Cytoskeletal rearrangements accompanying Salmonella entry into epithelial cells

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Summary

Salmonella bacteria can enter (invade) eukaryotic cells, and exist as intracellular parasites. Confocal, light immunofluorescence and electron microscopy were used to examine various cytoskeletal components of cultured Madin Darby canine kidney (MDCK) and HeLa epithelial cells after infection with Salmonella typhimurium. These bacteria entered and remained within membrane-bound vacuoles and were surrounded by large (5—10 μm) dense structures composed of various cytoskeletal components. These structures consisted of extensive aggregations of polymerized actin, α-actinin and tropomyosin above and beside the invading bacterium in both epithelial cell lines. These structures were evident soon after bacterial addition (maximal at 20 min for HeLa cells, 60 min for MDCK cells), and disappeared later in the infection as the cytoskeletal components returned to a more normal distribution after bacterial internalization. Surprisingly, tubulin also aggregated above internalized Salmonella although bacterial entry or penetration through polarized monolayers was not disrupted by the microtubule-inhibiting agent nocardazole (this treatment actually enhanced tubulin accumulation around these organisms). There were little if any rearrangements in intermediate filaments composed of keratin or vimentin. Large amounts of talin also accumulated above and around invading Salmonella, but there was only a minor accumulation of vinculin around a few organisms. Pretreatment of epithelial cells with the microfilament inhibitor cytochalasin D blocked bacterial internalization but did not prevent accumulation of polymerized actin and α-actinin directly beneath uninternalized bacteria, yet prevented accumulation of the other cytoskeletal components. These results suggest that Salmonella bind to the surface and trigger a signal in epithelial cells that causes marked rearrangements in various cytoskeletal components, including recruitment of actin filaments and α-actinin, which then generates the force necessary for bacterial uptake.

Key words: Salmonella, invasion, cytoskeleton, actin filaments, microbial pathogenesis.

Introduction

Many serious pathogenic organisms are capable of entering eukaryotic cells and existing as intracellular parasites (Moulder, 1985). This niche affords a desirable environment for growth and protection from host defences (Finlay and Falkow, 1988a; Moulder, 1985). However, in order to enter eukaryotic cells, the invading organism must devise a mechanism whereby it is internalized into the host cell. The particle size of such pathogenic organisms is usually so large (greater than 1 μm) that internalization requires alterations in the underlying host cytoskeletal structure, especially in non-professional phagocytic cells such as epithelial cells and fibroblasts, which have a rigid cytoskeleton. Many pathogenic organisms are capable of altering the host eukaryotic cytoskeleton. Invading Shigella flexneri are surrounded by polymerized actin and myosin (Clerc and Sansonetti, 1987). Cytochalasins inhibit the internalization of most pathogenic bacteria that enter eukaryotic cells (Finlay and Falkow, 1988; Moulder, 1985). Enteropathogenic Escherichia coli causes local disruption of microvilli and a proliferation of actin filaments under adherent organisms in HEP-2 cells (Knutton et al. 1987). Once free in the cytoplasm of host cells, both Listeria and Shigella species cause a dramatic rearrangement of intracellular actin, a process involved in intercellular transmission of these pathogens (Bernardini et al. 1989; Tilney and Portnoy, 1989). It has recently been shown that invasin, a protein from pathogenic Yersinia pseudotuberculosis that promotes bacterial invasion, binds to various β1 integrins (Isberg and Leong, 1990), suggesting that this may be the linkage between these bacteria, integrins and the host microfilament system.
Salmonella species are gram-negative bacteria that can cause a variety of diseases including gastroenteritis, typhoid fever and septicemia. These organisms gain entry into the host by oral ingestion of contaminated material, whereby they proceed through the stomach to the small intestine. They then penetrate through the intestinal epithelial cells lining the small bowel and are taken up by cells of the reticuloendothelial system, which are concentrated in the liver and spleen. Salmonella are capable of surviving and replicating inside macrophages (Buchmeier and Heffron, 1989), and further bacterial growth occurs in the liver and spleen, which can result in dissemination of organisms to other body tissue causing a systemic infection. A comprehensive descriptive study of Salmonella penetration of guinea pig intestinal epithelium was published by Takeuchi (1967). He observed that Salmonella typhimurium entered directly into epithelial cells via their apical surface and resided within membrane-bound vacuoles. We have found that Salmonella species can penetrate through polarized MDCK or Caco-2 (human intestinal) epithelial monolayers in a similar manner, disrupting apical microvilli and forming membrane-bound vacuoles (Finlay and Falkow, 1990; Finlay et al. 1988a). After a minimum of 2 h (Caco-2) or 4 h (MDCK) apically added bacteria are found in the basolateral medium. These bacteria also increase the transepithelial permeability of these monolayers, but the disruptions in tight junctions are not sufficient to allow non-invasive E. coli to penetrate. Salmonella species remain within membrane-bound vacuoles while inside eukaryotic cells, where they multiply extensively (Finlay and Falkow, 1989).

