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Intercellular Spread of *Shigella flexneri* through a Monolayer Mediated by Membranous Protrusions and Associated with Reorganization of the Cytoskeletal Protein Vinculin

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The spread of *Shigella flexneri* in a monolayer of infected Henle and HeLa cells was studied by using immunofluorescence and electron microscopy. Infected cells produced numerous bacterium-containing membranous protrusions up to 18 μm in length that penetrated adjacent cells and were subsequently phagocytosed. Fluorescence staining of actin and vinculin in infected cells with phalloidin and monoclonal antibody to vinculin, respectively, demonstrated that the protrusions containing the bacteria consisted of these cytoskeletal proteins. Actin accumulated predominantly at the poles of bacteria distal to the tip of protrusions and appeared as trails extending back towards the host cell cytoplasm. Vinculin, however, was distributed uniformly around the bacteria and throughout the protrusion. A profound rearrangement of vinculin occurred in Henle and HeLa cells following infection with shigellae; whereas in uninfected cells it was distributed mainly around the cell periphery, in infected cells it concentrated mainly around clusters of bacteria in the cytoplasm. This suggests a possible involvement of the vinculin cytoskeletal protein in the intercellular spread of shigellae during an infection.

Shigellae are enteroinvasive pathogens which are able to penetrate, multiply within, and lyse intestinal epithelial cells (19). The invasive ability of *Shigella* strains can be assessed in vitro by their ability to infect monolayers of cultured mammalian cell lines such as HeLa or Henle (11, 19). The entry process into mammalian cells involves actin polymerization and myosin accumulation, a mechanism similar to phagocytosis, and requires metabolically active bacteria and host cells (5, 10, 11). Bacteria initially present in phagocytic vacuoles lyse the vacuolar membranes and escape into the cytoplasm, where they undergo rapid multiplication. Their interaction with host cell microfilaments, intracellular spread, and subsequent infection of adjacent cells have been described previously (1, 5, 28). Intracellular movement of the nonmotile *Shigella flexneri* bacteria has been suggested to involve polymerization of actin, and a 120-kDa protein located on the bacterial outer membrane which appears to interact with microfilaments within eukaryotic cells has been proposed to play a role in these movements (1). Bacterial movement within infected cells leads to the formation of extracellular protrusions, which are thought to play a role in the spread of bacteria to adjacent cells (1, 23, 25). However, until now a detailed ultrastructural analysis of the lateral spread of shigellae within an infected monolayer has not been published in support of this proposal.

Bacterial penetration of eukaryotic cells, intracellular movement, and the formation of protrusions consisting of actin filaments associated with α -actinin and tropomyosin have been documented for another intracellular parasite, *Listeria monocytogenes*. This work has revealed a detailed sequence of events that take place during the bacterial

intracellular life cycle (6, 22, 37). The involvement of actin but not microtubules in the intracellular movement of both *L. monocytogenes* and *S. flexneri* has been demonstrated by the use of drugs which specifically interfere with the polymerization of these components of the cytoskeleton (1, 5, 6, 11, 22, 25, 37).

Several studies have reported the interaction of viruses with host cell cytoskeletal proteins during virus assembly and release through specialized microvilli or cytoplasmic protrusions from infected cells (12, 34). Reorganization of the cytoskeletal protein vinculin has been observed after infection of several cell lines with Rous sarcoma virus (16, 20, 27). Vinculin has been shown to be located in the focal adhesion plaques (areas in which cells adhere to the culture dish surface), anchoring actin filament bundles to the plasma membrane, and to be a cytoskeletal component of the fibronectin-actin transmembrane complex (8, 38). Parallels between viral and intracellular bacterial spread through intercellular protrusions have not, to our knowledge, been explored in any detail. We therefore decided to investigate whether such rearrangement of vinculin also occurs during infections of cultured cells with shigellae, since a role for this protein in bacterial infections has so far not been reported.

In this communication, we demonstrate the development of bacterium-containing protrusions in Henle and HeLa cells infected with shigellae and the subsequent transmission of bacteria to neighboring cells by these protrusions. We further show the association of actin and vinculin with *S. flexneri*-induced protrusions and a dramatic reorganization of vinculin distribution in infected cells.

MATERIALS AND METHODS

Bacterial strain and culture conditions. A virulent strain of *S. flexneri* serotype 5 (M90 T) (28) was grown on Congo red agar plates (26) at 37°C for 24 to 36 h. Tryptone soy broth (Oxoid, Hants, England) was inoculated with colonies show-

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ing red centers on Congo red plates, and the plates were incubated with shaking in an orbital shaker at 37°C. Bacteria were harvested in the exponential phase of growth.

