BACTERIAL PATHOGENICITY

Cell proliferation enhances entry of *Listeria monocytogenes* into intestinal epithelial cells by two proliferation-dependent entry pathways

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Bacterial entry into intestinal host cells is the result of a fairly sophisticated manipulation of host cell machinery by the pathogens. To study further the potential cell target of *Listeria* spp., the in-vitro entry of *L. monocytogenes* strains into intestinal cells was examined in relation to the metabolism, proliferation and differentiation of the cells by the alamarBlue assay, [³H] thymidine incorporation, and brush border-associated enzyme activities, respectively. The study showed that cell metabolism was not involved in the entry of *L. monocytogenes* in three cell models (two human and one porcine). On the other hand, entry was closely related to the proliferation process and poorly related to the differentiation state of the cells. The use of *L. monocytogenes* mutants lacking invasion proteins showed that InlA and InlB acted in synergy to mediate the entry of *L. monocytogenes* into proliferative cells, whereas InlA alone seemed to be involved in the entry into non-proliferative cells. These two entry pathways could correspond to the two cellular processes used by *L. monocytogenes* to enter proliferative and non-proliferative cells, as suggested by the use of cytochalasin D, nocodazole, chloroquine and monodansylcadaverine. Taken together, we propose a hypothesis in which the entry of *L. monocytogenes* is mediated by interaction between randomly distributed E-cadherin on the surface of proliferative cells. In contrast, the entry into non-proliferative cells may involve pp60^{c-src}, a proto-oncogenic tyrosine kinase signal that modifies E-cadherin localisation. In conclusion, these results suggest that *L. monocytogenes* may preferentially enter crypt cells *in vivo* by a microfilament-dependent process, whereas the few bacteria that infect villus cells enter by an E-cadherin-internalin interaction that mediates microtubule-dependent endocytosis.

Introduction

Listeria monocytogenes is a gram-positive bacterium that causes an uncommon but potentially serious type of food-borne infection, with a fatality rate that is even higher than that of *Clostridium botulinum* [1]. *L. monocytogenes* infections in healthy adults are usually asymptomatic or at most produce mild influenza-like symptoms. Less commonly, diarrhoea and abdominal discomfort can occur. In fact, 5–10% of the population carry *L. monocytogenes* in their intestinal tracts without apparent symptoms [1]. *L. monocytogenes* causes more serious infections in adults with underlying conditions that compromise their immune responses, such as AIDS, diabetes and old age [1]. The primary route of *L. monocytogenes* infection is the

gastrointestinal tract, through the consumption of contaminated food [2]. *L. monocytogenes* uses M cells as an entry point [3] or it can enter intestinal cells [4]. These observations *in vivo* are consistent with in-vitro infection by *L. monocytogenes* of several intestinal cell lines of human, murine and porcine origins (Caco-2, RPMI1650 and IPI-2I cells, respectively) [5–7].

Although animal models provide the best standard for research on mechanisms of bacterial virulence, animals present a complex system in which many variables cannot be controlled. Cultured mammalian cells are commonly used to provide a simpler, more easily controlled model for investigating the host–bacterium interaction. Several bacterial genes that allow entry of *Listeria* cells by mechanisms such as actin polymerisation, intracellular multiplication and cell to cell spread, have been identified with these cultured cell lines [8, 9]. To date, three bacterial surface proteins, internalin A (InlA) and InlB (encoded by the *inlA*

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and *inlB* genes) and p60, have been involved in the entry of *L. monocytogenes* into continuous cell lines [10–12]. However, only E-cadherin, the cell receptor for InlA, has been identified [13].

The fact that *L. monocytogenes* enters just confluent Caco-2 cells suggests that it may invade undifferentiated intestinal cells [5, 14, 15]. A previous study reported that few *L. monocytogenes* strains entered intestinal and kidney finite cell lines, but the same strains entered their low and high transformed cell counterparts in greater numbers [7]. The *L. monocytogenes* susceptibility phenotype was related to the loss of contact inhibition and anchorage-dependent growth. In addition, *L. monocytogenes* strains were able to induce cell foci in a mouse finite cell line [16], and mouse fibroblast cells transfected with a recombinant plasmid containing the haemolysin gene of *L. monocytogenes* exhibited both increased cell proliferation and cell foci [17]. Taken together, these results raise the question of whether the differentiation or proliferation states of the cells modify their susceptibility to *L. monocytogenes*.

The susceptibility of intestinal cells was studied in relation to the metabolism, proliferation and differentiation of cells by use of the alamarBlue assay, [³H] thymidine incorporation and brush border-associated enzyme activities, respectively. Three intestinal cell models were used for this purpose, namely ileal finite and continuous porcine cell lines and two human colonic adenocarcinoma cell lines, i.e., Caco-2 and several subpopulations of HT-29. These colonic epithelial cells have been shown to undergo morphological and functional differentiation characteristics of mature enterocytes but in different in-vitro culture conditions [18]. When cultured in the presence of glucose the HT-29 cell line is totally undifferentiated. However, when cultured in the absence of glucose the same cells exhibit typical enterocytic differentiation after several days at confluency [19]. Similar enterocytic differentiation, characterised by polarisation of the cell layer, with the presence of tight junctions, apical brush borders and associated hydrolases, can be obtained with Caco-2 cells after several days in the presence of glucose [20]. These cell lines are now widely used in the study of human intestinal cell function [20] and in the study of bacterium–enterocyte interactions [21, 22]. The mechanisms involved in the uptake of *L. monocytogenes* by intestinal epithelial cells were investigated with selected inhibitors of eukaryotic cell structures and processes and in terms of internalin–cadherin interaction.