The entry of Salmonella species into epithelial cells is a complex event (Finlay and Falkow, 1989b). We have shown that bacterial RNA and protein synthesis are required, and several new bacterial proteins must be synthesized before bacteria are internalized (Finlay et al. 1989b). Several bacterial genes that are required for invasion have been identified (Elingshors et al. 1989; Finlay et al. 1988b; Galan and Curtiss, 1989; Liu et al. 1988). Endosome acidification is not needed for bacterial entry or intracellular multiplication (Finlay and Falkow, 1988). The microfilament inhibitors cytochalasins B and D block Salmonella uptake, but the microtubule inhibitors vincristine, vinblastine and colchicine have no effect on bacterial invasion (Finlay and Falkow, 1988). We previously noted that there is a large condensation of actin surrounding invading bacteria in MDCK cells (Finlay et al. 1989a). Similar structures have been seen with the pathogenic organism Shigella flexneri entering HeLa cells (Clerc and Sansonetti, 1987). The eukaryotic receptor(s) of Salmonella has not been identified, but chemical modification of epithelial surface protein and sugar structures prevents Salmonella adherence and invasion (Finlay et al. 1989b). Salmonella species have a broad host range, and are able to enter most animal cells except yeast and red blood cells (unpublished observations).

The mechanisms whereby Salmonella or most other parasites are internalized into host cells are poorly understood. To further our understanding of this process, we examined the cytoskeleton of epithelial cells after S. typhimurium infection. These studies have led to some unexpected observations, and may provide new tools for studying the eukaryotic cytoskeleton. These studies also provide new clues as to how parasites enter non-phagocytic host cells.

Materials and methods

**Bacteria and tissue culture**

*S. typhimurium* SL1344 is mouse virulent, his"*, resistant to streptomycin, and was kindly provided by B. Stocker of the Department of Microbiology and Immunology, Stanford, California. pBR322 is a plasmid that contains the *Yersinia pseudotuberculosis* invasion (lux) gene cloned into pRH226 (Iaberg and Falkow, 1985). This clone, when transformed into *E. coli*, enables *E. coli* to enter into eukaryotic cells via binding to β1 integrins (Iaberg and Leong, 1990). Strain I Madin Darby canine kidney (MDCK) epithelial cells and HeLa (human epithelioid carcinoma) cells (ATCC no. CCL2) were maintained as described elsewhere (Finlay and Falkow, 1988; Finlay et al. 1988a).

**Antibodies and cell reagents**

*Salmonella* antisera with group B factors 1, 4, 5 and 12 from Difco Laboratories (Detroit, MI) was diluted 1/100 and used to label bacterial surface lipopolysaccharide (LPS). Rhodamine-phalloidin was purchased from Molecular Probes (Eugene, OR) and used as recommended. Monoclonal anti-human tubulin (NE1-052), monoclonal anti-human vimentin (NE1-853) and monoclonal anti-human keratin (NE1-053) were purchased from Immunologics (Burlingame, CA), and monoclonal IgG from Dakopatts, Glostrup, Denmark.

