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ORIGINAL INVESTIGATION

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Superinfection by *Listeria monocytogenes* of cultured human enterocyte-like cells infected with poliovirus or rotavirus

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Abstract A mixed infection with either rotavirus or poliovirus and *Listeria monocytogenes* was analysed in Caco-2 cells, a tumour-derived cell line, highly susceptible to these pathogens. The multiplication of these pathogens, whose usual site of entry and/or replication is the intestine, was also followed by electron microscopy. Results obtained showed an increase of *L. monocytogenes* internalisation in cells infected with rotavirus, whereas the preinfection with poliovirus had only a slight interfering effect on bacterial entry. Analysis of *L. monocytogenes* multiplication in virus-infected cells revealed that rotavirus also promoted bacterial replication, which poliovirus hampered replication. Concerning the effect of Caco-2 cell invasion by *L. monocytogenes* on viral replication, we observed an increase in rotavirus antigen synthesis but no significant effect on poliovirus yield under our experimental conditions.

Key words *Listeria monocytogenes* · Poliovirus · Rotavirus · Coinfection

Introduction

Listeria monocytogenes is a Gram-positive bacterium which can cause serious and sometimes fatal infections in pregnant women, infants, young children, and immunocompromised patients. Outbreaks of listeriosis have been shown to be associated with the ingestion of contaminated food, and there is evidence that a critical step in the pathogenesis of listeriosis is represented by the invasion of intestinal epithelial cells by this organism (Farber and Peterkin 1991; Schuchat et al. 1991).

Rotaviruses, double-stranded RNA viruses belonging to the *Reoviridae* family, infect the small intestine via the oral route. These viruses play an important role worldwide in the aetiology and epidemiology od diarrhoeal diseases of children between 6 and 24 months of age (Donelli and Superti 1994). Polioviruses, among the *Picornaviridae* family, replicate in the intestinal tract, and trivalent live poliovirus vaccine is orally administered to infants and young children up to 36 months of age. Therefore, both rotavirus and poliovirus are widely circulating in the population.

Cellular modifications induced by viruses whose normal habitat is the intestinal tract may modulate the ability of other pathogens, such as bacteria or viruses, to adhere to and invade epithelial cells, and may produce or favour more severe infections in humans (Sweet and Smith 1990). It has been reported that the invasiveness of *Salmonella typhimurium* was enhanced in HEp-2 cells preinfected with infectious or non-infectious Coxsackievirus B 1 (Bukholm and Degré 1984; Bukholm et al. 1985) and that preinfection of MA-104 by human rotavirus promoted enterobacteria internalisation (Bukholm 1988). Moreover, it has also been observed that coinfections of HEp-2 cells with enteroviruses and *Campylobacter* isolates resulted in an increase of bacterial invasion (Konkel and Joens 1990). Coxsackievirus B 1 has also been reported to induce an enhancement of invasion of *Shigella flexneri* in HEp-2 cells, which correlated with an increase of induced phagocytosis (Modalsli et al. 1990). Recently, we observed an increase in invasion ability of *S. flexneri* in different cell lines infected with poliovirus or other enteroviruses (Marchetti et al. 1992; Seganti et al. 1994). With regard to mixed viral infections, we have demonstrated an increase of poliovirus multiplication in cells infected with rotavirus, and an interfering effect of poliovirus on rotavirus replication (Superti et al. 1994).

Here we report results of a study on the infection of enterocyte-like cells by rotavirus or poliovirus and *L. mono-*

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cytogenes. For this purpose, Caco-2 cells, a gut tumour-derived cell line, was choosen as representing a suitable in vitro model for investigating the infectious process of pathogens for which the intestine is the usual site of entry or replication (Gaillard et al. 1987; Svensson et al. 1991; Tucker et al. 1993 a, b; Tucker and Compans 1993).

Materials and methods

Host cells

Caco-2 cells (a human colonic carcinoma cell line) were kindly provided by Dr. Lina Bernardini (Department of Cellular Biology and Development, Faculty of Science, Rome, Italy) and were utilised between passages 60 and 70. Cells were cultured in minimal essential medium (MEM), supplemented with 1.2 g/l $NaHCO_3$, 2 mM glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 20% heat-inactivated foetal calf serum in a 5% CO_2 incubator.

