Med Microbiol Immunol (1995) 184: 123–127

ORIGINAL INVESTIGATION

Jo H. A. J. Curfs · Jacques F. G. M. Meis
Jack A. M. Fransen · Hein A. L. van der Lee
Jacomina A. A. Hoogkamp-Korstanje

Interactions of *Yersinia enterocolitica* with polarized human intestinal Caco-2 cells

Received: 8 June 1995

Abstract The in vitro interactions of *Yersinia enterocolitica*, *Salmonella typhimurium* and *Escherichia coli* with polarized human colonic carcinoma (Caco-2) cells are described. Invasion of a confluent Caco-2 cell monolayer by *Yersinia* and *Salmonella* took place within 4 h after contact, which was in marked contrast to *E. coli* which did not invade Caco-2 cells. Cytoplasmic extrusions developed on the apical membrane and indicated the site of entrance of bacteria into the Caco-2 cells. Intracellular *Yersinia* and *Salmonella* were surrounded by a vacuolar membrane. Single as well as multiple bacteria were enclosed within a single vacuole. At 6 h after contact some of the intracellular yersiniae were found free in the cytoplasm. Furthermore, morphological signs of degeneration of Caco-2 cells such as vacuolization and autophagy were observed. Caco-2 cells infected with *Salmonella* also showed degenerative changes but the salmonellae resided within membrane-bound vacuoles in contrast to *Yersinia*. These observations are in contrast to those described for the invasion of other cell lines (not derived from intestinal epithelium) by *Yersinia* and may reflect more closely the interactions between *Yersinia* and the intestinal epithelium during gastrointestinal infection.

Key words *Yersinia* · *Salmonella* · *Escherichia* · Caco-2 · Invasion, ultrastructural

Introduction

An important determinant of virulence of pathogenic bacteria is the invasion of normally nonphagocytic host cells. Binding to host cells can result in extracellular adhesion followed by internalization of the pathogens. Many enteropathogenic organisms such as *Salmonella, Shigella* and *Yersinia* species have the ability to adhere to and penetrate epithelial cell barriers (Moulder 1985; Finlay and Falkow 1988, 1989; Falkow et al. 1992; Conconnier et al. 1994). Oral infection with *Y. enterocolitica* is presumably followed by penetration of the intestinal epithelium permitting the bacteria access to the underlying lamina propria, where they may be internalized by macrophages and/or leukocytes. After invasion multiplication and translocation to other tissues where they inflict inflammatory responses may follow. Therefore, clinical symptoms may not be limited to gastroenteritis or terminal ileitis but may also include extraintestinal symptoms such as mesenteric lymphadenitis. Additionally, a variety of postinfectious sequelae such as arthritis and erythema nodosum may develop.

Initial interactions between host and pathogens have been studied in animal models and in vitro. In the intestine of orally infected mice invasion of *Y. enterocolitica* O:8 predominantly occurs in ileal Peyer's patches, although some invasion into the remaining mucosa can be observed (Hanski et al. 1989). Grützkau et al. (1990) demonstrated in mice that uptake of *Y. enterocolitica* takes place by microfold (M) cells of the follicle-associated epithelium of the Peyer's patches. However, oral infection with *Y. enterocolitica* O:8 is usually lethal for mice and conclusions made from observations made in mice may not apply to human yersiniosis. In vitro studies with *Yersinia* and/or *Salmonella* have used CHO, HEp-2, MDCK, HeLa, A431 and Henle cells and human fibroblasts (Brunius 1980; Schiemann and Crane 1987; Finlay and Falkow 1988, 1989; Rosenshine et al. 1994). These studies show similar results for both organisms and, therefore, *Yersinia* is supposed to enter its host by invading the epithelium of the intestine through a mechanism similar to that used by *Salmonella*. However, although some of the studied cell lines are polarized, none of them develop a well-defined brush border and, thus, may not resemble the intestinal epithelium.