**Immunofluorescence**

MDCK or HeLa cells were split and 1x10⁶ cells were added to each well of a 24-well tissue culture plate containing a glass coverslip. Cells were grown overnight and infected with approximately 5x10⁶ freshly grown *S. typhimurium* for 20 min with HeLa cells or 60 min with MDCK cells. Coverslips were washed extensively with phosphate-buffered saline (PBS), and fixed in 2% paraformaldehyde, pH 7.2, for 1 h, at 4°C, except for tubulin preparations, which were incubated for 5 min in 0.2 mg/ml 1-dithio-bis(succinimidyl proprionate) followed by 5 min in 100 mM Pipes, pH 6.9, 1 mM EDTA, 4% polyethylene glycol 6000 prior to paraformaldehyde fixation. After washing, cells were permeabilized in 0.1% Triton X-100 for 5 min (HeLa) or 15 min (MDCK). Coverslips were washed and incubated in primary and then secondary antibodies for 1 h each, mounted in mounting medium (Sigma), and sealed with nail polish. Nocodazole disruption of microtubules (Pareyko et al. 1989) was carried out by preincubating cells in 10 μg/ml nocodazole (stock was made in DMSO) for 1 h on ice followed by warming to 37°C for 30 min. Cytochalasin D

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was used at a final concentration of 1 μg ml⁻¹ (Finlay and Falkow, 1988), and cells were preincubated for 30 min. Indirect immunofluorescence was examined using a Zeiss Axioskop microscope under oil immersion, and photographed using Kodak T-Max film with an ASA of 400 pushed to 1600. Confocal microscopy was performed using a BioRad MRC 500 system.

**Transmission electron microscopy**
MDCK monolayers grown in Lux Permanox culture dishes (Miles Scientific, Naperville, IL) were washed with PBS and fixed in cold (4°C) 2% glutaraldehyde, 0.1 M sodium phosphate buffer (pH 7.4) overnight. After washing with phosphate buffer, samples were postfixed in cold 1% OsO₄ in 0.1 M phosphate buffer for 90 min, and then stained with cold 0.25% uranyl acetate overnight. Samples were dehydrated in an ethanol series and embedded in a firm Spurr’s plastic. Samples were sectioned and stained with uranyl acetate and lead citrate before examination.

**Invasion and penetration assays**
The number of intracellular bacteria was determined using a previously described method that uses gentamicin to kill extracellular bacteria (Finlay and Falkow, 1988). Samples were done in triplicate. Inhibition studies with anti-integrin antibodies were done by preincubating cells in antibody for 30 min prior to bacterial addition. Penetration rates through polarized monolayers were performed as described elsewhere (Finlay and Falkow, 1990; Finlay et al. 1988c).

**Results**

**Actin**
We used phase-contrast microscopy to examine MDCK and HeLa cells that had been infected with *S. typhimurium*. As shown in Fig. 1A, a dense structure surrounds organisms as they enter MDCK cells. These structures ranged in size from 5 to 10 μm and always contained a bacterium at the center. They were not observed in uninfected cells. The number of these structures peaked about 1 h after bacterial addition, and by 3 h only a small number could be found. The structures observed in HeLa cells were not as well defined as in MDCK cells, and consisted of many string-like structures originating from the center where the bacterium was located (Fig. 1B). The presence of these structures in HeLa cells was expected, as they were seen shortly after bacterial infection. Penetration rates through polarized monolayers were performed as described elsewhere (Finlay and Falkow, 1990; Finlay et al. 1988c).

![Fig. 1. Phase-contrast micrographs of *S. typhimurium* invading: (A) MDCK or (B) HeLa cells. In MDCK cells 60 min after infection a large dense structure surrounding a bacterium is visible and marked by an arrow in A. In HeLa cells 20 min after infection stringy structures surrounding bacteria are visible, also marked by arrows in B. These structures are representative of those seen shortly after bacterial infection. The presence of *S. typhimurium* within these structures was confirmed by fluorescent labelling of the bacteria and examining them by indirect immunofluorescence. Bar, 10 μm.](image-url)

When an infection time course was followed in MDCK cells (samples were collected every 10 min), we could see no such actin structures 10 min after addition of *S. typhimurium*. By 30 min there were several such structures visible, which were always associated with internalized bacteria. At this time, nearly all intracellular bacteria were surrounded by such structures, and at no time could we detect bacteria bound to the surface but not internalized. One hour after infection the largest number of visible structures of condensed actin could be seen, although there were bacteria already inside cells that were no longer surrounded by such structures (Fig. 2A). There were few structures visible from 1.5 h to 3.5 h.