Materials and methods

Cell culture

The porcine ileal cell lines were established from histocompatible miniature pigs. Two small intestine cell lines were used, an epithelial finite cell line (I35) and its SV40 T antigen transformed counterpart (IPI-2I)

[7, 23]. The colonic carcinoma cell line HT-29 (passage 10–40) was obtained from ECACC (No. 91072201) and a subclone (passage 15–30) adapted to inosine was obtained from C. Sapin (INSERM, U410 Faculté de Médecine X. Bichat, Paris, France). The cells were maintained in D10 medium: Dulbecco's Modified Eagle's Medium, 25 mM glucose (DMEM) supplemented with inactivated fetal calf serum (FCS) (Gibco) 10%, 2 mM L-glutamine and insulin 10 µg/ml. Differentiated HT-29 cells were obtained by culture in a glucose-free medium supplemented with dialysed FCS and with 25 mM galactose or 2.5 mM inosine. The culture medium was changed daily in all experiments. The adenocarcinoma cell line Caco-2 (ECACC No. 86010202) was grown in D10 medium supplemented with non-essential amino acids 1%. To prevent common microbial contamination, antibiotics (penicillin 100 IU/ml, streptomycin 100 µg/ml) were added routinely to the cell culture media, except for the invasion assay. Mycoplasma contamination was routinely monitored by Hoechst 33258 stain [24].

Assessment of the proliferation and differentiation levels of the intestinal cells

Brush border enzyme activities were determined after partial purification of the membranes. At the designated times, cultures from each treatment were washed twice with phosphate-buffered saline (PBS), pH 7.4, scraped off with a plastic cell scraper and centrifuged at 270 *g* for 10 min at 4°C. The pellet was resuspended and maintained for 30 min at 4°C in 5 mM Hepes buffer, pH 7.6, supplemented with protease inhibitors: 200 µM phenylmethylsulphonyl fluoride (PMSF), leupeptin and aprotinin 10 µg/ml. Cell lysates were disrupted at 4°C with a Sonifier 250 (Branson Ultrasonics, Danbury, CT, USA) in pulse mode and centrifuged at 19 000 *g* for 20 min with an MR 1822 centrifuge (Jouan, Saint-Nazaire, France). Pellets containing membrane extracts were resuspended with 20 mM Hepes buffer. The protein concentration of cell fractions was determined by performing a micro-BCA (bicinchoninic acid) protein assay (Pierce, Rockford, IL, USA). Sucrase activity was determined according to Messer and Dahlqvist [25], alkaline phosphatase activity according to Garen and Levinthal [26] and dipeptidyl-peptidase IV activity according to Nagatsu *et al.* [27].

Incorporation of tritiated thymidine was used to assess DNA synthesis as a marker of cell proliferation. At the designated time points, cells grown in P96-wells were washed and cultured for 4 h at 37°C in a CO₂ incubator in a medium without FCS but supplemented with glucose, galactose or inosine and [³H] thymidine (DuPont de Nemours) 1 µCi/ml. Non-incorporated thymidine was washed off in PBS and plates were frozen at –20°C. After thawing, cells were resuspended and transferred on to filters by a cell harvester. Dried filters were counted in scintillation vials.

The alamarBlue assay (Alamar Biosciences, Sacramento, CA, USA) was used to assess the oxidation–reduction potential of the cells as a marker of cell metabolism activity [28]. AlamarBlue fluoresces in response to the chemical reduction of growth medium by cells. Cells were grown in P96-wells in the same conditions as were used to assess [³H] thymidine incorporation. The culture medium was removed and 100 µl of culture medium supplemented with alamarBlue 10 µl were incubated for 2 h at 37°C. The fluorescence measurements were collected from the cytofluor (Millipore).

Anchorage independence assay

Anchorage independent growth was determined with Noble agar (Difco) or agarose (Serva, Saint Germain en Laye, France) as described by MacPherson and Montagnier [29] with some modifications [16]. Briefly, cells were diluted to 5×10^4 /ml of culture medium containing agar 0.4% and agarose 0.5%. Two ml of cell suspension were then plated in a 6-well culture plate containing a solidified bottom layer of agar 0.75% or agarose 1% in culture medium. After 1 week, the agar or agarose layer was overlaid with 1 ml of culture medium. Colonies larger than 12 cells were scored under an inverted microscope following incubation for 2 or 3 weeks at 37°C.

Detection of E-cadherin of intestinal cells

To study all the E-cadherin of the cells, E-cadherin was immunoblotted with specific antibody (Transduction Laboratories) from the ionic detergent-soluble cell fraction [30]. Intestinal cells, cultured as previously indicated, were washed four times with PBS and lysed in buffer A (10 mM Tris HCl (pH 7.4), SDS 1%) and then boiled for 10 min. Solubilised proteins were collected after sonication and centrifugation for 5 min at 14 000 g. The same amount of protein (100 µg) was electrophoresed on a polyacrylamide 10% gel and transferred to nitrocellulose. E-cadherin was revealed by incubation with anti-E-cadherin monoclonal antibody overnight, at 4°C, followed by incubation for 45 min at 20°C with biotinylated anti-mouse immunoglobulins and then streptavidin horseradish peroxidase (30 min at 20°C) and subsequently developed with 3-3'-diaminobenzidine.