Listeria monocytogenes

A clinical isolate of *L. monocytogenes* (L 37), kindly provided by Dr. Paolo Aureli (Istituto Superiore di Sanità, Rome, Italy), was routinely subcultured on "Listeria selective agar base" (Oxoid) and grown in "brain heart infusion" (BHI, Oxoid).

Viruses

Simian rotavirus SA-11 was grown in LLC-MK 2 cells. Virus was preactivated with 20 μg/ml trypsin (type IX, Sigma) for 30 min at 37 °C, diluted tenfold in 199 medium and inoculated into confluent cell monolayers grown in roller bottles at a multiplicity of infection (MOI) of 5 plaque-forming units (PFU)/cell. After 90 min at 37 °C, the inoculum was removed, and the monolayers were washed once with phosphate-buffered saline (PBS), pH 7.4 and then incubated at 37 °C in 199 medium containing 1 μg/ml trypsin. When an extensive cytopathic effect was observed, infected cultures were frozen and thawed three times, centrifuged (3000 rpm for 10 min) and supernatants were stored at –70 °C.

Poliovirus type 1, Mahoney strain, was grown in Vero cells. Confluent cell monolayers were infected at an MOI of 1 PFU/cell. After 60 min at 37 °C, the inoculum was removed, monolayers were washed once with PBS and MEM supplemented with 2% foetal calf serum was added. After 48-h incubation at 37 °C, the cultures were frozen and thawed, centrifuged (3000 rpm for 10 min) and supernatants were stored at –70 °C.

Invasion assays

The entry of *L. monocytogenes* into Caco-2 cells was tested according to Conte et al. (1994). Briefly, semiconfluent monolayers of Caco-2 cells, propagated without antibiotics in Eagle's MEM, were infected for 1 h at 37 °C at an MOI of approximately 100 exponentially grown bacteria per cell. After infection, cells were washed five times with Eagle's MEM, and 1 ml of fresh medium containing 5 μg/ml gentamicin was added to each well. After a 1-h incubation period at 37 °C, the cells were washed in MEM, trypsinised, counted by haemacytometer, and lysed by addition of cold 0.1% Triton X-100 and plated on Listeria-selective agar base to determine viable intracellular bacteria.

For the kinetic intracellular growth assays, Caco-2 cells were infected for 1 h at 37 °C [100 colony-forming units (CFU)/cell], and incubated in gentamicin containing medium for an additional period of 8 h at 37 °C. At various time intervals [3, 5 and 7 h post infection (p.i.)], cell monolayers were washed with MEM, counted by haemacytometer, and lysed with Triton X-100 for CFU counts.

Viral infection

Viral infections were performed in Caco-2 cells grown in 24-well Nunc cultures dishes (3×10^5 cells/well) by incubating rotavirus (0.4 or 4 PFU/cell) or poliovirus (0.1 or 1 PFU/cell) suspensions with cell monolayers for 1 h at 37 °C. Non-adsorbed viral particles were then removed by washing with PBS, and fresh MEM was added for different intervals before *L. monocytogenes* invasion assays. Intracytoplasmic rotavirus antigen synthesis was determined by indirect immunofluorescence (Superti and Donelli 1991). Poliovirus release in the supernatants of infected Caco-2 cells was measured by PFU assay in Vero cells (Marchetti et al. 1992).

Coinfected monolayers were also washed five times with MEM, fixed in methanol, and stained by Giemsa stain.