**α-Actinin**
Two different antibody preparations (one monoclonal and one polyclonal) were used to examine α-actinin structure in infected MDCK and HeLa cells. Infected cells stained...
with either of these antibodies exhibited a large accumulation of α-actinin around invading organisms. These structures were similar to those seen with rhodamine–phalloidin-stained cells, and were tightly intertwined with either of these antibodies exhibited a large accumulation of tropomyosin (Fig. 6A–C). HeLa cells were also altered, with the bacteria causing the formation of a 'stringy' accumulation of tropomyosin (Fig. 6D–F). Stress fibers were visible in HeLa cells (Fig. 6D) but were below the bacteria and the associated tropomyosin accumulation (Fig. 6D versus E).

**Tubulin**

Tubulin We have previously shown that colchicine, vincristine and vinblastine do not affect *Salmonella* invasion. Thus when we examined the organization of microtubules with two different monoclonal anti-tubulin antibodies we were surprised to see a large condensation of tubulin surrounding and above internalized bacteria with both antibodies. In MDCK cells these new tubulin-containing structures were filamentous, but of a larger diameter than microtubules (Fig. 7A and B). When MDCK cells were pretreated with nocadazole (10 μg ml⁻¹, 4°C, 1 h), a potent but specific microtubule inhibitor (Parczyk et al. 1989), microtubules were completely disrupted (Fig. 7D). However, the condensation of tubulin around the invading bacteria was actually enhanced by this treatment, yielding larger accumulations of tubulin above internalized bacteria (Fig. 2C, 7D). Pretreatment in nocadazole for up to 4 h also increased the amount of tubulin accumulation above internalized organisms (data not shown).

To determine if the tubulin was associated with the observed actin structures, MDCK and HeLa cells were stained with phalloidin and anti-tubulin. Although they stained their appropriate but different cytoskeletal components, both stains were localized to the same structures above internalized bacteria (Fig. 7E and F), and bacteria could be seen at the center of these structures by phase-contrast microscopy.

Since tubulin exhibited such a marked rearrangement, we tested bacterial invasion in the presence of nocadazole-treated cells. We found that equal numbers of *S. typhimurium* entered HeLa cells when they were treated with nocadazole (1.5±0.2% of the initial inoculum) or an equal amount of dimethyl sulfoxide (DMSO; 2.0±1.1%). Invasion levels of *S. typhimurium* in MDCK cells treated with either nocadazole (1.0±0.6%) or DMSO (0.5±0.2%) were also similar, again confirming that functional microtubules are not required for bacterial internalization. We also tested the penetration rates of *S. typhimurium* through polarized MDCK and Caco-2 epithelial monolayers grown on permeable filters. Nocadazole did not affect the penetration rates of these organisms through these monolayers (2 h for Caco-2, 4 h for MDCK), nor did it alter the transepithelial resistance of uninfected monolayers, indicating that microtubules are not required for *Salmonella* penetration through polarized epithelial monolayers.

**Talin and vinculin**

Three different antibodies to talin (anti-chicken monoclonal, anti-chicken monoclonal and anti-human polyclonal) were used to examine talin distribution in infected cells. As shown in Fig. 8, large amounts of talin also condensed around invading bacteria in both cell lines when stained with anti-human polyclonal serum. Focal contacts were also visible in both cells in a lower focal plane when stained with this antibody (data not shown). Most of the altered talin was localized above the bacteria, as illustrated by the two different focal planes shown in Fig. 8A and B. These structures were similar to that described above for actin, α-actinin, tropomyosin and tubulin, except they appeared less filamentous. Similar structures were seen in MDCK cells using the anti-chicken monoclonal antibodies (Fig. 8C). However, these antibodies did not stain HeLa cells. The anti-chicken talin polyclonal antibodies stained both cells faintly, but similar structures were still visible in both cell lines (data not shown).