Bacterial strains and growth media

The INRA 76 strain, serotype 4b, was a gift from Dr Bille (Centre National de Référence des *Listeria*, CHU Vaudois, Lausanne). This strain is a human isolate from an outbreak in Switzerland that expresses a haemolytic titre of 100 [31]. Strain Bug 949, a chromosomal mutant of *L. monocytogenes* EGD with an in-frame deletion in the *inlA* and *inlB* genes, expresses neither InlA nor InlB. The chromosomal mutants $\Delta inlA$ and $\Delta inlB$ with an in frame *inlA* or *inlB* deletion were also

used [12]. These strains and the EGD strain were the gift of P. Cossart (Pasteur Institute, Paris, France). *L. monocytogenes* strains were grown in Brain Heart Infusion (BHI) Broth (Difco) and then in a BHI agar tube. For each experiment the number of cfu was checked by plating the appropriate dilution on tryptic soy agar (TSA) plates.

Infection of intestinal cells

The method for determination of entry of *Listeria* strains into intestinal cells has been described elsewhere [32]. Briefly, cell monolayers were grown on 24-well tissue culture plates (Falcon). Cells were incubated in medium without antibiotics overnight before use. Cell monolayers were infected for 2 h at 37°C with 200 µl of bacterial suspension at a density of 5×10^7 cfu/ml in appropriate medium without FCS. After washing, cell monolayers were overlaid with fresh medium containing gentamicin 100 µg/ml. After incubation for 1.5 h at 37°C, cells were washed twice and lysed by adding 1 ml of cold distilled water. The number of viable bacteria released from the cells was assessed on TSA plates by counting appropriate dilutions.

Infection of cells in the presence of biochemical inhibitors

Inhibition assays were conducted by adding individual inhibitors to the monolayers 1.5 h before the addition of bacteria. Inhibitors were maintained throughout the 2-h infection period, and the infection assay was performed as described previously. Low concentrations of each inhibitor used were chosen, to avoid the interference often described at high concentrations [33] and to retain bacterial and cell viability. Epithelial cell viability over the assay period was measured by the trypan blue exclusion assay. All inhibitors, from Sigma, were used from stock solutions: cytochalasin D 500 µg/ml and nocodazole 10 mg/ml in dimethylsulphoxide (DMSO), 25 mM monodansylcadaverine in methanol and chloroquine 100 mg/ml in distilled water. Controls of bacterial entry were performed in the presence of DMSO alone.

Results

Characteristics of porcine intestinal cells

To understand how the porcine intestinal finite cell line was resistant to entry of *L. monocytogenes* in contrast to its SV40 T antigen transformed counterpart, certain cell characteristics were compared. These two cell lines exhibited numerous phenotypic differences. The greatest differences between these two cell lines were the proliferation level and metabolic activity (Table 1). The I35 cells were weakly differentiated intestinal cells. Table 1 shows that I35 cells had some brush borders as previously demonstrated [7], a high level of alkaline phosphatase but a weak level of dipeptidyl peptidase IV

Table 1. Characteristics of porcine intestinal cells

Cell characteristics	Intestinal finite cell line I35	Intestinal continuous cell line IPI-2I
<i>L. monocytogenes</i> entry rate	3.24 SD 0.02*	5.95 SD 0.04
Alkaline phosphatase	9.1 SD 0.1 [†]	1.01 SD 0.1
Dipeptidyl peptidase IV	43.3 SD 1.7 [†]	8.2 SD 0.1
Cell proliferation	3437 SD 1906 [‡]	80977 SD 29680
Metabolic activity	2982 SD 143 [§]	7836 SD 356
Contact inhibition	+	—
Anchorage-dependent growth	+	—
E-cadherin**	—	+
Cytokeratine 18 ^{††}	—	+
Villin ^{††}	—	—
Brush border ^{‡‡}	Some	Some
Desmosomes ^{‡‡}	No	Some

*Mean number (log₁₀) and SD of gentamicin surviving INRA 76 strain of *L. monocytogenes* bacteria 3.5 h after infection. Similar results were obtained with the EGD strain (not shown).

[†]Expressed in mIU/mg of protein from membrane extract.

[‡][³H]-labelled thymidine incorporated after 4 h.

[§]Arbitrary fluorescence units.

^{||}Determined by growth capacity in both agar and agarose [16].

**Determined by Western blotting of total cell extract and revealed by anti-E-cadherin monoclonal antibody (Transduction Laboratories).

^{††}Determined by immunofluorescence staining with monoclonal antibodies raised either against cytokeratin peptide 18 (Sigma) or against villin (Bioscience Int., Kennebunkport, ME, USA) [23].

^{‡‡}Determined by electron microscopy [7, 23].

activity compared to results obtained with HT-29 cells (Table 2). The IPI-2I cells had no brush border enzyme activity but had some brush borders and desmosomes [23]. These results showed that the markers of proliferation, differentiation and metabolic activity towards *L. monocytogenes* invasion were different in the susceptible and the resistant cells. To identify whether proliferation or differentiation states are involved in *L. monocytogenes* susceptibility, Caco-2 cells, which can spontaneously differentiate upon reaching confluence in normal culture conditions, were used.