Phalloidin-tetramethyl rhodamine isothiocyanate staining

Caco-2 cells, infected with rotavirus (4 PFU/cell) or poliovirus (1 PFU/cell) for 3 h at 37 °C, were incubated with *L. monocytogenes* (100 CFU/cell) for 3 h at 37 °C and then stained by tetramethyl rhodamine isothiocyanate (TRITC)-conjugated phalloidin (Sigma). Prior to the staining assay, the cells were washed three times with PBS and fixed with paraformaldehyde in the same buffer containing 2% sucrose for 10 min at room temperature. Cells were then washed in PBS and incubated for 10 min at room temperature in 0.5% Triton X-100 (Sigma) in the same buffer. Cells, washed five times in PBS, were then incubated with TRITC-phalloidin for 30 min at 37 °C in a moist chamber. After extensive washing, cells were examined with a Leitz Dialux epifluorescence microscope. Control cells, incubated with *L. monocytogenes* for 3 h at 37 °C were processed as above.

Transmission electron microscopy

Infected cell monolayers were fixed in 3% cacodylate-buffered (0.1 M, pH 7.2) glutaraldehyde for 1 h at room temperature and post-fixed in 1% OsO_4 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, UK). Serial ultrathin sections were collected on 200-mesh grids and then counterstained with uranyl acetate and lead citrate. Sections were observed on a Zeiss 902 at 80 kV. Samples of Caco-2 cells coinfected with rotavirus and *L. monocytogenes* were taken at 5 h p.i. while Caco-2 coinfected with poliovirus and *L. monocytogenes* were observed at 8 h. Rotavirus and poliovirus were added to cell monolayers at an MOI of 4 PFU/cell and 10 PFU/cell, respectively, and *L. monocytogenes* was inoculated 3 h p.i. (100 CFU/cell).

Results

Invasiveness of *L. monocytogenes* in Caco-2 cells preinfected with SA-11 rotavirus

In a first series of experiments, Caco-2 cells were incubated with *L. monocytogenes* suspensions (100 CFU/ml) 1, 3, and 5 h after rotavirus infection (4 PFU/cell). As shown in Table 1, preincubation of cells with rotavirus increased bacterial invasion efficiency in a time-dependent manner. The difference between virus-infected cells and control cells was more evident 5 h after virus exposure, when the percentage of bacterial internalisation reached

values sixfold higher in infected cells with respect to mock-infected cells.

In these experiments, controls were performed using rotavirus which had been incubated with rabbit anti-rotavirus hyperimmune serum for 2 h at 37 °C before infection. Neutralised virions were unable to induce any variation in bacterial internalisation (data not shown).

Table 1 Invasiveness of *Listeria monocytogenes* in Caco-2 cells preinfected with SA-11 rotavirus

Preincubation of cells with SA-11 rotavirus (h)[a]	Invasion efficiency (%)[b]
Control	3.0±0.3
1	4.3±0.5
3	5.7±0.5
5	18.1±3.0

[a] Caco-2 cells were infected with SA-11 rotavirus at a multiplicity of infection (MOI) of 4 PFU/cell for different intervals
[b] Invasion assays were performed by adding to cell monolayers *L. monocytogenes* (100 CFU/cell) as described in Materials and methods. Invasion efficiency is the percent of the inoculated CFU which were internalized into Caco-2 cells. Data are presented as means (±standard deviation) of at least four experiments

Table 2 Invasiveness of *L. monocytogenes* in Caco-2 cells preinfected with poliovirus type 1

Preincubation of cells with poliovirus type 1 (h)[a]	Invasion efficiency (%)[b]
Control	2.8±0.3
1	1.4±0.2
2	1.0±0.1
3	0.8±0.1

[a] Caco-2 cells were infected with poliovirus type 1 at an MOI of 1 PFU/cell for different intervals
[b] Invasion assays were performed by adding to cell monolayers *L. monocytogenes* (100 CFU/cell) as described in Materials and methods. Invasion efficiency is the percent of the inoculated CFU which were internalized into Caco-2 cells. Data are presented as means (±standard deviation) of at least four experiments

Invasiveness of *L. monocytogenes* in Caco-2 cells preinfected with poliovirus

In other experiments, cells were infected with poliovirus (1 PFU/cell) before the addition of *L. monocytogenes*. As shown in Table 2, virus-infected cells were less susceptible to bacterial invasion than mock-infected cells since a reduction in the bacterial entry was achieved. As observed for rotavirus infection, the effect of poliovirus on bacterial internalisation was a time-dependent process, with inhibition being more pronounced 5 h after viral infection.