Focal adhesion plaques were stained in both cell lines by anti-vinculin (Fig. 9A), but usually no rearrangement was seen near bacteria, as illustrated in Fig. 9A. A small accumulation of vinculin (much less than the other components described) occurred near invading bacteria in a few cases, but this was quite rare.

**Intermediate filaments**

Intermediate filaments were examined in infected cells by using monoclonal antibodies directed against keratin and vimentin. These antibodies stained the intermediate filaments of both MDCK and HeLa cells. There was no disruption in keratin filaments in either cell line near internalized bacteria (Fig. 9C). There was a small amount of accumulation of vimentin filaments near bacteria in a few isolated cases, but this accumulation was minor compared to that seen with the other cytoskeletal components described here. As a control, non-immune rabbit polyclonal sera was used to stain both cell lines, but no structures were stained near internalized bacteria. Additionally, a monoclonal antibody directed against clathrin, rabbit polyclonal sera raised against the cytoplasmic soluble protein dihydrofolate reductase, and a monoclonal specific for the Golgi apparatus did not stain the structures surrounding internalized bacteria.

**Transmission electron microscopy of cytoskeletal structures surrounding internalized bacteria**

MDCK and HeLa cells infected with *S. typhimurium* (1 h or 20 min, respectively) were examined by transmission electron microscopy and focal planes.
Fig. 2. Double exposure of indirect immunofluorescence micrographs of MDCK cells 1 h after infection with *S. typhimurium* (labelled with FITC). (A) Rhodamine-phalloidin was used to stain actin filaments. Actin-containing stress fibers are visible, as are large accumulations of polymerized actin surrounding an invading bacterium (center). Also visible are several bacteria that have already entered these cells, and the actin aggregation is no longer present. (B) Monoclonal anti-α-actinin (BM-75.2) followed by secondary rhodamine labelling was used to illustrate α-actinin accumulation around invading bacteria. (C) Nocadazole-treated cells (see Materials and methods) stained with a monoclonal against tubulin (NEI-052). Microtubules were completely disrupted by this procedure, but the tubulin accumulation surrounding bacteria was actually enhanced. In all three cases the bacteria were inside the cell in a focal plane below the cytoskeletal components.
Fig. 3. Indirect immunofluorescence micrographs of MDCK (A–C) and HeLa (D–F) cells infected with *S. typhimurium* followed by staining with phalloidin (A,B,D and E) and polyclonal sera against *S. typhimurium* (C and F). (A) Lower focal plane, below the large accumulations of actin surrounding bacteria. Several stress fibers are visible. (B) Upper focal plane, focussed on the large accumulations of actin. (C) Corresponding bacterial stain for A and B. (D) Lower focal plane, illustrating the staining of stress fibers below the areas of altered actin accumulation. (E) Upper focal plane, illustrating the filamentous accumulation of actin above internalized bacteria. (F) Corresponding bacterial stain for D and E. Bar, 10 μm.
Fig. 4. Indirect immunofluorescence micrographs of HeLa (A–B) and MDCK (C–D) cells infected with S. typhimurium followed by staining with monoclonal anti-α-actinin (BM-75.2) (A, B and C) and polyclonal sera against S. typhimurium (D). (A) Lower focal plane illustrating the presence of α-actinin in focal contacts and stress fibers in HeLa cells. These structures are not affected by bacterial invasion. (B) Upper focal plane, showing the filamentous structure (arrow) near the cell's apical surface. Although not shown, there was a single bacterium just below this structure. (C) Filamentous structures above internalized bacteria (D) in MDCK cells. Bar, 10 μm.
Fig. 5. Confocal micrograph of a MDCK cell infected for 1 h with *S. typhimurium* and then labelled with a monoclonal antibody against α-actinin, illustrating the distribution of α-actinin around and above, but not below, an internalized bacterium. Four sections were taken, starting with the basolateral region of the cell (A), and moving 0.4 μm upward towards the apical surface (B, C and D). The bacterium is visible at the center of a large accumulation of α-actinin in sections A and B. Sections C and D are above the bacterium as well as most of the rest of the cell’s α-actinin. Bar, 10 μm.

electron microscopy. All bacteria that were visible were internalized, and no bound bacteria were observed. Many small filamentous structures (actin filaments) were visible above and beside the vacuoles containing bacteria (Fig. 10). These structures ran parallel to the surface of the cell. Linkage of these filaments to the vacuole containing the bacterium was not obvious, nor were any visible intact microtubules associated with the filamentous network, although microtubules were visible in other areas of the cell.