Three human adenocarcinoma cell lines (Caco-2, HT-29 and T84) have been shown to form polarized mono-

J. H. A. J. Curfs (✉) · J. F. G. M. Meis · H. A. L. Van der Lee
J. A. A. Hoogkamp-Korstanje
Department of Medical Microbiology,
University Hospital Nijmegen, P.O. Box 9101, 6500 HB Nijmegen,
The Netherlands
Tel.: (31) 80 614336; Fax: (31) 80 540216

J. A. M. Fransen
Department of Cell Biology and Histology, Faculty of Medicine,
University of Nijmegen, Nijmegen, The Netherlands

layers and well-defined brush borders mimicking human intestinal epithelium (Pinto et al. 1983; Dharmsathaphorn et al. 1984; Huet et al. 1987). Previous studies of Caco-2 cells with *Salmonella* sp. (Finlay and Falkow 1990) and *Campylobacter jejuni* (Russel and Blake 1994) have shown that bacteria interacted with apical microvilli and cause disruptions in the brush border followed by internalization. Recently Coconnier et al. (1994) have shown that *Y. pseudotuberculosis* is able to enter Caco-2 cells by an $\alpha_5\beta_1$ integrin-dependent mechanism. However, the behavior and morphology of *Yersinia* inside Caco-2 cells remains unclear. Here we present a comparative study of the interactions of *Y. enterocolitica* and *S. typhimurium* with Caco-2 cells.

Materials and methods

Caco-2 cells

Human colon carcinoma-derived Caco-2 cells (Pinto et al. 1983) were grown to confluence on surfactant-free nitrocellulose membrane filters (12-mm diameter, 0,4-µm pore size, Costar, Cambridge) placed in 24-well tissue culture plates (Costar, Cambridge) in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis) without antibiotics, supplemented with 20% heat-inactivated fetal calf-serum, and 1% non-essential amino acids. Cells were incubated at 37 °C/90% relative humidity in air containing 5% CO_2. The medium was added to both the apical and basolateral sides of the cells and was changed daily.

The cells were used only when they satisfied two conditions. (1) They had to form a tight monolayer. The tightness of the monolayer was tested by inducing a higher level of medium in the apical chamber (Klumperman et al. 1991). The cells were only used when this difference was maintained for at least 4 h. (2) Caco-2 cells had to be fully differentiated which was assessed by positive immunofluorescence labeling for sucrose-isomaltase, a marker for the functional differentiation of Caco-2 cells (Semenza 1986). Cells were ready for experiments when they were in culture for 12 or more days.

Bacteria

Y. enterocolitica serotype O:8 strain WA (ATCC 9610), serotype O:3 (NCTC 11 176) and serotype O:9 (NCTC 11 174) all harboring the virulence plasmid were grown overnight at 26 °C in brain heart infusion (BHI) broth followed by 4 h growth at 37 °C. *S. typhimurium* (ATCC 13 311) and *Escherichia coli* (ATCC 25 922) were grown at 37 °C in BHI.

Monolayer penetration assay

After washing with PBS, bacterial suspensions of 2×10^8 CFU/ml in DMEM without supplements were made and 0.5 ml of this suspension was added to the apical side (brush border) of Caco-2 cells. Cells with bacteria were incubated at 37 °C for 4 and 6 h and prepared for electron microscopy. Additionally, 6 h after incubation Giemsa-stained preparations were examined by light microscopy for determination of the percentage of infected cells (100 cells/filter were counted). All experiments for electron microscopy were performed in triplicate.

Scanning electron microscopy

Filters containing bacteria and cells were washed thoroughly with DMEM followed by overnight fixation in 2% glutaraldehyde in 0.1 M phosphate buffer (PB) at 4 °C. Dehydration was performed in an ascending series of alcohols followed by critical point drying with CO_2 in a critical point drying apparatus. After mounting and coating with gold, samples were examined in a JEOL 6310 scanning electron microscope.

Transmission electron microscopy

Monolayers were fixed as described above and after washing with PB, cells were post-fixed in 1% OsO_4 in PB for 1 h and dehydrated in an ascending series of alcohols and embedded in Epon 812. Ultrathin sections were cut with a Diatome diamond knife on a Reichert-Jung microtome. Sections were stained with 3% uranyl acetate (10 min) and lead citrate (10 min) at ambient temperature. Sections were examined in a JEOL 1010 transmission electron microscope.

