BACTERIAL PATHOGENICITY

The effects of inhibitors of vacuolar acidification on the release of *Listeria monocytogenes* from phagosomes of Caco-2 cells

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To evaluate the role of the acidic pH of phagosomes on the invasive ability and fate of *Listeria monocytogenes* within host cells, entry and replication of this gram-positive bacterium in a human enterocyte-like cell line (Caco-2) were investigated by a combination of biochemical and ultrastructural approaches. The effects of inhibitors of vacuolar acidification – the lipophilic weak base ammonium chloride, the carboxylic ionophore monensin and the vacuolar proton ATPase inhibitor bafilomycin A₁ – on the bacterial invasion pathway were analysed. These agents, which raise the intracellular vesicle acidic pH of living cells by different mechanisms, affected *L. monocytogenes* replication in Caco-2 cells. Bacteria internalised by bafilomycin-treated cells were unable to escape from phagosomes, as demonstrated by electron microscopy. The results provide evidence that low pH is required for efficient intracellular growth of *L. monocytogenes*.

Introduction

Listeria monocytogenes is an intracellular, facultative, gram-positive bacterium that causes serious and sometimes fatal infections in pregnant women, neonates and immunocompromised patients [1]. Most outbreaks of listeriosis are clearly associated with the ingestion of contaminated food and there is evidence that the intestine is the usual site of entry of *L. monocytogenes* [2, 3].

This micro-organism can enter and multiply in professional and non-professional phagocytes [4, 5] and its invasion strategy in enterocyte-like cells has been well characterised [6, 7]. Each step of the invasive process of *L. monocytogenes* is controlled by different genes [5, 8]. Entry into epithelial cells is mediated by internalin, an 80-kDa bacterial protein coded by the *inlA* chromosomal gene, interacting with an unknown receptor on the cell surface [9]. Once inside the phagosome, *L. monocytogenes* produces the exotoxin listeriolysis O, a 58-kDa protein coded by the *hly* gene, which participates in the lysing of the phagolysosomal membrane allowing the invading bacterium free access to the host cytoplasm [10–12].

Then a 90-kDa bacterial protein, coded by the *actA* gene, induces actin polymerisation, leading to bacterial movement, eventually spreading to adjacent cells [13, 14].

Listeriolysin needs a low pH for its activity *in vitro* [15], and it has been demonstrated recently that phagosome acidification of murine macrophages unequivocally occurs during infection by *L. monocytogenes* [16]. Nevertheless, it has also been reported that weak bases do not prevent *L. monocytogenes* or *Bacillus subtilis* expressing listeriolysin gaining access to the host cytoplasm [17, 18].

Since acidification of vesicles is considered to be an important part of several endocytic pathways [19] and the role of phagosome acidic pH on the invasion and the fate of *L. monocytogenes* within host cells remains controversial, entry and replication of this micro-organism into a human enterocyte-like cell line (Caco-2 cells) were studied by a combination of biochemical and ultrastructural approaches. The effects of different inhibitors of vacuolar acidification – the lipophilic weak base ammonium chloride, the carboxylic ionophore monensin and the antibiotic bafilomycin A₁ – on the invasion pathway and intracellular replication of a *L. monocytogenes* wild-type strain were studied. All these agents, raising the intracellular vesicle acidic pH by different mechanisms [20–22], affected *L.*

monocytogenes intracellular replication. Moreover, by electron microscopy, bacteria internalised in bafilomycin-treated Caco-2 cells were shown to be unable to escape from phagosomes.

Materials and methods

Bacterial strains and growth conditions

L. monocytogenes L37, a clinical isolate provided by Dr P. Aureli (Istituto Superiore Sanità, Rome, Italy) and *L. monocytogenes* Bof 299 EGDsm^r provided by Dr P. Cossart (Institut Pasteur, Paris, France) were the wild-type strains used. The strains were subcultured routinely on Listeria Selective Agar Base (LSAB, Oxoid) and grown in Brain Heart Infusion Broth (BHI, Oxoid). The haemolytic activity of bacterial strains was assessed as described previously [23].

Cell cultures

The human colon carcinoma cell line Caco-2 (kindly provided by Dr Bernardini, Department of Cellular Biology and Development, University La Sapienza, Rome, Italy) and epithelioid carcinoma of human cervix cells (HeLa S3) were cultured in minimum essential medium (Eagle's MEM) as described previously [23, 24].