Entry of *L. monocytogenes* into Caco-2 cells in relation to proliferation and differentiation states

The cells infected by *L. monocytogenes* were characterised in terms of proliferation, metabolic activity

and brush border-associated enzymes (Fig. 1). The expression of enzyme activities, i.e., sucrase and alkaline phosphatase, the most common markers used for the functional differentiation of intestinal absorptive cells, increased as a function of the days in culture (Fig. 1a). It is notable that the levels of enzyme activities in the cell homogenate were in agreement with those reported by Pinto [18]. According to the expression of differentiation-associated hydrolases, the differentiation process began only at day 8 of culture. At days 17 and 20 of culture a differentiated cell monolayer was obtained.

The entry of *L. monocytogenes* was markedly modified by the number of days in culture (Fig. 1b). The bacterial entry rate was expressed as the number of cfu/viable cell because the number of cells/well was lower in the case of semiconfluent cells than with cells at day 20. The number of intracellular bacteria which were recovered 2 h after infection with a multiplicity of infection (MOI) of 100 bacteria/cell, corresponded to 29 bacteria/cell (2.2×10^6 bacteria/well) for semiconfluent monolayers (second day) compared to 0.7 bacteria/cell (2.7×10^5 bacteria/well) for monolayers at days 15–20 of culture.

Other cell markers were modified during the differentiation process of Caco-2 cells. [³H] thymidine incorporation diminished with the age of the cell culture (Fig. 1c), suggesting a decrease in cell proliferation, as already reported by the assessment of protein concentration in wells during cell culture [19]. Cell proliferation was minimal at days 15–20 of culture, corresponding to the minimal entry rate (similar numbers of cfu were obtained between days 15 and 20). Taken together, these results showed that proliferative, undifferentiated intestinal cells were more susceptible to entry of *L. monocytogenes* than non-proliferative, differentiated cells.

Cell susceptibility to invasion by *L. monocytogenes* was enhanced by the loss of contact inhibition and anchorage-dependent growth [16]. Therefore, the study

Table 2. Invasion of HT29 cells cultured in different conditions

Culture medium	Duration of culture (days)	Culture medium 18 h before infection	Entry rate* log ₁₀ cfu (SD)	Cell proliferation [†] Mean (SD) cpm	Alkaline phosphatase [‡] Mean (SD) mIU/mg	Dipeptidylpeptidase IV [‡] Mean (SD) mIU/mg
Glucose	6	Glucose	7.16 (0.07)	100 558 (12332)	0.57 (0.02)	55 (3)
	6	Inosine	5.90 (0.03)	55 461 (3832)	0.6 (0.02)	50 (5)
Galactose	6	Galactose	4.14 (0.09)	10 829 (2173)	1.73 (0.03)	96 (4)
	21	Galactose	4.48 (0.09)	14 147 (6420)	0.6 (0.01)	118 (5)
Inosine	21	Glucose	6.61 (0.01)	83 119 (18147)	ND	ND
	6	Inosine	5.15 (0.10)	28 964 (6072)	0.98 (0.02)	110 (11)
	6	Glucose	6.95 (0.05)	104 902 (20168)	ND	104 (4)
	40	Inosine	5.30 (0.02)	28 619 (9454)	6.03 (0.11)	119 (10)
	40	Glucose	6.20 (0.05)	103 215 (48535)	6.43 (0.05)	114 (9)

ND, not done.

*Mean number (log₁₀) (SD) of gentamicin-surviving bacteria 3.5 h after infection.

[†][³H]-labelled thymidine incorporated (cpm) after cell culture for 3.5 h in the presence of radiolabelled compound.

[‡]Expressed in mIU/mg of protein from membrane extract.

Fig. 1. Entry of *L. monocytogenes* strain into Caco-2 cells in relation to state of differentiation and proliferation. (a) The degree of Caco-2 cell differentiation was evaluated according to both alkaline phosphatase (◆) and sucrase (□) activities. (b) The entry of *L. monocytogenes* INRA 76 strain was studied in relation to the duration of Caco-2 cell culture in days. (c) The degree of cell proliferation was evaluated by thymidine incorporation (◇), and metabolic activity was evaluated according to the reduction of the oxidation–reduction indicator alamarBlue (■).

investigated whether anchorage-dependent growth was different in susceptible undifferentiated Caco-2 cells and their resistant differentiated counterparts. The relative effects of days in culture on anchorage-

dependent growth were compared with both standard soft agar and agarose. At day 20 of culture, anchorage-dependent growth was greater than that obtained for cells at day 7 of culture. The percentage

of colony-forming efficiency was increased by 0.023 SD 0.009 to 0.17 SD 0.06 in agar and by 0.08 SD 0.008 to 0.89 SD 0.12 in agarose. These differences were highly significant ($p < 0.01$) and were consistent with previous observations in SV40 transformed cell lines and 5-azacytidine-treated finite cell lines [16]. However, the fact that differentiated Caco-2 cells proliferate very little could explain these results. To disentangle further the relationship between proliferation and differentiation, several subpopulations of HT-29 cells were used. The HT-29 cell model is particularly attractive, as it is the only cell line of intestinal origin to display reversible structural and functional features of mature intestinal epithelial cells [34].