Results

Determination of number of infected Caco-2 cells

Light microscopic examination of Caco-2 cells after 6 h of incubation revealed that more than 90% of the cells contained one or more *Yersinia* bacteria. This was independent on the serotype. In the experiments with *Salmonella* 87% of the Caco-2 cells were infected. No bacteria were found in Caco-2 cells incubated with *E. coli*.

Scanning electron microscopy

Yersinia enterocolitica

After 4 h of incubation *Y. enterocolitica* O:8WA can be seen in close contact with the brush border of the Caco-2 cells, and at the interaction site of some of the bacteria with the brush border the formation of cytoplasmic extrusions (blebs) can be observed (Fig. 1 A). The formation of blebs may indicate entrance of bacteria into Caco-2 cells. Apart from these extrusions the brush border remains largerly intact without any indication of cell degeneration.

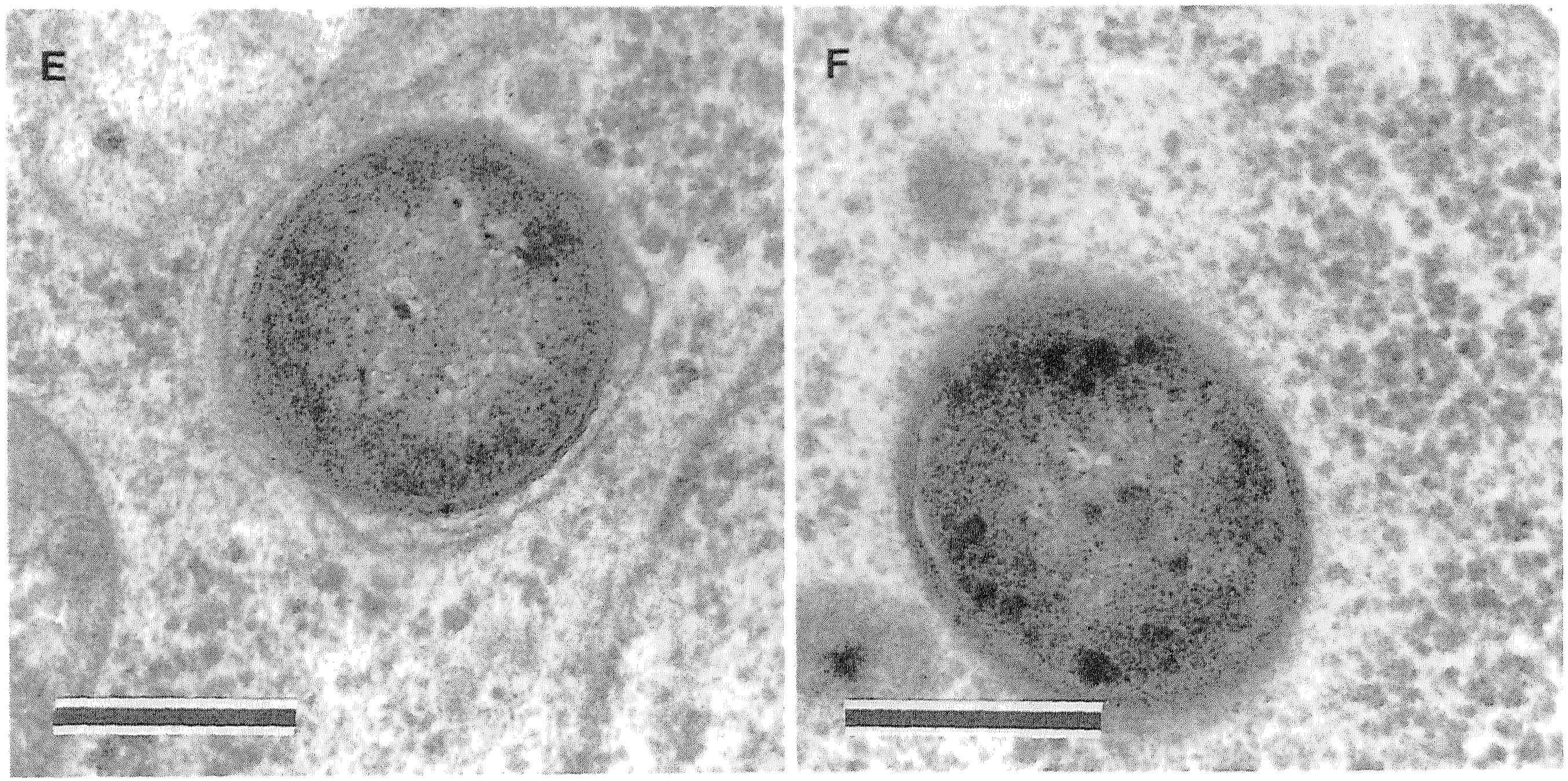
At 6 h after incubation, extracellular bacteria are mainly located at the region of contact between adjacent cells (Fig. 1 B). Protoplasmic extrusions are rarely observed. Furthermore, the surface of the Caco-2 cell monolayer (brush border) appears to be undamaged, similar to that seen at 4 h.