Infection of cells

The method described by Gaillard *et al.* [6] with minor modifications was used for cell infection. Caco-2 and HeLa cells were trypsinised 48 h and 24 h before infection, respectively, and the cell concentration was adjusted to 2×10^5 cells/ml. Semi-confluent cell monolayers grown without antibiotics in Eagle's MEM were inoculated with bacterial suspensions (100 bacteria/cell) in logarithmic phase, corresponding to a subculture of an overnight culture incubated for 60 min at 37°C. After an infection period of 1 h at 37°C, the cells were washed extensively with PBS and 1 ml of fresh medium containing gentamicin 5 µg/ml was added to each well. At different time points, cells were lysed by the addition of 0.5 ml of cold Triton X-100 0.1%, diluted in H₂O and plated on LSAB to determine the number of viable intracellular bacteria (cfu/ml). Data concerning invasion assays were derived from four independent experiments, performed in triplicate. The mean values and standard deviations were determined for each assay. In some experiments, infected monolayers were fixed in methanol and stained with Giemsa. The number of cells invaded and of internalised bacteria/invaded cell were evaluated by random counting of 400 cells.

Chemicals

The following inhibitors of vacuolar acidification (all from Sigma) were used: ammonium chloride (NH₄Cl)

(a lipophilic weak base), monensin (a carboxylic ionophore) and bafilomycin A₁ (an inhibitor of vacuolar type H⁺-ATPase). NH₄Cl was dissolved as a stock solution in water, monensin in absolute methanol and bafilomycin A₁ in dimethylsulphoxide. Serial dilutions of these drugs were incubated for different time intervals (up to 24 h) with cells or bacteria to establish the non-cytotoxic, non-bactericidal and non-bacteriostatic concentrations: 20 mM NH₄Cl, 0.1 µM monensin and 0.1 µM bafilomycin A₁ did not affect the viability of either bacteria (as determined by viable counts) or eukaryotic cells (as determined by neutral red uptake assays).

Transmission electronmicroscopy

Infected and uninfected cell monolayers were fixed in cacodylate-buffered glutaraldehyde 3% (0.1 M, pH 7.2) for 1 h at room temperature and post-fixed in OsO₄ 1% for 1 h. Fixed specimens were dehydrated through a graded series of ethanol solutions. They were then scraped off the surface of culture dishes and embedded in Agar 100 (Agar AIDS, Cambridge, Essex). Serial ultra-thin sections were collected on 200-mesh grids and then counterstained with uranyl acetate and lead citrate, and examined by electron microscopy (Zeiss 902 at 80 kV). To investigate the number of *L. monocytogenes* in bafilomycin-treated and untreated infected cells, 50 separate cytoplasmic areas for both cases to be studied were selected at random, each area being 20 µm². Bacteria within selected areas were counted and the average number of *L. monocytogenes*/20 µm² was determined for each of the groups of 50 areas. Statistical analysis was by Student's *t* test.

Results

Invasion of epithelial cell lines by wild-type *L. monocytogenes* strains L37 and EGD

Both *L. monocytogenes* strains possessed haemolytic activity and were able to invade Caco-2 and HeLa S3 cells, although *L. monocytogenes* L37 showed a higher invasion efficiency than *L. monocytogenes* EGD (Table 1). To study the intracellular replication of these strains, after infection for 1 h, a bactericidal concentration of gentamicin (5 µg/ml) was added to monolayers to kill extracellular bacteria and, at different time

Table 1. Invasion of epithelial cell lines by *L. monocytogenes* wild-type strains

Bacterial strains	Host cells	Percentage of invasion*
<i>L. monocytogenes</i> EGD	Caco-2	4.3(0.2)
<i>L. monocytogenes</i> L37	Caco-2	7.0(0.3)
<i>L. monocytogenes</i> EGD	HeLa S3	4.6(0.4)
<i>L. monocytogenes</i> L37	HeLa S3	6.2(0.2)

Data are means (SD) for four experiments.

