, panels e and g). Bar = 10 μm.

without EGTA, whereas it was reduced 12 times in the presence of EGTA. Entry into postconfluent monolayers grown on plastic dishes was enhanced 20 times by treatment with Ca^{2+}-free medium without EGTA and only 3 times in the presence of EGTA. Entry into postconfluent monolayers grown on filters was enhanced 5 and 20 times, respectively, after treatment with Ca^{2+}-free medium and Ca^{2+}-free medium added with EGTA. A number of factors may account for these results. Treatments aimed at opening intercellular junctions appeared more effective with nonconfluent than confluent monolayers. However, cells from nonconfluent monolayers also detached more easily, especially following treatment with EGTA. Intercellular junctions were likely to be more difficult to disrupt with cells grown on filters than on plastic dishes. Similarly, Madin-Darby canine kidney cells, which are also able to form polarized monolayers, are less polar when grown on plastic than on filters (10). Finally, EGTA treatment appeared to have contradictory effects. As assessed by phase-contrast examination (monolayers grown on plastic dishes or on glass coverslips) and measurement of transepithelial resistance (monolayers grown on filters), this agent opened intercellular junctions better than Ca^{2+}-free medium alone. However, EGTA markedly inhibited the process of *L. monocytogenes* entry into Caco-2 cells.

The inhibitory effect of EGTA treatment on *Listeria* entry was reversible. When monolayers pretreated with EGTA were infected using Ca^{2+}-containing tissue culture medium, invasion levels were equal to or even greater than those reached with monolayers pretreated and infected using Ca^{2+}-free medium alone. Thus, the inhibitory effect of EGTA was probably due to depletion of Ca^{2+} or other divalent cations in the extracellular medium and not to any irreversible damage caused to Caco-2 cells. Also, immunofluorescence studies showed that the reduced *Listeria* uptake observed with EGTA resulted, at least partially, from the reduction of bacterial adhesion. Listeriae adhered four to eightfold less to monolayers if EGTA was added to Ca^{2+}-free medium. This inhibitory effect was also reversible. This suggests that the binding of *L. monocytogenes* to Caco-2 cells, which is the first step of the entry process, is Ca^{2+} dependent. In addition, the Ca^{2+} concentration must likely reach a certain threshold for completion of *Listeria* uptake. The adverse effect of EGTA might explain previous contradictory results about the effects of disruption of intercellular junctions on *Listeria* entry. Karunasagar et al. utilized Ca^{2+}-free medium with EGTA to open intercellular junctions of Caco-2 cells and found that this treatment reduced *Listeria* entry about 10-fold (20). Thus, these investigators concluded that a basolateral route of invasion was unlikely. Given our results, it is probable that the decrease in invasion in their studies was due to the inhibitory effect of EGTA.

Karunasagar et al. also presented scanning electron micrographs suggesting that *L. monocytogenes* is able to enter Caco-2 cells through the apical surface (20). Listeriae adhered to microvilli and triggered their uptake by inducing the formation of lamellipodium-like projections. The subsequent steps of invasion were not analyzed. Our results suggest that such an apical route of entry is infrequent. About 95% of bacteria internalized by 3- to 4-day-old monolayers were seen at the outer edges of islets, the only basolateral surface accessible to bacteria, and most of the remaining 5% of invasion occurred in sites appearing as intercellular spaces. Also, with postconfluent monolayers, only one cell per 10 fields (magnification of 400) contained listeriae coated with F-actin after 4 h of infection.

The role of the *inlAB* locus in entry of *L. monocytogenes* into Caco-2 cells was examined by using a transposon *inlAB* mutant (strain BUG8). The *inlAB* mutant was severely attenuated for entry, with invasion levels that were reduced 50- to 100-fold compared with its parent, irrespective of the cell monolayer age. However, it was still able to invade Caco-2 cells in small numbers. Similarly, only a partial defect in invasive capacity of an *inlAB* deletion mutant obtained from strain EGD was recently reported (5). This suggests the existence of at least another *L. monocytogenes* invasion locus besides *inlAB*, possibly also belonging to the *inl* gene family. In contrast to *inlAB*, the invasion process mediated by this putative locus does not seem to be affected by the presence of EGTA in the extracellular medium. We are currently studying the genetic basis for this invasion system and the cell receptor(s) involved in *Listeria* uptake.

We previously reported that *inlA* promotes listerial entry into Caco-2 cells grown at semiconfluency (2 to 3 days of culture) (11). As shown here, this is valid also for 1- to 21-day-old Caco-2 monolayers. Thus, the *inlA*-encoded protein, internalin, is indicated as the main component mediating invasion of Caco-2 cells, regardless of the state of cell polarization and differentiation. Conversely, the receptor for internalin is probably distributed uniformly on nonpolarized cells and basolaterally on polarized cells. Considering the inhibitory effect of EGTA on *Listeria* adhesion and entry, it is also likely that internalin-cell receptor binding is a Ca^{2+}-dependent process. Ca^{2+}-dependent adhesion to human intestinal cells has been reported for other gram-positive organisms (2, 22). Di- and trivalent cations play a role in many adherence systems. Ca^{2+} might act as an ionic bridge between surfaces of bacteria and epithelial cells (22).

By extrapolating our results to the in vivo setting, *L. monocytogenes* may enter nonpolarized enterocytic cells (undifferentiated dividing crypt cells) from the intestinal lumen but not polarized cells (nondividing villus cells). However, after oral

TABLE 1. Adhesion of *L. monocytogenes* to Caco-2 monolayers treated with Ca^{2+}-free medium with or without EGTA[a]

Treatment	Mean no. (SD) of adherent bacteria	
	3-day-old monolayers	14-day-old monolayers
No treatment	42 (12)	5 (3)
Ca^{2+}-free medium	190 (31)	82 (19)
Ca^{2+}-free medium + EGTA	26 (8)	20 (8)

[a] Monolayers treated or not with Ca^{2+}-free medium or Ca^{2+}-free medium plus EGTA were infected with EGD-SmR for 90 min at 4°C. Cells were then washed, fixed, and processed for immunolabeling of bacteria. Adherent bacteria were counted by examining 500 (3-day-old monolayers) or 2,000 (14-day-old monolayers) Caco-2 cells. Results are expressed as the mean numbers of adherent bacteria (and standard deviation) per 100 cells.

administration, listeriae are not found in crypt cells but in villus absorptive cells (32). Because invasion of these highly differentiated cells is thought to be exclusively basolateral, *L. monocytogenes* must utilize another site of entry. This could be the M cell, as reported for other bacterial pathogens (18, 23, 34, 37). This hypothesis is in agreement with previous results showing that listeriae given to rodents penetrate mostly into the Peyer's patches (26). *Listeria* infection of M cells might be followed by basolateral infection of enterocytes. Similar mechanisms have been postulated for shigellae (34). Once inside enterocytes, listeriae could multiply and propagate locally by cell-to-cell spreading (27, 36). This scenario is supported by recent work, in which the *inlAB* mutant BUG8 and the parental strain EGD-SmR were studied in mice infected by the oral route (15). BUG8 retained the ability to invade the host, but initial counts in the liver and spleen were substantially lower for this strain than for its parent, indicating a defect in initial invasion and penetration into the host.

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