Controls were performed using poliovirus which had been incubated with human anti-poliovirus hyperimmune serum for 2 h at 37 °C before infection. Under these experimental conditions, no variation in bacterial internalisation was observed (data not shown).

Viral replication in Caco-2 cells postincubated with *L. monocytogenes*

Caco-2 cells were infected for 5 h with rotavirus (0.4 PFU/cell) or poliovirus (0.1 PFU/cell) prior the addition of *L. monocytogenes* suspensions (100 CFU/cell). After 1-h incubation at 37 °C, cells were washed and further incubated in gentamicin containing medium for 1 h. Rotavirus antigen synthesis and poliovirus yield were then measured as described in Materials and methods. The addition of bacteria in virus-infected cells resulted in an enhancement of rotavirus replication, whereas poliovirus yield was not affected (Fig. 1 a, b). In fact, we observed a two-fold increase in the percentage of rotavirus-infected cells, whereas no significant differences in the release of poliovirus in supernatants were detected.

Intracellular multiplication of *L. monocytogenes* in Caco-2 cells preinfected with rotavirus or poliovirus

To verify whether viral infection had any effect on intracellular bacterial multiplication, the number of *L. mono-*

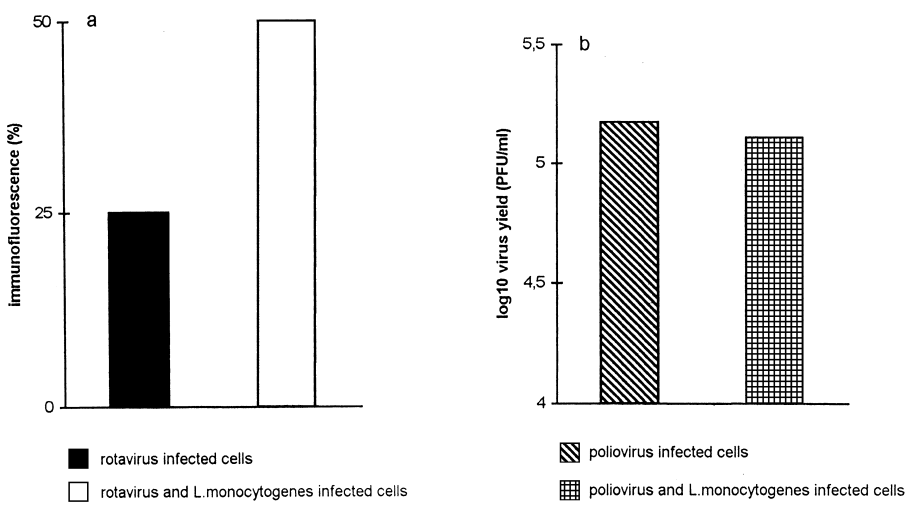

Fig. 1 a, b Viral replication in Caco-2 cells postincubated with *Listeria monocytogenes*. **a** SA-11 rotavirus antigen synthesis in Caco-2 cells postincubated with *L. monocytogenes*. Bacteria (100 CFU/cell) were added to Caco-2 cells 5 h after rotavirus infection (0.4 PFU/cell) and viral antigen synthesis was determined 7 h post infection (p. i.). **b** Poliovirus yield in Caco-2 cells postincubated with *L. monocytogenes*. Bacteria (100 CFU/cell) were added to Caco-2 cells 5 h after poliovirus infection (0.1 PFU/cell) and virus yield was determined 7 h p. i.