*Values represent the number of internalised bacteria and are expressed as the ratio of intracellular cfu/inoculum cfu $\times$ 100.

intervals, infected cells were lysed to determine the number of viable intracellular bacteria. The results of these experiments showed that *L. monocytogenes* L37 was capable of multiplication in both cell lines and its growth rate was similar to that of *L. monocytogenes* EGD (data not shown). The following experiments were undertaken only with *L. monocytogenes* L37 strain and Caco-2 cells.

Effects of inhibitors of vacuolar acidification on *L. monocytogenes* L37 entry and growth in Caco-2 cells

The requirement of a low-pH step during *L. monocytogenes* invasion of Caco-2 cells was investigated with three inhibitors of vacuolar acidification of living cells (NH_4Cl , monensin and bafilomycin A_1) which act on the acidification of intracellular vesicles by different mechanisms. NH_4Cl , a lipophilic weak base, enters the vesicles readily and to accumulates, preventing endosome acidification [20]; the monovalent ion-selective ionophore monensin facilitates the transmembrane exchange principally of sodium ions for protons [21]; the antibiotic bafilomycin A_1 is a powerful and selective inhibitor of the vacuolar proton-ATPases [22].

These compounds were tested in preliminary experiments with both bacteria and Caco-2 cells. NH_4Cl (20 mM), 0.1 μM monensin and 0.1 μM bafilomycin A_1 were added to Caco-2 cells for 30 min before the addition of the bacterial inoculum and also kept in contact during the infection step (1 h at 37°C) and the treatment with gentamicin. Infected monolayers were lysed after different time intervals to determine the number of viable intracellular bacteria.

Fig. 1 indicates that the invasion efficiency of bacteria in Caco-2 cells exposed to the vacuolar acidification inhibitors was not significantly affected. In fact, 2 h after the addition of bacteria, the susceptibility of NH_4Cl - and monensin-treated colonic cells to infection with *L. monocytogenes* L37 was similar to that of untreated cells and, in bafilomycin-treated cells, the bacterial internalisation was slightly decreased. These results indicate that endosome acidification is not required for the internalisation step of the listeria invasion pathway. When incubation of Caco-2 cells with the drugs was prolonged, the intracellular growth of *L. monocytogenes* exhibited a completely different behaviour from that observed in untreated controls. The number of intracellular bacteria within cells exposed to the agents determined at 4 and 6 h after infection remained unaltered whereas bacteria multiplied in untreated cells.

These experiments were also performed in HeLa cells and intracellular bacteria were evaluated by viable counts and Giemsa-stained preparations. Results were consistent with those presented in Fig. 1.

Fig. 1. Effect of inhibitors of vacuolar acidification on invasion of Caco-2 cells by *L. monocytogenes* L37. The cells were infected at a multiplicity of infection of 100 bacteria/cell for 1 h at 37°C. The agents were added to Caco-2 cells for 30 min before the addition of bacterial inoculum, during the infection step (1 h at 37°C) and during treatment with gentamicin. At different time intervals after the incubation in gentamicin containing medium, Caco-2 cells were lysed to determine the number of viable intracellular bacteria. Results are expressed as the mean $\log_{10}$ of viable bacteria/well. Data represent the average of five independent experiments performed in duplicate. Error bars represent SD. ■, untreated Caco-2 cells; □, Caco-2 cells treated with 20 mM NH_4Cl ; △, Caco-2 cells treated with 0.1 μM monensin; ○, Caco-2 cells treated with 0.1 μM bafilomycin A_1 .

From these results it would appear that multiplication of *L. monocytogenes* in Caco-2 cells was blocked in the presence of the different compounds that elevated the vacuolar pH. To establish which phase of the invasion pathway was affected, drugs were incubated with the cells before, during and after infection. No inhibition of invasion was observed when the drugs were pre-incubated with the cells and removed before the addition of bacterial inoculum, nor when they were added only during the infection step.

To assess which step of the intracellular infective cycle was affected, experiments were performed in which the compounds were added before (30 min), during the infection step (1 h), and together with gentamicin immediately after the monolayer infection for different periods of time (1, 3, 5 and 7 h). After incubation the compounds were removed and fresh medium containing gentamicin was added to cell monolayers and the degree of intracellular bacterial multiplication was determined 7 h after the infection step.