*Cytochalasin D treatment does not block accumulation of polymerized actin filaments beneath adherent bacteria*

We were unable to find bacteria bound to epithelial cell surfaces using light microscopy, indicating that once bound they are rapidly internalized. To dissect the steps associated with internalization, MDCK and HeLa cells were treated with cytochalasin D (1 μg ml⁻¹) for 1 h prior to infection. After this treatment, bacteria were visible only on the cell surface. When these epithelial cells were stained with rhodamine-phalloidin or antibodies directed against α-actinin, stress fibers and other actin filaments were disrupted. However, there was still a large condensation of polymerized actin and α-actinin beneath the bacteria on the cell surface (Fig. 11A and C). This condensation was not as filamentous nor as large as that seen without cytochalasin D, but was more globular in appearance. The location of the bacteria above the condensed actin and α-actinin was confirmed by using different fluorescent sections in the confocal microscope. Antibodies to many other cytoskeletal components were also examined by immunofluorescence microscopy of MDCK and HeLa cells that were infected and treated with cytochalasin D. Cytochalasin D treatment abolished any observable accumulation of tropomyosin (Fig. 11E), talin and tubulin below surface-bound *S. typhimurium*. Keratin, vinculin and vimentin staining patterns also remained independent of the location of these bound bacteria.

*Integrin antibodies do not inhibit Salmonella invasion*

Talin is known to be associated with focal contacts and involved in linking integrins to actin filaments (Burridge and Fath, 1989). Because of the large alterations that we observed in talin distribution, we examined whether integrins are involved in *Salmonella* internalization. HeLa cells were preincubated in various anti-integrin antisera, and then infected with *S. typhimurium*. The numbers of internalized bacteria in HeLa cells were then quantitated as described in Materials and methods. Antibodies tested included anti-fibronectin receptor polyclonal sera (1/2 minimal dilution), anti-β1 (1/2), anti-α2 (1/10), anti-α5 (1/2), anti-α6 (1/2), anti-β2 (1/6), anti-αv (1/10) and anti-epidermal growth factor receptor (1/2) as a
Fig. 6. Indirect immunofluorescence micrographs of MDCK (A–C) and HeLa (D–F) cells infected with S. typhimurium followed by staining with a monoclonal against tropomyosin (TM311) (A,B,D and E) and polyclonal sera against S. typhimurium (C and F). (A) Lower focal plane, below the three large accumulations of tropomyosin surrounding bacteria. (B) Upper focal plane, focussed on the large accumulations of tropomyosin. (C) Corresponding bacterial stain for A and B. (D) Lower focal plane, illustrating the staining of stress fibers below the areas of disrupted tropomyosin. (E) Upper focal plane, illustrating the filamentous accumulation of tropomyosin above internalized bacteria (arrow). (F) Corresponding bacterial stain for D and E. Bar, 10 μm.