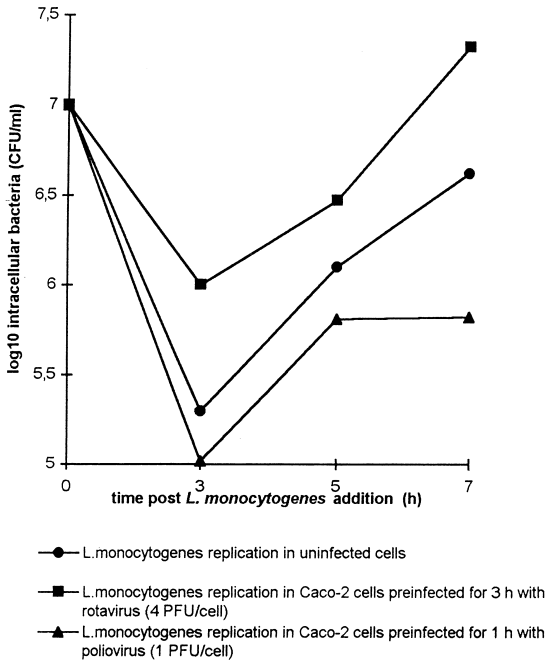

Fig. 2 Intracellular multiplication of *L. monocytogenes* in Caco-2 cells preinfected with SA-11 rotavirus or poliovirus type 1. Caco-2 cells were infected with SA-11 rotavirus (4 PFU/cell) or poliovirus type 1 (1 PFU/cell). At 3 h p. i., cells were incubated with *L. monocytogenes* (100 CFU/cell, see Materials and methods for details). Results are expressed as the $\log_{10}$ of viable bacteria/ml (mean of three determinations; standard deviation 0.21). *Ordinate*: $\log_{10}$ intracellular bacteria (CFU/ml). *Abscissa*: hours post infections

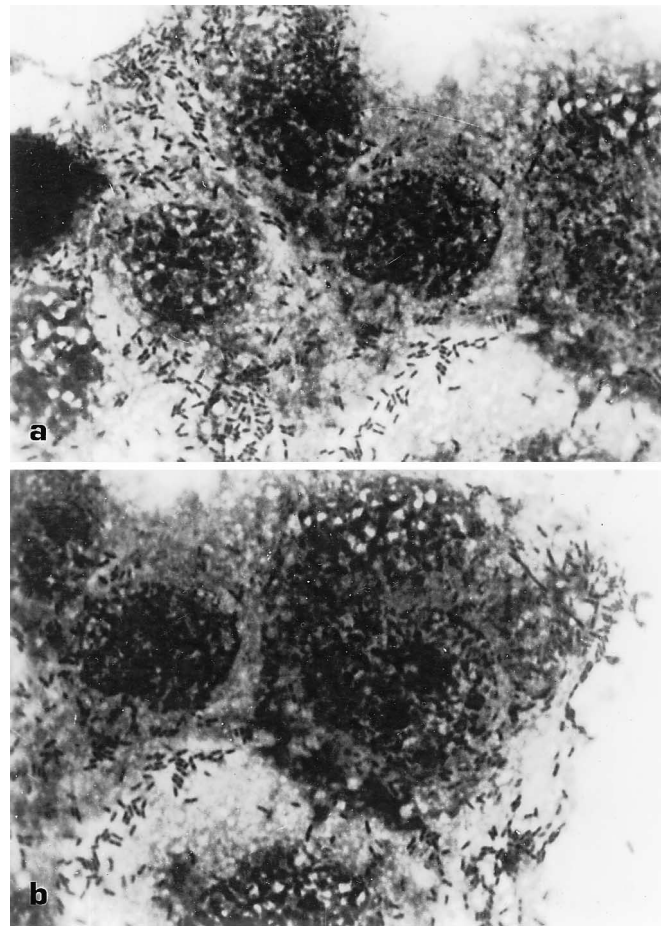

Fig. 3 Light micrographs of islets of Caco-2 cells coinfected with rotavirus (4 PFU/cell) and *L. monocytogenes* (**a**) or poliovirus (1 PFU/cell) and *L. monocytogenes* (**b**). Bacteria (100 CFU/cell) were added to Caco-2 cells 5 h after viral infection and cells were incubated at 37 °C for 1 h. After washing, cells were seeded with gentamicin containing medium for 1 h and then fixed and stained with Giemsa. **a** ×3000; **b** ×3800

cytogenes per cell was measured at different intervals (3, 5 and 7 h after bacterial addition) in Caco-2 cells preinfected for 3 h with either rotavirus (4 PFU/cell) or poliovirus (1 PFU/cell). Results obtained demonstrated that *L. monocytogenes* replication was enhanced by rotavirus infection, whereas in cells infected with poliovirus a decrease in bacterial multiplication was noticed (Fig. 2).