The results of the kinetics of action of inhibitors of vacuolar acidification on *L. monocytogenes* replication, shown in Fig. 2, are expressed as the percentage of cfu reduction by comparison with untreated controls. Fig. 2 shows that NH_4Cl , monensin and bafilomycin A_1 strongly inhibited bacterial multi-

plication and that this action took place in the earliest stages of the invasion process. In other experiments, after the bacterial infection stage, Caco-2 infected cells were incubated in medium containing gentamicin for different lengths of time (1, 3 and 5 h) before the addition of the agents, and in these conditions the intracellular replication of bacteria was not affected (results not shown).

Electron microscopic examination of bafilomycin A₁-treated and untreated L. monocytogenes-infected cells

As the inhibitor of vacuolar proton-ATPases had the greatest effect on bacterial multiplication, electron microscopy was used to investigate the fate of *L. monocytogenes* in bafilomycin A₁-treated and untreated Caco-2 cells. *L. monocytogenes* was incubated for 1 h with Caco-2 cells pretreated for 30 min at 37°C with 0.1 µM bafilomycin A₁ at a multiplicity of infection of 100 bacteria/cell in the presence or absence of the agent. Samples were taken after extensive washes at the end of a re-incubation period of 1 h and 3 h in fresh medium containing gentamicin 5 µg/ml either with or without 0.1 µM bafilomycin A₁.

Fig. 2. Effect of inhibitors of vacuolar acidification on intracellular multiplication of *L. monocytogenes*. The cells were infected at a multiplicity of infection of 100 bacteria/cell for 1 h at 37°C. The agents were added to the cells for 30 min before the addition of bacterial inoculum, during the infection step (1 h at 37°C), and together with gentamicin immediately after the monolayer infection for different periods of time (1, 3, 5 and 7 h). After incubation the compounds were removed and fresh medium containing gentamicin was added to cell monolayers. Intracellular viable bacteria were determined 7 h after the infection step. Results are expressed as the percentage of cfu reduction by comparison with untreated controls. Data represent the average of five independent experiments performed in duplicate. Error bars represent SD. □, Caco-2 cells treated with 20 mM NH₄Cl; Δ, Caco-2 cells treated with 0.1 µM monensin; ○, Caco-2 cells treated with 0.1 µM bafilomycin A₁.

Untreated cells are shown in Fig. 3. At 1 h after infection, occasional bacteria (< 1 bacteria/cell) were still in the process of entry (Fig. 3a) whereas cell-associated bacteria were intact and located within cell phagosomes (Fig. 3b); about 10 bacteria/cell were observed. After infection for 3 h, most bacteria were free within the cytoplasm (Fig. 3c), demonstrating the ability of *L. monocytogenes* L37 to lyse the phagocytic vacuole membranes during the infective process. Intracytoplasmic bacteria appeared to be undamaged. Electron microscopy showed that intracellular movement and cell-to-cell spread occurred at this stage. Fig. 3d shows bacterial cells projecting into the neighbouring cells through a pseudopodium. Protrusion is limited by a double membrane.

Results obtained by treating *L. monocytogenes*-infected cells with bafilomycin A₁ are shown in Fig. 4. Fig. 4a shows the process of ingestion. After 1 h bacterial cells still appeared to be enclosed within a membrane-bound vacuole (Fig. 4b). This observation is in agreement with previous results obtained with inhibitors of vacuolar acidification showing that the inhibitory effect of these agents takes place after the process of entry. In contrast to the untreated cells, electron microscopy revealed that 3 h after infection, all bacteria remained enclosed within phagosomes. Many phagosomes had fused to form larger pockets in which dense lysosomal material could be observed (Fig. 4c) and the remaining micro-organisms were in the process of being degraded, as determined by breaks in the cell wall (Fig. 4d) or bacterial distortions.

Discussion

The usual in-vivo site of *L. monocytogenes* entry is the intestine. The mechanism of invasion of this micro-organism has been clarified [6] by means of the human colon carcinoma cell line (Caco-2), which displays the remarkable property of expressing typical enterocytic differentiation under standard culture conditions.