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Fig. 7. Indirect immunofluorescence micrographs of MDCK cells infected with *S. typhimurium* followed by staining with a monoclonal against tubulin (NEI-052) (A,B,D,E) and polyclonal sera against *S. typhimurium* (C). (A) and (B) are two focal planes illustrating microtubules and the brightly staining filamentous structures above internalized bacteria (C). These structures were thicker in diameter and shorter than microtubules, and were only seen in infected cells. (D) Large structures accumulating around internalized bacteria after treatment with the microtubule-disrupting agent nocadazole. Microtubules appear disrupted by this treatment. (E) and (F) Cells stained for microtubules (E) or with phalloidin (F). Both panels are at the same focal plane, and illustrate the different staining patterns of microtubules (E) and microfilaments (F), except for the condensation that occurs above bacteria, as marked by the two arrows.
Fig. 8. Infected HeLa (A,B) and MDCK (C) cells stained with anti-talin serum. (A) Immunofluorescence micrograph of an upper focal plane (apical surface), illustrating the large accumulation of talin above internalized bacteria after staining with polyclonal anti-mammalian talin serum. The talin accumulation was not as filamentous in nature as seen with the other cytoskeletal stains. (B) Lower focal plane, with internalized S. typhimurium visible below condensed talin. (C) Confocal micrograph showing talin aggregation in MDCK cells after staining with monoclonal anti-chicken talin. Bacteria were not stained, but were visible under all the brightly staining areas of talin accumulation. Bars, 10 μm.

Discussion

S. typhimurium causes marked changes in the epithelial cell as it enters. We have shown previously that it causes degeneration of microvilli on polarized epithelial monolayers (Finlay and Falkow, 1990; Finlay et al. 1988a). In the guinea pig epithelium and polarized Caco-2 human intestinal epithelial cells Salmonella also causes bulging and protrusion of the apical cell surface (Finlay and Falkow, 1990; Takeuchi, 1967), presumably because of localized disruption of the underlying cytoskeleton. We have shown here that large amounts of actin, α-actinin, tropomyosin, tubulin and talin accumulate when infected with S. typhimurium. All of these molecules occur in filamentous structures, which are located beside and above internalized bacteria. The formation of these filamentous actin-rich structures is correlated with initial bacterial entry, after which they disappear and their components assume their normal patterns of distribution leaving the bacterium within its vacuole relatively free of cytoskeletal proteins.

We found that the filamentous structures caused by invading bacteria were more tightly clustered in MDCK cells than in HeLa cells, although the same cytoskeletal components were affected in both cell lines. This difference is probably due to the generally more organized cytoskeleton in MDCK cells. Salmonella also entered HeLa cells faster than MDCK cells, again presumably because of the relatively 'looser' organization of their cytoskeleton. The large accumulation of actin, α-actinin and tropomyosin found in our study suggests that Salmonella use a phagocytic-like uptake mechanism in epithelial cells. It has been shown that Shigella flexneri, another invasive organism, causes actin polymerization and myosin condensation around invading organisms in HeLa cells (Clerc and Sansonetti, 1987).

The accumulation of tubulin around invading Salmonella was unexpected. We have shown here and elsewhere (Finlay and Falkow, 1988) that microtubule inhibitors do not block Salmonella invasion or penetration through polarized epithelial monolayers. However, nocodazole did not decrease the accumulation of tubulin around the bacteria: instead it enhanced it, even though cell microtubules were completely disrupted. These data suggest that tubulin associated with these structures is in either a monomeric form or an altered (nocodazole-resistant) control. None of these integrin antibodies decreased the number of intracellular Salmonella (data not shown). However, anti-fibronectin receptor, anti-β1, anti-α5 and anti-α6 antibodies at these dilutions decreased internalization of E. coli containing the cloned invasion gene from Yersinia pseudotuberculosis (Inv, which encodes invasin) from 10- to 100-fold (data not shown), indicating that these antibodies inhibit invasin binding. Invasin is known to use several β1 integrins as receptors, and integrin antibodies inhibit its adherence and promotion of bacterial uptake (Isberg and Leong, 1990). Interestingly, when we examined HeLa cells infected with E. coli containing cloned invasin for cytoskeletal rearrangements, no rearrangement of actin or α-actinin was visible. Addition of the synthetic peptide, GRGDSP, at concentrations up to 1 μg/ml (which inhibits fibronectin binding to its receptor; Ruoslahti and Pierschbacher, 1987), or fibronectin did not affect S. typhimurium uptake into MDCK or HeLa cells (data not shown).
Fig. 9. Indirect immunofluorescence micrograph of infected HeLa cells stained with a monoclonal directed against vinculin (VIN-11-5) (A), keratin (NEI-054) (C), or polyclonal sera against S. typhimurium (B and D). (A) Basolateral focal plane, showing focal contacts stained with vinculin. These focal contacts were not affected by bacterial invasion (B). (C) Keratin intermediate filaments were not affected by internalized bacteria (D). Bars, 10 μm.
Bacteria internalized within vacuoles. These organisms may share some components that would be needed for subsequent internalization. The use of cytochalasin D has allowed us to begin to determine the order of cytoskeletal recruitment that occurs during bacterial uptake. Actin and α-actinin both accumulate beneath the surface of bound bacteria, yet we could see no accumulation of the other cytoskeletal components such as tropomyosin, talin or tubulin. This suggests that polymerized actin and α-actinin molecules are recruited first, directly beneath the bound bacteria, and that the other components such as tropomyosin accumulate once actin and α-actinin filaments are in place. Since the actin structures beneath bacteria can be stained with phalloidin (stains only polymerized actin), the bacteria must engage and hold the actin in some polymerized state so that it resists disruption by cytochalasin D. The other significant point of the cytochalasin D studies is that actin and α-actinin condensation can occur beneath the bacteria, while it is still bound to the outside of the cell and not internalized. In agreement with the time-course studies, this suggests that this process is essential to internalization and begins upon bacterial binding to the epithelial surface, rather than a consequence of the bacteria being inside the cell. The accumulation of polymerized actin beneath surface-localized bacteria is very similar to that reported for enteropathogenic E. coli (Knutton et al. 1987). However, this organism is not particularly invasive, and usually remains bound to the surface of the cell, resting on a pedestal-like structure. These organisms may share some components that would be needed for triggering actin accumulation beneath the organism, although S. typhimurium would need additional components that would be needed for subsequent internalization.