No difference is observed between serotypes O:3, O:8 and O:9. All three strains show a comparable interaction with the apical surface of the Caco-2 cells.

Salmonella typhimurium

At 4 and 6 h after incubation, numerous bacteria can be seen interacting with the apical surface of the Caco-2 cells. Degenerative changes of the brush border have become apparent and comprise elongation, thinning and broadening of the villi. Cytoplasmic blebs are a prominent feature (Fig. 2 A).

Bars: A–C 5 μm, D–F 0.5 μm



Escherichia coli

The *E. coli* strain used in our experiments does not induce any changes in the brush border at either 4 or 6 h after incubation (Fig. 2 C).

Transmission electron microscopy

Yersinia enterocolitica

Intracellular bacteria can be demonstrated 4 and 6 h after inoculation of the Caco-2 cells (Fig. 1 C). At 4 h after in-

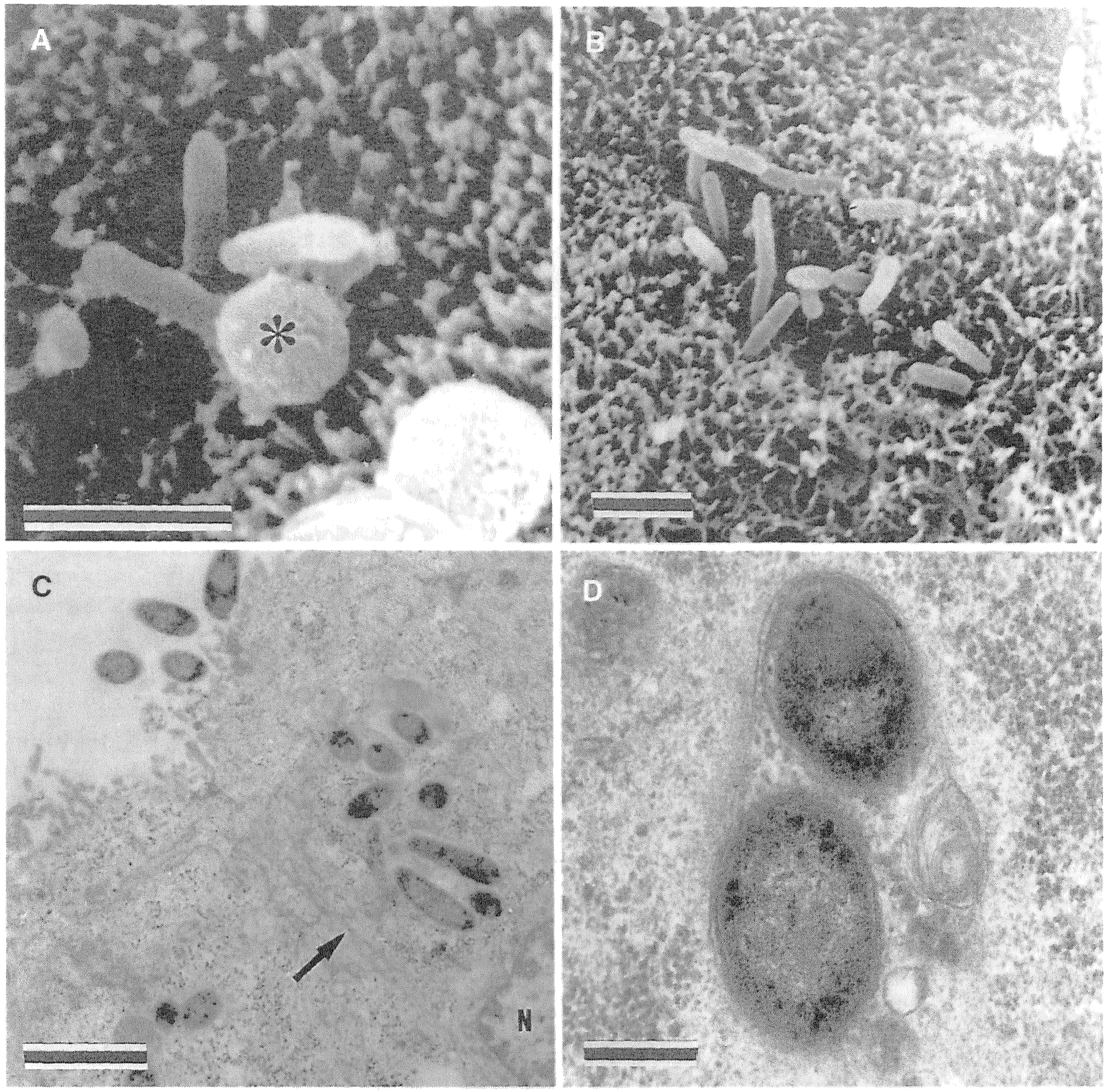


Fig. 1A–F Interaction of *Yersinia enterocolitica* O:8WA with Caco-2 cells. **A** Scanning electron micrograph of the apical surface of Caco-2 cells 4 h after addition of bacteria. Note that apart from cytoplasmic extrusions (*blebs, arterisk*), no degenerative changes of the brush border, as described for *Salmonella*, have occurred. Several bacteria can be seen in close contact to the villi of the brush border. **B** Scanning electron micrograph of the brush border of Caco-2 cells 6 h after incubation. Extracellular bacteria are predominantly located at the borders between adjacent cells. Note the absence of cytoplasmic extrusions. **C** Transmission electron micrograph of two Caco-2 cells with multiple intracellular bacteria. Note that the brush border is intact. (*N* nucleus, *arrow* cell border). **D** Transmission electron micrograph of multiple intracellular bacteria enclosed by a single membrane resulting from either coalescence of vacuoles or inclusion of several bacteria by one membrane during invasion. Note membrane whorl. **E** Transmission electron micrograph of single intracellular bacterium surrounded by a vacuolar membrane. **F** Transmission electron micrograph of *Yersinia* free in the cytoplasm. *Bars*: **A**, **C** 5 μm; **D**, **E** 0.5 μm