Entry of L. monocytogenes into human intestinal cells in relation to metabolic activity

The alamarBlue assay showed that chemical reduction of growth medium occurred in parallel with the expression of the differentiation markers and contrary to the proliferation level of Caco-2 cells (Fig. 1c). In addition, entry of *L. monocytogenes* was poorly related to metabolic activity with HT-29 cells (Fig. 2). These results are the opposite of those obtained with porcine cells, where the entry of *L. monocytogenes* was related to metabolic activity (Table 1). It is not known why chemical reduction of growth medium was inversely proportional to intestinal human cell growth compared to intestinal porcine cells, but it could explain the daily medium change necessary with differentiated inosine-adapted HT-29 cells.

Entry of L. monocytogenes into HT-29 cells in relation to culture conditions

As observed with Caco-2 cells, the entry of *L. monocytogenes* into inosine-adapted HT-29 cells was closely related to days of culture and to the proliferation rate of cells, assessed by [^3H] thymidine incorporation (Fig. 3). Thus, the entry and proliferation rates decreased by factors of 100 and 4, respectively, between cells grown in glucose ($t = 0$) and cells grown for 6 days in inosine medium ($t = 6$). Between days 6 and 40 in inosine medium, there was no significant difference either in the cell proliferation rate or in the *L. monocytogenes* entry rate. Cell differentiation began from day 6 in inosine medium, as demonstrated by the brush border-associated enzyme activities, i.e., alkaline phosphatase and dipeptidylpeptidase IV activities (Table 2). The HT-29 cells were only terminally differentiated at day 40 in inosine medium, as demonstrated by the alkaline phosphatase activity (Table 2), and by the expression of villin (data not shown). No terminally differentiated HT-29 cells were obtained in galactose at day 21. The proliferation levels of the galactose-adapted cells (at days 6 and 21) were lower than those obtained with inosine-adapted cells and were related to lower *L. monocytogenes* entry rates (Table 2).

The results show that HT-29 cells were undifferentiated and proliferative in glucose medium, whereas they were undifferentiated and non-proliferative in inosine or galactose medium at day 6 of culture. As the *L. monocytogenes* entry rate was lower at day 6 in

Fig. 2. Entry of *L. monocytogenes* strain into inosine-adapted HT-29 cells in relation to cell metabolism. The entry of *L. monocytogenes* INRA 76 strain was studied in relation to the duration of inosine-adapted HT-29 cells grown in glucose medium ($t = 0$) or for several days in inosine medium. The number of bacteria that survived exposure to gentamicin on account of their intracellular location was assessed after bacteria-cell contact for 3.5 h (◆). The chemical reduction of growth medium was evaluated by alamarBlue assay (□). Values represent means and SD of six wells. A representative experiment is shown which was reproduced in two independent trials.

Fig. 3. Entry of *L. monocytogenes* strain into inosine-adapted HT-29 cells in relation to state of cell proliferation. The entry of *L. monocytogenes* INRA 76 strain was studied in relation to the duration of inosine-adapted HT-29 cells grown in glucose medium ($t=0$) or several days in inosine medium. The number of intracellular bacteria that survived exposure to gentamicin was assessed after bacteria-cell contact for 3.5 h (◆). The degree of cell proliferation was evaluated by [3 H] thymidine incorporation (□). Values represent means and SD of six wells. A representative experiment is shown which was reproduced in three independent trials.

inosine medium than in glucose medium, our hypothesis is that cell proliferation plays a major role in the susceptibility to *L. monocytogenes*.

Entry of L. monocytogenes into HT-29 cells in relation to cell proliferation level

To demonstrate further the relationship between cell proliferation level and *L. monocytogenes* entry rate, the proliferation state of the cells was modified without modifying the differentiation markers. This was achieved by adding either glucose to the culture medium of cells cultured with inosine, or inosine to the culture medium of cells cultured with glucose. In these conditions, [3 H] thymidine incorporation decreased from 100 000 cpm for HT-29 cells cultured in glucose medium to 55 500 cpm for cells cultured for 18 h in inosine medium. In the same conditions, the entry of *L. monocytogenes* decreased from 7.2 to 5.9 log₁₀ cfu, without modification of brush border enzyme activities (0.57 versus 0.6 and 55 versus 50 mIU/mg for alkaline phosphatase and dipeptidyl-peptidase IV activity, respectively) (Table 2). Similar results were obtained when glucose was substituted for galactose (data not shown). In contrast, when cell proliferation of differentiated cells was induced by the substitution of inosine for glucose, brush border enzyme activities were not modified (at day 40 of culture, alkaline phosphatase and dipeptidylpeptidase IV activities varied from 6.03 to 6.43 and 119 to

114 mIU/mg, respectively), but the entry of *L. monocytogenes* was markedly increased, reaching values close to those obtained at day 6 in glucose medium (Table 2). Similar effects on the entry of *L. monocytogenes* were obtained when non-proliferative and undifferentiated cells cultured for 6 days in inosine medium were cultured for 18 h in a medium in which inosine or galactose was substituted for glucose. These results show that modification of cell proliferation without modifying cell differentiation altered the entry of *L. monocytogenes* into intestinal cells.