Giemsa staining

To reveale the degree of viral cytopathic effect, coinfected Caco-2 cells were stained by Giemsa. As shown in Fig. 3, no significative differences in cell morphology between rotavirus-*L. monocytogenes* (Fig. 3 a) and poliovirus-*L. monocytogenes* (Fig. 3 b) coinfected cells were observed. Moreover, rotavirus-infected cells seems to contain an higher number of bacteria than poliovirus-infected cells.

Fluorescence microscopy

To visualise actin polymerization and rearrangement into tails, TRITC-phalloidin stain was performed in *L. monocytogenes*-coinfected cells. Figure 4 shows control cells incubated with *L. monocytogenes* (Fig. 4 a), rotavirus-*L. monocytogenes*-coinfected cells (Fig. 4 b), and poliovirus-*L. monocytogenes*-coinfected cells (Fig. 4 c). Actin tails were more evident in rotavirus-infected cells comparing to poliovirus-infected cells.

Electron microscopy

Caco-2 cell monolayers, infected with either rotavirus (4 PFU/cell) or poliovirus (10 PFU/cell), were postincubated with *L. monocytogenes* and examined by electron microscopy. At 5 h p. i., *L. monocytogenes* and rotavirus were both located in Caco-2 cell cytoplasm as shown in Fig. 5 a. *L. monocytogenes* appeared to be enclosed within a membrane-bound vacuole. Figure 5 b shows a Caco-2 cell coinfected with poliovirus and *L. monocytogenes.* After 8 h on infection, viral aggregates were located free in the cytoplasm, while *L. monocytogenes* still appeared laying close to the cell membrane.