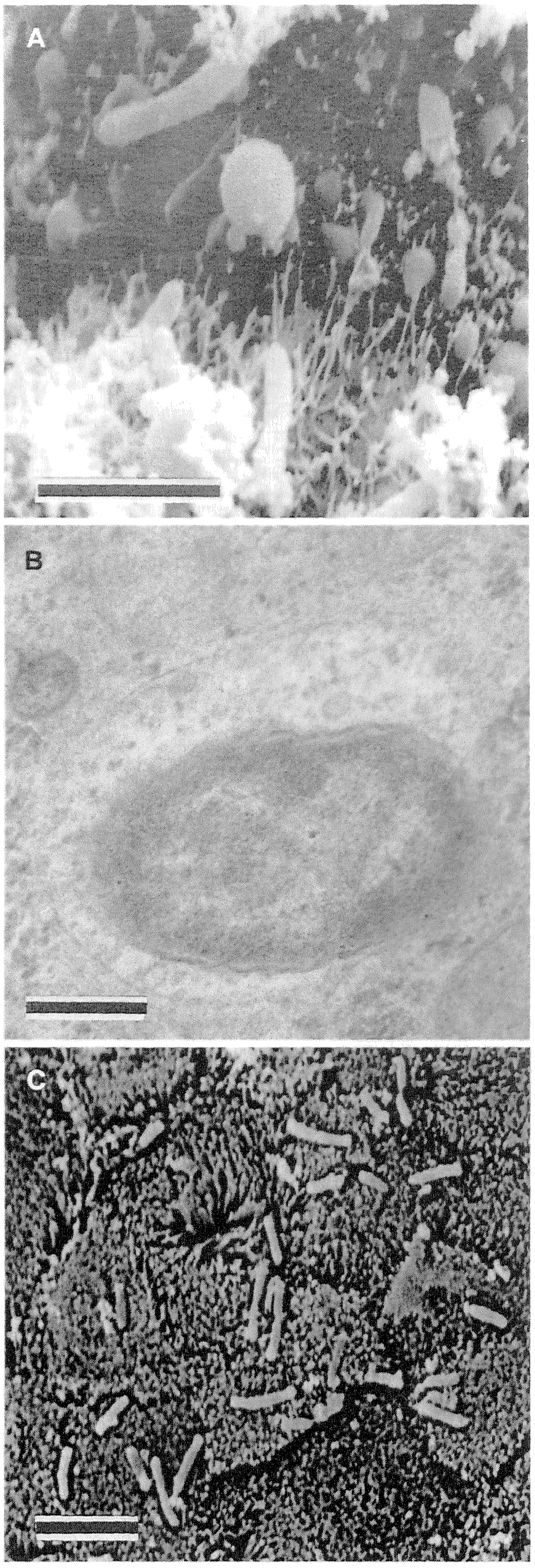


Fig. 2 A–C Interaction of *Salmonella typhimurium* and *Escherichia coli* with Caco-2 cells. **A** Scanning electron micrograph of the apical surface of Caco-2 cells 4 h after addition of *Salmonella*. Degenerative changes of the brush border have become apparent and comprise elongation, thinning and broadening of the villi. Cytoplasmic blebs is a prominent feature. **B** Transmission electron micrograph showing *S. typhimurium* surrounded loosely by a vacuolar membrane. **C** Scanning electron micrograph of the apical surface of Caco-2 cells showing that *E. coli* does not induce any pathological changes. *Bars*: **A**, **C** 5 μm; **B** 1 μm

cubation, the vast majority of intracellular bacteria are tightly enclosed by a vacuolar membrane (Fig. 1 D, E). Vacuolar membranes enclose both multiple (Fig. 1 D) and single (Fig. 1 E) bacteria. After 6 h bacteria are found both inside vacuoles and free in the cytoplasm (Fig. 1 F). No evidence for transmigration of bacteria through Caco-2 cells was found within 6 h of incubation.

Some pathological alterations of Caco-2 cells can be observed 6 h after contact and comprise the presence of membrane whorls, indicating autophagy (Fig. 1 D), as well as increased vacuolization (not shown). The integrity of the monolayer, however, remains unaffected. No differences between the three tested serotypes can be demonstrated.

Salmonella typhimurium

After 4 and 6 h of incubation salmonellae are found inside Caco-2 cells and the bacteria are always surrounded by a vacuolar membrane (Fig. 2 B). This membrane encloses the bacterium loosely in contrast to the observations with *Yersinia* where the vacuolar membrane is tightly wrapped around the bacterium (Fig. 1 E). As for *Yersinia* no evidence for transmigration can be observed and pathological changes such as membrane whorls are prominent.

Escherichia coli

No intracellular bacteria are found indicating that this strain does not enter Caco-2 cells.