Many pathogenic bacteria have the ability to invade host tissues by inducing their own endocytosis. Upon bacterial internalisation, generally, the newly formed phagosome is acidified by means of a protein pump ATPase and lysosomal enzymes are incorporated to form a degradative environment. Some bacteria remain entrapped in these compartments and are digested, whereas others have evolved a specialised strategy enabling them to escape into the cytoplasm and to multiply [25, 26].

The role of low pH in phagosome disruption by the exotoxin listeriolysin O is controversial. While it is accepted that listeriolysin O, necessary for the disruption of the phagosome membrane and intracellular growth of *L. monocytogenes*, is active only at

Fig. 3. Electron micrographs of thin sections of Caco-2 cells infected with *L. monocytogenes*. **a**, After infection for 1 h a few bacteria are still in the process of entry (bar = 0.6 μ m); **b**, bacteria enclosed in phagosomal membrane (bar = 0.3 μ m); **c**, 3 h after invasion bacteria are free in cytoplasm (bar = 0.24 μ m); **d**, bacterium invading a neighbouring cell (transverse section of bacterium inside a pseudopodium); the membrane of the protrusion and the plasma membrane of the adjacent cell are both visible (arrowheads) (bar = 0.3 μ m).

low pH of 5.5 [15, 16] and that the optimum for lysis of erythrocytes by listeriolysin O is pH 5.6 [15], it has been reported that the weak base chloroquine does not prevent access of the bacterial cells to the cytoplasm and, in addition, haemolysin mutants are able to grow in some cell lines [17].

In this study an attempt was made to evaluate the role of vacuolar pH on the entry and growth of *L. monocytogenes* within enterocyte-like cells by use of different agents capable of raising the pH of intracellular acidic compartments. These agents, which readily enter the vesicles, have frequently been used to analyse the requirement of a low pH step during the

entry of parasites [19]: NH_4Cl is an 'acidotropic' weak base that accumulates in vesicles [20], monensin is a monovalent ion-selective ionophore that intercalates into membranes and mediates exchange of monovalent cations through the membrane thereby effecting a rise in vacuolar pH [21], and bafilomycin A_1 is a selective inhibitor of the vacuolar proton-ATPases [22]. Results showed that all these agents were capable of preventing the intracellular growth of *L. monocytogenes*. Experiments in which the compounds were present before, during and for different time periods after the infection step showed that this inhibition took place at an early stage of *L. monocytogenes* invasion of Caco-2 cells: inhibition

Fig. 4. Thin sections of bafilomycin A_1 -treated *L. monocytogenes*-infected cells. **a**, Bacteria in the process of entry (bar = $0.5\ \mu\text{m}$); **b**, after incubation for 1 h bacterial cells appeared to be enclosed within a membrane-bound vacuole (bar = $0.4\ \mu\text{m}$); **c**, after infection for 3 h bacteria remained enclosed within a large phagosome in which dense lysosomal material is visible (bar = $0.5\ \mu\text{m}$); **d**, one bacterium is under process of degradation and breaks in the cell wall are visible (arrowheads) (bar = $0.47\ \mu\text{m}$).

of bacterial growth had reached maximal values when the agents were present in the culture medium for 120 min after the infection step, suggesting that raising the intracellular vesicle pH influenced the delivery of bacteria from phagosomes.

To verify this hypothesis, the fate of *L. monocytogenes* in bafilomycin A_1 -treated and untreated Caco-2 cells was followed by transmission electron microscopy. In both experimental conditions, *L. monocytogenes* was able to invade the enterocyte-like cells, confirming that the inhibitory effect of bafilomycin A_1 took place after bacterial internalisation. Different events were observed after the infection step in untreated Caco-2

cells; release from the phagocytic vacuole allowed bacteria to escape phagolysosomal fusion and cell-to-cell spread was observed, whereas in bafilomycin A_1 treated cells, bacteria initially remained enclosed within phagosomes and subsequently appeared in phagolysosomes. These findings confirm that inhibitors of vacuolar acidification prevent the escape of bacteria from the phagosome compartment and are consistent with the hypothesis that an acidic pH is necessary for listeriolysin O to be active.

These agents represent a useful tool for the investigation of pH requirement in the endocytic pathways [19, 22] but as so far there have been only a few

reports on their use *in vivo*, a choice between these different compounds for the potential treatment of *L. monocytogenes* infection will be possible only after an evaluation of their pharmacological properties in animal models.

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