Isolation and preparation of antisera to LPS. Cells from a 1-liter culture were killed by treatment with formalin, 4% (vol/vol), for 15 min at room temperature. Washed cells were resuspended in 20 ml of distilled water and broken by sonication (10 60-s pulses on ice with 30-s cooling intervals). Unbroken cells were removed by centrifugation at $5,000 \times g$ for 10 min. The extract was incubated for 18 h at 37°C with 200 μg of DNase I (D-5025; Sigma Chemical Co., St. Louis, Mo.) and 50 μg of RNase A (R-4875; Sigma) per ml and then for a further 18 h with 200 μg of protease type XIV (P-5147; Sigma) per ml. The suspension was heated to 67°C, mixed with an equal volume of 90% (wt/vol) phenol at 67°C, and stirred for 15 min at 67°C. Following centrifugation ($10,000 \times g$ for 15 min at 4°C), the aqueous phase was removed and dialyzed against water for 18 h. The solution was centrifuged at $100,000 \times g$ for 3 h, and the resulting lipopolysaccharide (LPS) pellet was desiccated. Antibodies to LPS were raised in rabbits (Dakopatts, Glostrup, Denmark) by injecting 25 μg subcutaneously four times at 2-week intervals. Two weeks after the last injection, serum was collected and used for immunological studies.

Tissue culture methods and infection procedures. The human intestinal epithelial cell line Henle 407 (ATCC strain CCL-6) or HeLa S₃ (ATCC strain CCL-2.2) was maintained in Dulbecco's modified Eagle medium (GIBCO laboratories, Eggenstein, Germany) with 10% fetal calf serum (GIBCO), 5 mM glutamine (Flow Laboratories Inc., McLean, Va.), 1 mM pyruvate (Flow), 100 IU of penicillin per ml, and 100 μg of streptomycin per ml (Flow) in an atmosphere containing 5% CO₂ at 37°C. Trypsinized cells were seeded at a concentration of approximately 2×10^5 cells per coverslip (15 by 15 mm) in tissue culture plates (12 by 4.5 cm²; Flow) or 5×10^6 cells per 75-cm² flask (Sterilin, Middlesex, England). Plates or flasks were incubated for 18 to 24 h in 5% CO₂ and washed three times in phosphate-buffered saline (PBS; NaCl, 8.0 g/liter; KCl 2.0, g/liter; Na₂HPO₄ · 2H₂O, 2.0 g/liter; KH₂PO₄, 2.0 g/liter; pH 7.4). For infection of monolayers, bacteria were harvested in the exponential phase, resuspended to a density of 10^8 CFU/ml in Dulbecco's modified Eagle medium without antibiotics, and overlaid onto the monolayer of cells on either coverslips (1 ml) or flasks (10 ml). Contact between bacteria and cells was facilitated by centrifugation ($145 \times g$ for 10 min) (Heraeus Omnifuge 2.0 RS, Osterode, Germany), followed by incubation for 30 min at 37°C with 5% CO₂. Extracellular organisms were removed by washing three times with PBS. Dulbecco's modified Eagle medium containing gentamicin (100 $\mu\text{g}/\text{ml}$) was then added; this concentration of gentamicin killed extracellular bacteria and thereby prevented any reinfection of cells. Monolayers were incubated for an additional 4 to 5 h. Minimal lysis of the monolayers occurred during this time interval. The development of extracellular protrusions was monitored by using an inverted microscope with a $\times 20$ objective (Zeiss, Oberkochen, Germany). Cellular multiplication was stopped 5 h after infection by carefully washing