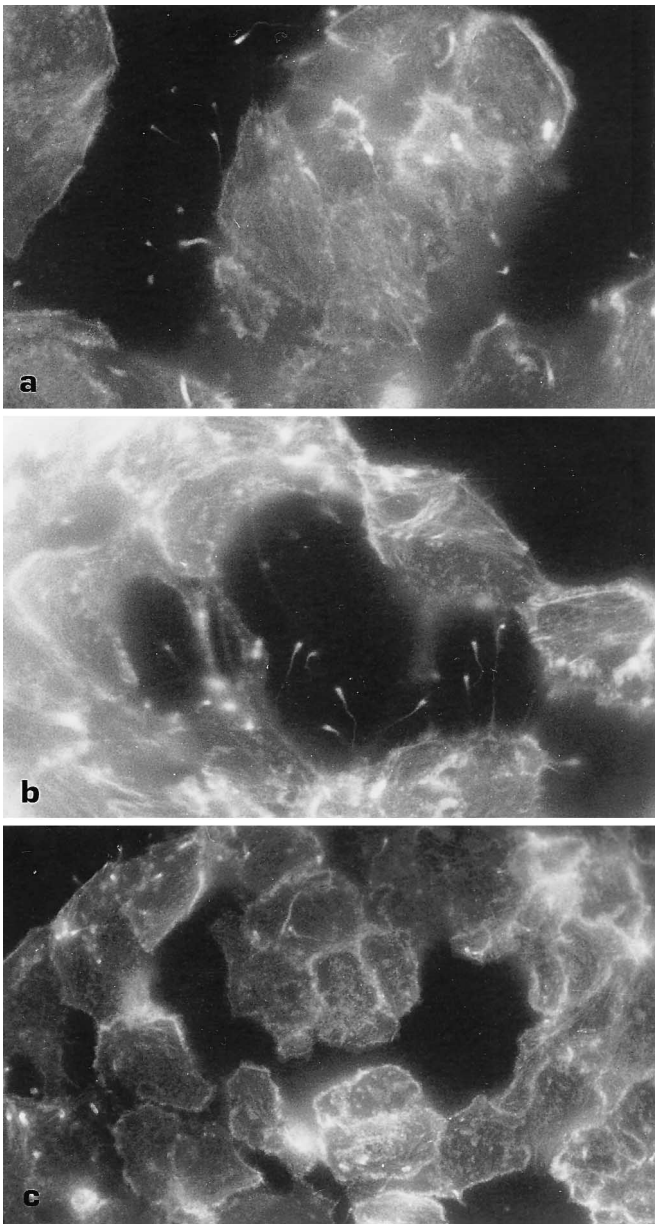

Fig. 4 a–c Phalloidin-TRITC staining of Caco-2 cells coinfected with rotavirus or poliovirus and *L. monocytogenes*. **a** Control cells incubated with *L. monocytogenes* (100 CFU/cell). **b** Caco-2 cells infected with rotavirus (4 PFU/cell) and postincubated with *L. monocytogenes* (100 CFU/cell). **c** Caco-2 cells infected with poliovirus (1 PFU/cell) and postincubated with *L. monocytogenes* (100 CFU/cell). Several listeriae followed by actin tails can be seen in control (**a**) and rotavirus preinfected (**b**) cells. **a** ×4000; **b, c** ×2500

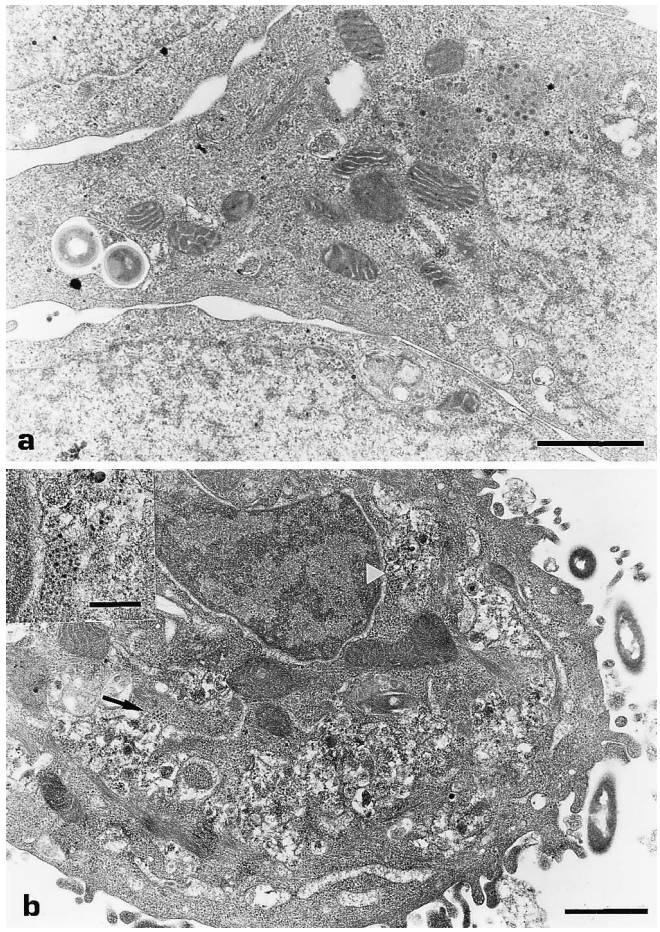

Fig. 5 a, b Electron micrograph of thin sections of Caco-2 cells infected with rotavirus or poliovirus and *L. monocytogenes*. **a** Cell monolayers were previously infected with SA-11 rotavirus and then with *L. monocytogenes* as described in Materials and methods. After 5 h of infection, rotavirus and *L. monocytogenes* can be seen in the cytoplasm of a Caco-2 cell. Bacteria are enclosed in a phagosomal membrane. **b** Cell monolayers were previously infected with poliovirus type 1 and then with *L. monocytogenes* as described in Materials and methods. At 8 h p.i., electron-dense viral aggregates (*black arrow*) are located in the cytoplasm. *White arrowhead* highlights the enlarge area (*inset*) showing a poliovirus aggregate. *L. monocytogenes* can be seen laying close to the cell membrane. *Bars* **a, b** = 1 μm, *inset* = 0.3 μm

Discussion

Combined infection with viral and bacterial agents often results in a more severe disease than does infection with either agent alone. One possible explanation is that the host becomes compromised and is, therefore, non-specifically susceptible to bacterial disease (Davison and Sanford 1981). However, investigators have proposed that specific, rather than non-specific, interactions may lead to bacterial colonisation and penetration of virus-infected cells (Degré and Glasgow 1968).