Discussion

Y. enterocolitica is able to enter animal cells, as demonstrated earlier for several non-intestinal cell lines such as CHO, Hep-2, MDCK and Henle cells (Brunius 1980; Schiemann and Crane 1987; Finlay and Falkow 1988) and it is assumed that after oral infection *Yersinia* initially follows the same route as *Salmonella* (Finlay and Falkow 1989). Entrance of *Yersinia* and *Salmonella* into these cell lines can be blocked by microfilament inhibitors (Buckholm 1984; Finlay and Falkow 1988), indicating active uptake of bacteria by the host cell. Bacteria are internalized singly (Finlay and Falkow 1989) and are surrounded by a membrane-bound inclusion. We demonstrate that after entrance into Caco-2 cells *Y. enterocolitica* is able to liberate itself from the surrounding vacuolar membrane, in contrast to that previously described for *Y. enterocolitica* inside non-intestinal cell lines (Brunius 1980; Finlay and Falkow 1988, 1989) and in contrast to *Salmonella* which remains within the vacuole (Finlay and Falkow 1990). The demonstration of *Yersinia* bacteria free in the cytoplasm indicates that, although initial uptake is likely to occur through an endocytosis-like process, *Yersinia* is able to lyse the enclosing membrane, thereby releasing itself into the cytoplasm of the Caco-2 cell. In addition, the presence of

single as well as multiple bacteria inside one vacuole suggests coalescence of vacuoles or inclusion of several bacteria by a single vacuole during uptake, which is contradictory to what has been described for *Yersinia* in non-intestinal cell lines (Brunius 1980; Finlay and Falkow 1988). The differences in the way the vacuolar membrane surrounds *Yersinia* compared to *Salmonella* may arise from different internalization strategies as hypothesized by Isberg and Tran Van Nhieu (1994). They suggest that *Yersinia* uptake occurs through an "exclusive" or "zippering" mechanism, whereas uptake of *Salmonella* is "inclusive" or "triggering".

Our observation that after 6 h of incubation yersiniae are found intra- and extracellularly without indications of invasion or blebs suggests that at this timepoint further invasion or endocytosis of bacteria has stopped. The majority of the Caco-2 cells already harbor several bacteria per cell and one can imagine that if invasion indeed depends on the microfilament rearrangement of the Caco-2 cells, as described by Buckholm (1984) for *Yersinia* invasion in HEp-2 cells, internalization of additional bacteria may be limited.

No indications have been found that *Yersinia* might be able to disrupt the tight junctions between adjacent Caco-2 cells within 6 h of contact, suggesting that in vivo bacteria may be able to transmigrate through the cells of the epithelial layer of the intestine rather than between the cells. However, no evidence for transcytosis was found within 6 h after incubation of *Yersinia* with Caco-2 cells. It remains to be established with the help of non-lethal in vivo models if, in addition to uptake by and passage through M cells, *Yersinia* can indeed transmigrate through absorptive cells of the epithelium as described for *Salmonella* (Kohbata et al. 1986) or that bacterial dispersion may also occur through lysis of epithelial cells as described for *Shigella* (Sansonetti et al. 1986; Sansonetti and Mounier 1987). Supportive for the latter mechanism is the demonstration of membrane whorls (autophagy) and increased vacuolization in *Yersinia*-infected cells indicating the degeneration of Caco-2 cells that may ultimately lead to cell lysis. Lysis of epithelial cells of the intestine in vivo will allow access to the underlying lamina propria and could result in bacterial translocation to other tissues.

References

Brunius G (1980) Proliferation of *Yersinia pseudotuberculosis* in HeLa cells. FEMS Microbiol Lett 7:45–46

Buckholm G (1984) Effect of cytochalasin B and dihydrocytochalasin B on invasiveness of entero-invasive bacteria in HEp-2 cell cultures. Acta Pathol Microbiol Immunol Scand 92:145–149

Coconnier M-H, Bernet-Camard M-F, Servin AL (1994) How intestinal epithelial cell differentiation inhibits the cell-entry of *Yersinia pseudotuberculosis* in colon carcinoma Caco-2 cell line in culture. Differentiation 58:87–94