Role of internalin in the entry of L. monocytogenes into proliferative and non-proliferative HT-29 cells

L. monocytogenes requires the *inl* locus to enter epithelial cells [10]. The study tested whether InlA and InlB were involved in the entry of *L. monocytogenes* into intestinal cells. As shown in Fig. 4a, the *inlAB* mutant was markedly attenuated for entry into HT-29 cells, with entry levels reduced 100–3300-fold compared to the wild-type EGD. With intestinal porcine cells the entry level of the *inlAB* mutant was reduced 250-fold (Fig. 4b). These data confirmed that the *inlAB* locus was necessary for entry into intestinal epithelial cells. However, the decrease in entry rate was only partial. Indeed, up to 0.1% of the bacteria deposited entered cells (Fig. 4a). The respective role of the InlA and InlB proteins was

Fig. 4. Entry into intestinal cells of *L. monocytogenes* strains carrying deletions in the *inlAB* locus. HT-29 cells were cultured in glucose medium (proliferative undifferentiated HT-29 cells: HT29-P-UD) or for 6 days in inosine medium (non-proliferative undifferentiated HT-29 cells: HT29-NP-UD) or for 40 days in inosine medium (non-proliferative differentiated HT-29 cells: HT29-NP-D) or for 40 days in inosine medium and 18 h in glucose medium (proliferative differentiated HT-29 cells: HT29-P-D). Cells were then infected for 2 h with 3×10^7 bacteria: (█) wild-type EGD, (▨) *inlA* negative mutant, (▩) *inlB* negative mutant, (■) *inlAB* negative mutant. This figure summarises two-to-six independent gentamicin survival assays, performed in duplicate. (a) Each value represents the mean (log₁₀) of gentamicin-surviving bacterial counts (cfu) and SD. (b) Values along the vertical axis are given in relation to the invasion of the wild-type strain EGD which is arbitrarily fixed at 100.

then studied with proliferative or differentiated HT-29 cells, or both. The study demonstrated that *InlA* is essential for entry into non-proliferative HT-29 cells whereas *InlB* is not (Fig. 4b). The weak entry rate of the $\Delta inlA$ mutant was irrespective of the cell differentiation state. In contrast, the entry of *L. monocytogenes* into proliferative cells was dependent on the expression of both *InlA* and *InlB* proteins. Indeed, the *inlAB* mutant entry was markedly attenuated whereas the single $\Delta inlA$ and $\Delta inlB$ mutants expressed only a partial reduction in entry capacity (Fig. 4b). The entry of *L. monocytogenes* strains carrying deletions in the *inlAB* locus into the proliferative porcine intestinal cells confirmed the synergy between *InlA* and *InlB* for entry into proliferative intestinal cells (Fig. 4b).

*Role of cytoskeletal proteins in the entry of *L. monocytogenes* into proliferative and non-proliferative HT-29 cells*

To determine whether the entry of *L. monocytogenes* into proliferative and non-proliferative HT-29 cells is associated with the same entry mechanism, cells were treated with specific inhibitors before and during bacterial infection. Experiments were performed with the human intestinal HT-29 cell line cultured in glucose or inosine and with porcine intestinal cell lines (Table 3). Disruption of actin filaments by cytochalasin D at a low concentration (0.1 $\mu\text{g/ml}$) significantly ($p = 0.001$) blocked the entry of *L. monocytogenes* into non-proliferative HT-29 cells (93.2% inhibition) in a dose-dependent manner. The inhibitory effect was

Table 3. Inhibition of intestinal cell invasion by *L. monocytogenes* after treatment of cells with inhibitors of microfilaments, microtubules and endocytosis*

Inhibitors	Concentration ($\mu\text{g/ml}$)	Human intestinal cells		Porcine intestinal cells	
		Non-proliferative HT29 cells $\log_{10}$ cfu (SD)	Proliferative HT29 cells $\log_{10}$ cfu (SD)	Finite cell line I35 $\log_{10}$ cfu (SD)	Continuous cell line IPI-21 $\log_{10}$ cfu (SD)
Control	...	5.15 (0.01) [†]	7.42 (0.01)	3.24 (0.02)	5.95 (0.04)
Cytochalasin D	0.05	4.90 (0.01)	7.18 (0.01)	2.73 (0.04)	5.22 (0.07)
	0.1	3.98 (0.01)	5.93 (0.06)	2.53 (0.06)	3.44 (0.10)
Nocodazole	1	4.19 (0.04)	7.02 (0.14)	<1.5	4.84 (0.13)
	5	3.18 (0.15)	7.03 (0.15)	<1.5	4.49 (0.14)
Monodansyl	8.5	4.56 (0.11)	7.22 (0.01)	ND	5.41 (0.03)
Cadaverine	34	2.99 (0.15)	6.92 (0.03)	ND	4.41 (0.19)
Chloroquine	10	4.07 (0.01)	7.15 (0.03)	2.43 (0.03)	ND
	50	3.79 (0.05)	7.16 (0.11)	Cytotoxic	4.84 (0.07)
	100	2.92 (0.05)	7.09 (0.01)	Cytotoxic	3.81 (0.11)

ND, not done.

*Entry assay was performed in the continuous presence of each reagent after pre-incubation of cells for 30 min with each reagent.