In this report we describe the effect of rotavirus or poliovirus infection on *L. monocytogenes* invasiveness in Caco-2 cells, a human enterocyte-like cell line. As intestinal epithelial cells constitutes the primary barrier of the host which must be traversed prior to *L. monocytogenes* dissemination from the infected organism, Caco-2 cells represent a useful in vitro tool for the investigation of the infectious process of this pathogen for which the gut is the usual site of entry.

The results presented in this study show that infection of enterocyte-like cells with different enteric viruses influ-

ences the cell susceptibility to a secondary bacterial invasion. In fact, rotavirus infection made these cells more permissive to *L. monocytogenes*, with the invasion efficiency increasing with the length of the virus preincubation time, whereas poliovirus replication slightly reduced bacterial entry. Moreover, *L. monocytogenes* replication was enhanced by rotavirus infection and reduced in poliovirus-infected cells. Light microscopy, fluorescence microscopy, and transmission electron microscopy observations confirmed these findings.

It is well known that different mechanisms are involved in the interactions between viruses, bacteria and host cells. With respect to rotavirus, our results are in agreement with those of other authors who noticed a general increase of the invasive ability of several intestinal bacteria towards various cell lines infected with different viruses such as enteroviruses, measles virus, rotavirus and vesicular stomatitis virus (Bukholm and Degré 1984; Bukholm et al. 1985, 1986, 1988; Bukholm 1988; Marchetti et al. 1992; Seganti et al. 994).

The increase of the invasiveness of *L. monocytogenes* in rotavirus-infected cells could reflect the expression of virally synthesized membrane receptors. Alternatively, rotavirus adsorption to cell receptors might trigger a cell-mediated event responsible for the phagocytosis of invasive bacteria or, more likely, may render the membrane receptor E-cadherin (Mengaud et al. 1996) more easily accessible to the internalin protein of *L. monocytogenes*.

Under our experimental conditions, poliovirus infection did not induce significant differences in bacterial internalisation or replication in host cells. Similar results have been obtained by Konkel and Joens (1990), who observed that poliovirus infection had no effect on the ability of different strains of *Campylobacter jejuni* to adhere to or to invade HEp-2 cells. The slight reduction in *L. monocytogenes* entry observed in poliovirus-infected cells is likely to be related to the virus-induced modifications of cell membrane, allowing gentamicin penetration into Caco-2 cells. In fact, it has been reported that enterovirus multiplication in susceptible cells correlates with altered ionic conditions and consequent changes in membrane electric potential and permeability (Schaefer et al. 1982; Carrasco 1981).

Rotavirus antigen synthesis is increased when virus-infected cells are exposed to *L. monocytogenes*. After entry into cells, *L. monocytogenes* organisms are rapidly released from the phagocytic vacuole and replicate within the cytosol (Portnoy 1994). In this compartment, they become covered with filamentous actin (Mounier et al. 1990) which is rearranged into tails by the bacterial surface ActA protein, allowing the intracellular movement of bacteria. Whether these deep modifications in the cytosol can be responsible of the enhancement of rotavirus antigen synthesis remains to be explained.

In conclusion, infection of cells with viruses may be an important contributing factor for *L. monocytogenes* internalisation. Our data indicate that the enhancement of bacterial invasion is the result of specific rather than non-specific events because the two different enteric viruses tested exert different effects on *L. monocytogenes* invasiveness in Caco-2 cells.

Since polymicrobial infections of the gastrointestinal tract by bacteria and viruses frequently occur in vivo (Konkel and Joens 1990), further studies are in progress to provide additional insights into the mechanisms of the relationship between *L. monocytogenes* and enteric viruses.

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