Dharmsathaphorn K, McRoberts JA, Mandel KG, Tisdale LD, Masui H (1984) A human colonic tumor cell line that maintains vectorial electrolyte transport. Am J Physiol 246:204–208

Falkow S, Isberg RR, Portnoy DA (1992) The interaction of bacteria with mammalian cells. Annu Rev Cell Biol 8:333–363

Finlay BB, Falkow S (1988) Comparison of the invasion strategies used by *Salmonella cholerae-suis*, *Shigella flexneri* and *Yersinia enterocolitica* to enter cultured animal cells: endosome acidification is not required for bacterial invasion or intracellular replication. Biochimie 70:1089–1099

Finlay BB, Falkow S (1989) Common themes in microbial pathogenesis. Microbiol Rev 53:210–230

Finlay BB, Falkow S (1990) *Salmonella* interactions with polarized human intestinal Caco-2 epithelial cells. J Infect Dis 162: 1096–1106

Grützkau A, Hanski C, Hahn H, Riecken EO (1990) Involvement of M cells in bacterial invasion of Peyer's patches: a common mechanism shared by *Yersinia enterocolitica* and other enteroinvasive bacteria. Gut 31:1011–1015

Hanski C, Kutschka U, Schmoranzer HP, Naumann M, Stallmach A, Hahn H, Menge H, Riecken EO (1989) Immunohistochemical and electron microscopic study of interaction of *Yersinia enterocolitica* serotype O:8 with intestinal mucosa during experimental enteritis. Infect Immun 57:673–678

Huet C, Sahuquillo MC, Coudrier E, Louvard D (1987) Absorptive and mucus-secreting subclones isolated from a multipotent intestinal cell line (HT-29) provide new models for cell polarity and terminal differentiation. J Cell Biol 105:345–357

Isberg RR, Tran Van Nhieu G (1994) Two mammalian cell internalization strategies used by pathogenic bacteria. Annu Rev Genet 27:395–422

Klumperman J, Fransen JAM, Boekestijn TC, Oude Elferink RPJ, Matter K, Hauri H-P, Tager JM, Ginsel LA (1991) Biosynthesis and transport of lysosomal α-glucosidase in the human colon carcinoma cell line Caco-2: secretion from the apical surface. J Cell Sci 100:339–347

Kohbata S, Yokoyama H, Yabuuchi E (1986) Cytopathogenic effect of *Salmonella typhi* GIFU 10007 on M cells of murine ileal Peyer's patches in ligated ileal loops: an ultrastructural study. Microbiol Immunol 30:1225–1237

Moulder JW (1985) Comparative biology of intracellular parasitism. Microbiol Rev 49:298–337

Pinto M, Robine-Leon S, Appay M-D, Kedinger M, Triadou N, Dussaulx E, Lacroix B, Simon-Assmann P, Haffen K, Fogh J, Zweibaum A (1983) Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. Biol Cell 47:323–330

Rosenshine I, Ruschowski S, Foubister V, Finlay BB (1994) *Salmonella typhimurium* invasion of epithelial cells: role of induced host cell tyrosine protein phosphorylation. Infect Immun 62: 4969–4974

Russel RG, Blake DC (1994) Cell association and invasion of Caco-2 cells by *Campylobacter jejuni*. Infect Immun 62: 3773–3779

Sansonetti PJ, Mounier J (1987) Metabolic events mediating early killing of host cells infected by *Shigella flexneri*. Microb Pathog 3:53–61

Sansonetti PJ, Ryter A, Clerc P, Maurelli AT, Mounier J (1986) Multiplication of *Shigella flexneri* within HeLa cells: lysis of the phagocytic vacuole and plasmid-mediated contact hemolysis. Infect Immun 51:461–469

Schiemann DA, Crane MR (1987) Inhibition of epithelial cell invasion in vitro by *Yersinia enterocolitica* with anticellular antibody. FEMS Microbiol Lett 41:19–22

Semenza G (1986) Anchoring and biosynthesis of stalked brush border membrane proteins: glycosidases and peptidases of enterocyte and tubuli. Annu Rev Cell Biol 2:255–314