[†]Mean number of bacteria ($\log_{10}$) and SD of duplicates.

higher with proliferative HT-29 cells (96.7% inhibition; $p < 0.001$). Inhibition assays with nocodazole, monodansylcadaverine and chloroquine showed that the entry of *L. monocytogenes* into HT-29 proliferative cells was little affected (59.2, 68.0, 52.6% inhibition respectively) and was not dose-dependent. In contrast, the entry of *L. monocytogenes* was significantly inhibited ($p < 0.05$) in non-proliferative cells by the addition of the above inhibitors in a dose-dependent manner (98.3, 99.3 and 99.4% inhibition respectively) (Table 3). The same inhibition assay was conducted with proliferative IPI-21 cells and their non-proliferative non-transformed parental I35 cells. As observed with HT-29 cells, entry of *L. monocytogenes* into proliferative IPI-21 cells was significantly ($p < 0.01$) more affected by cytochalasin D than entry into non-proliferative I35 cells (99.7 versus 80.7% inhibition respectively). Moreover, disruption of microtubules by nocodazole showed a greater decrease in the entry of *L. monocytogenes* into non-proliferative than entry into proliferative cells (99.5 versus 96.3% inhibition respectively). Nocodazole showed a greater inhibitory effect on entry of *L. monocytogenes* into IPI-21 cells than into proliferative HT-29 cells. Infection of IPI-21 cells was significantly ($p < 0.01$) reduced in a dose-dependent manner by both monodansylcadaverine and chloroquine. No results were obtained with I35 cells treated by monodansylcadaverine because of its cytotoxicity, as observed by trypan blue exclusion assay. Chloroquine exhibited the same inhibitory level with I35 cells as with non-proliferative HT-29 cells at the same concentration. Cytotoxicity against I35 cells was observed at higher concentrations.

Discussion

Penetration into the intestinal epithelium is a critical step in the pathogenesis of *L. monocytogenes*. Unfortunately, the natural cell target of entry of *L. monocytogenes* into the intestine is still unknown. A

previous study reported that the loss of contact inhibition and anchorage-dependent growth enhanced the entry of *L. monocytogenes* strains into non-phagocytic cells [16]. One explanation might be that the main route of entry *in vivo* involves parts of the enterocytes that are at a defined state of differentiation or proliferation. To demonstrate further the relationship between differentiation, proliferation, cell metabolism and the *L. monocytogenes* entry rate, three intestinal cell models were used: one porcine, used as finite cell lines (I35) and its SV40 TAg-transformed cell line (IPI-21), and two human (Caco-2 and HT-29). These cells showed marked differences in the *L. monocytogenes* entry rate related to the cell cycle and also involving different entry pathways.

This study on the cell states enhancing *L. monocytogenes* entry showed that metabolic activity did not modify cell permissivity. Indeed the alamarBlue assay showed a considerable chemical reduction of growth medium in susceptible porcine cells, whereas a highly oxidised environment was observed with susceptible human cells. It also showed (with Caco-2 and HT-29 cells) that proliferative undifferentiated cells were susceptible to *L. monocytogenes* entry, unlike non-proliferative differentiated cells. The level of intracellular *L. monocytogenes* was thus decreased by 98% between Caco-2 cells cultured for 2 or 20 days. These results agree with those of Gaillard *et al.*, who reported a greater infection rate of semiconfluent monolayers of Caco-2 cells by *L. monocytogenes* [5, 15].

However, differentiation is a growth-related phenomenon. The differentiation process starts as soon as confluence is reached, i.e., when the cells stop dividing. This process has been described with both Caco-2 and HT-29 cells [20]. This study provides evidence that cell proliferation (but not cell differentiation) enhances the entry of *L. monocytogenes*. This was suggested with HT-29 cells between days 1

and 5 of culture in inosine where the cell proliferation diminished five-fold, the cell infection diminished 100-fold and the brush border enzyme activities were not modified. The role of cell proliferation was demonstrated when proliferation and differentiation levels of HT-29 cells were independently modified according to data showing that growth and intestinal differentiation may be independently regulated in HT-29 cells [34] and that the differentiation process of HT-29 cells is reversible *in vitro* only after several passages [18]. After the addition of glucose to medium of non-proliferative differentiated and undifferentiated inosine-adapted cells, the entry of *L. monocytogenes* increased eight- and 78-fold respectively, whereas cell proliferation increased in both cases without modification of brush border enzyme activities. When glucose was substituted for inosine or galactose, cell proliferation and *L. monocytogenes* entry rates were markedly decreased (20- and 66-fold respectively) without modification of brush border enzyme activities. These results show that proliferative differentiated cells are as susceptible to entry of *L. monocytogenes* as proliferative undifferentiated cells, in contrast to non-proliferative undifferentiated cells. Taken together, these data indicate that the entry of *L. monocytogenes* into human intestinal cells is a proliferation-dependent process. These results agree with the 23-fold greater proliferation rate of susceptible IPI-2I porcine intestinal cells compared to I35 cells and might indicate a proliferation cell-specific mechanism of bacterial entry.

Proliferation-dependent entry is supported by the demonstration of two bacterial entry mechanisms according to the proliferation state of the cells. As previously reported with other epithelial and endothelial cells, it has been shown that the *inlAB* locus of *L. monocytogenes* is involved in entry into both human and porcine intestinal cells [10, 12, 35, 36]. The entry of *L. monocytogenes* into non-proliferative HT-29 cells was strictly dependent on the InlA protein (98.6% inhibition compared to the wild-type strain), whereas the entry into proliferative cells implied a synergy between InlA and InlB proteins.