On the basis of our observations, we propose that Salmonella bind to a cell surface molecule and somehow cause the transmission of a signal that initiates actin-based internalization of the pathogen. This signal may be provided by the bacteria, since bacteria have to be metabolically active to enter host cells (Finlay et al. 1989b). This signal would then link the bound receptor (perhaps via a series of unknown intermediates) with α-actinin. These observations are similar to those reported here on Salmonella internalization, except for the involvement of tropomyosin. However, we were unable to find any association between known integrin molecules and Salmonella internalization, in contrast to that reported for Yersinia (Isberg and Leong, 1990). It is possible that we did not use the correct conditions necessary for blocking a required integrin, or there may be some other molecule, integrin or otherwise, that spans the host membrane, linking the cytoskeleton and the bacteria.
Fig. 11. Indirect immunofluorescence micrographs of MDCK cells pretreated with cytochalasin D (1 μg ml⁻¹) for 1 h before infection with *S. typhimurium*. This treatment blocked bacterial uptake but not binding. Samples were stained with rhodamine-phalloidin (A), anti-α-actinin (C), or anti-tropomysin (E) and polyclonal sera against *S. typhimurium* (B,D,F). Accumulation of polymerized actin (A) or α-actinin (C) is visible beneath bound bacteria (marked by arrows), but was not visible with tropomysin (E). Bar, 10 μm.
microfilaments and their associated binding proteins α-actinin, tropomyosin, and probably myosin. As in phagocytosis, myosin may then generate the force required to internalize the receptor and its bound microfilaments and their associated binding proteins talin and other cytoskeletal components similar to that observed with S. typhimurium might occur with invading Yersinia and, for that matter, with other intracellular parasites. However, no cytoskeletal rearrangements were visible with the cloned Yersinia invasin, and antibodies to various β1 integrins did not decrease S. typhimurium invasion, emphasizing the differences in invasion mechanisms between these two organisms. It would seem that pathogenic bacteria use various host cell proteins for receptors that have the capacity to generate linkages between the membrane and cytoskeleton that are necessary for entry of the pathogen into the eukaryotic cell. Whether binding to the receptor stimulates the formation of these linkages, or whether the bacterium synthesizes a product necessary for the creation of these linkages remains to be defined, as does the nature of the signal. These questions are currently under investigation in our laboratories. In addition to providing clues to the important question of determining how intracellular parasites enter host cells, those parasites may prove to be novel tools for studying the dynamic interactions of the eukaryotic cytoskeleton.

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