Similarly, the present study showed that two cell pathways were used by *L. monocytogenes* to enter proliferative and non-proliferative cells. The entry of *L. monocytogenes* into proliferative undifferentiated HT-29 cells and into proliferative poorly differentiated IPI-2I cells seemed to be strictly microfilament-dependent. Thus, the level of *L. monocytogenes* entry into porcine cells decreased 328-fold after cytochalasin D treatment and 29-fold after nocodazole treatment. In contrast, the entry of *L. monocytogenes* into non-proliferative porcine and human cells was strongly microtubule-dependent and was, to a lesser extent, significantly reduced by microfilament depolymerisation. The entry of *L. monocytogenes* into non-proliferative HT-29 cells was also strongly reduced by inhibitors of coated-pit formation (144-fold) and inhibitors of endosome

acidification (170-fold). These inhibitors exhibited weak effects on proliferative HT-29 cells (three- and two-fold respectively). Monodansylcadaverine inhibits transglutaminase activity and prevents receptor clustering [37]. Chloroquine, a lysosomotropic agent, prevents receptor–ligand dissociation. Thus, these results suggest that the entry process of *L. monocytogenes* into non-proliferative HT-29 cells involved receptor-mediated endocytosis with microtubules of the cells. This entry process has already been described for *Citrobacter freundii* and *Ehrlichia* spp., whose entry into INT407 intestinal cells is blocked by inhibitors of coated-pit formation and endosome acidification [38]. Microtubule-dependent entry was also consistent with the inhibition of entry of *L. monocytogenes* into dendritic cells after nocodazole treatment [39].

E-cadherin was recently identified as the cellular receptor involved in the entry of *L. monocytogenes* into epithelial cells [13]. E-cadherin is a member of the cadherin family, which plays a central role in cell sorting during morphogenesis and also mediates the formation of functional complexes and cell polarisation [40]. Western blots of total cell extracts with monoclonal antibody against E-cadherin revealed a 120-kDa band with human Caco-2 and HT-29 cells and with porcine IPI-2I cells (data not shown). However, spatial restriction of E-cadherin to the lateral surface of cells occurs during the phases of polarity/differentiation development [41]. There is thus a discrepancy between the high entry rate of *L. monocytogenes* into proliferative undifferentiated cells that express a reduced level of surface membrane E-cadherin and the low entry rate into non-proliferative differentiated cells that express a high level of intercellular E-cadherin. On the basis of the current results and of findings from other laboratories, we speculate that entry of *L. monocytogenes* into intestinal cells occurs as follows.

L. monocytogenes may enter proliferative cells through the entire cell surface. This was previously suggested for non-polarised cells by Gaillard and Finlay [15]. Proliferation and polarisation are closely related and often a proliferative cell is not polarised. We suggest that *L. monocytogenes* selectively enter proliferative cells because non-confluent HT-29 cells grown in inosine were weak proliferative cells, and were poorly polarised (there is no tight junction). However, few *L. monocytogenes* entered these cells compared to non-confluent HT-29 cells grown in glucose which were proliferative and poorly polarised cells. Our results also indicate that InlA and InlB act in concert to mediate a microfilament-dependent entry process. The entry of *L. monocytogenes* may be mediated by interaction between randomly distributed E-cadherin molecules on the surface of proliferative cells. Indeed, the compaction-specific redistribution of E-cadherin is reversed in adult tissues during mitosis. Distribution is diffuse over the cell surface in all M-phase cells [41].

In addition, the entry process may involve microfilaments, because E-cadherin is poorly associated with catenin and actin in proliferative cells [42].

Actin is closely related to the E-cadherin–catenin complex within non-proliferative cells and E-cadherin is spatially restricted to the lateral surface [41]. This could explain the low entry rate observed with non-proliferative/polarised cells. *L. monocytogenes* seems to enter into these cells by an internalin–E-cadherin interaction involving receptor-mediated endocytosis and microtubules. This entry process is in agreement with the demonstration that 18 h after glucose substitution by inosine, entries of bacteria markedly decreased. Indeed, Nathke *et al.* [40] suggested that E-cadherin becomes associated with the cytoskeleton 10 min after stable cell–cell contact and 1 h after this contact there is an increase in the amount of E-cadherin at the contact site. Two models could adequately account for this entry. First, *L. monocytogenes* enters through the lateral surface of cells but not by the apical surface as previously described [15]. However, semi-confluent HT-29 cells grown in inosine were infected in the same range as confluent cells (data not shown). Secondly, *L. monocytogenes* was able to induce an src-related tyrosine kinase signal that modified E-cadherin localisation. This hypothesis is in agreement with the role of tyrosine phosphorylation in the disruption of cadherin–catenin complexes [30]. In addition, a previous study demonstrated that protein tyrosine kinases play an important role in the entry of *L. monocytogenes* into epithelial cells [32].

This study used cultured cell lines that were carcinoma cells or finite cell lines and, therefore, differed from normal small intestine epithelial cells. The infection of these cell lines by *L. monocytogenes* may not necessarily be identical to in-vivo infection of the intestine. However, both in HT-29 and Caco-2 cells, the time course of the differentiation process closely mimics the situation found in the small intestine [20]. There is a particular situation in the intestine due to rapid epithelial cell renewal, with proliferating cells being undifferentiated and with the differentiation occurring during the crypt to villus migration of non-dividing cells [43, 44]. The results of this study strongly suggest that *L. monocytogenes* may preferentially enter crypt cells of the intestine *in vivo* by a microfilament-dependent process involving the bacterial *inlAB* locus. On the other hand, few *L. monocytogenes* may enter villus cells by an internalin–E-cadherin interaction involving microtubule-dependent and receptor-mediated endocytosis. Further studies are needed to elucidate cell receptors involved in the internalisation of *L. monocytogenes* into proliferative intestinal cells and the mechanisms of entry into proliferative and non-proliferative cells.

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