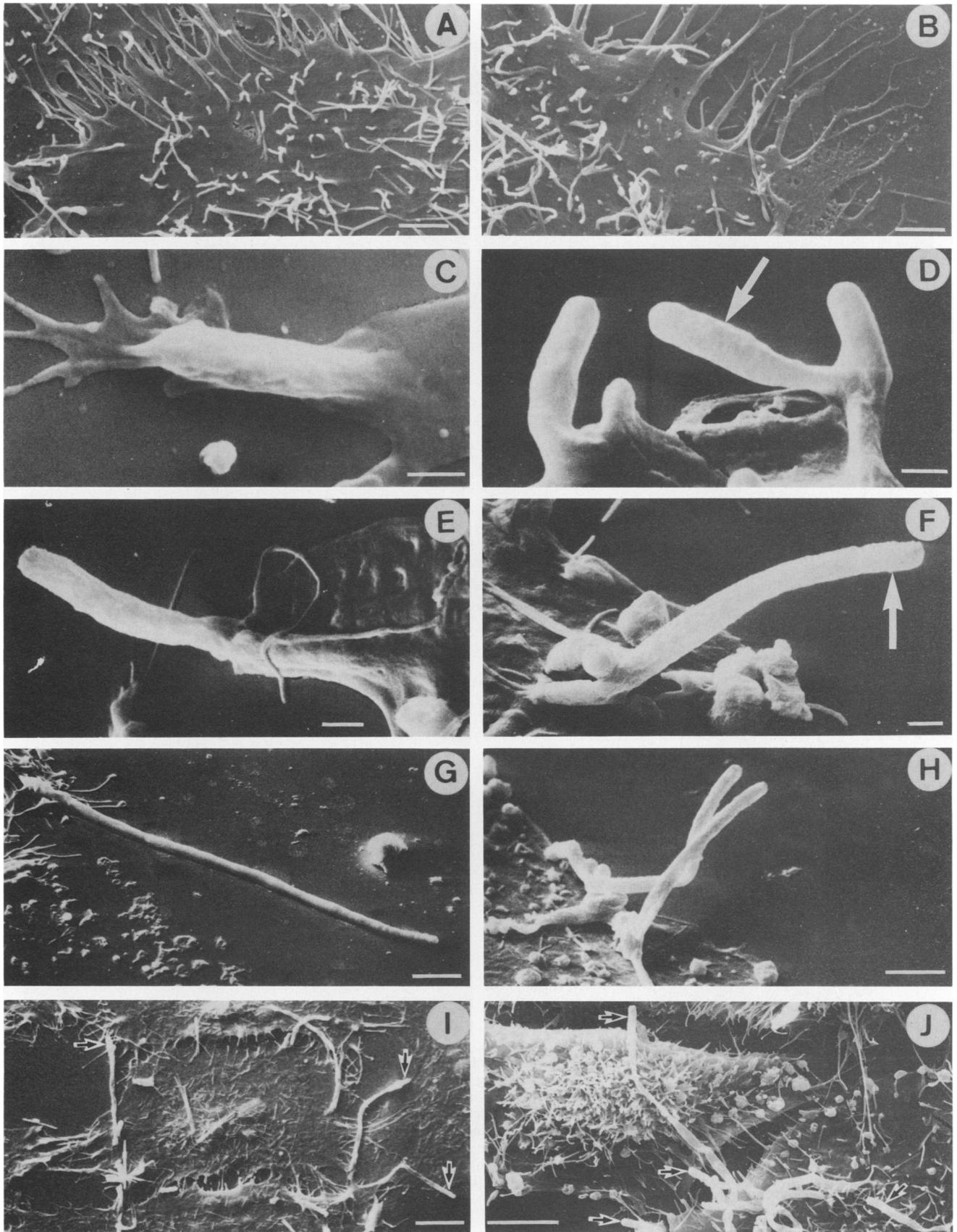
the monolayers three times with ice-cold HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (GIBCO), followed by fixation for electron microscopy or fluorescence microscopy.

Fluorescence staining of F-actin, vinculin, and LPS of bacteria. The washed cells were fixed in situ with 3.7% formaldehyde in PBS containing 1 mM MgCl₂ and 0.1 mM CaCl₂ (solution A). Coverslips were then treated for 10 min with 50 mM NH₄Cl in solution A and washed once in PBS. Unless otherwise specified, cells were rendered permeable to the antibody reagent by a brief (5-min) treatment with 0.2% Triton X-100 in solution A. To visualize actin, permeabilized cells were labeled with rhodamine-labeled phalloidin (a phallotoxin which binds F-actin specifically; Molecular Probes Inc., Junction City, Ore.) diluted 1/50 in PBS containing 10% fetal calf serum (solution B) for 30 min. To reveal vinculin, permeabilized cells were labeled with mouse monoclonal antibody (MAb) to vinculin (VIN-11-5; Sigma) diluted 1/100 in solution B for 1 h, rinsed in solution A, and subsequently treated with a 1/20 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Dianova, Hamburg, Germany) in solution B for 30 min. Coverslips were washed three times in solution A and mounted. Double fluorescence staining of actin and bacteria was performed on nonpermeabilized cells to study the nature of the membrane in protrusions. After being washed in ice-cold HEPES buffer, coverslips were incubated for 30 min on ice with rabbit antiserum to the LPS of *S. flexneri* serotype 5, diluted 1/50 in solution B. After the usual washing procedure, cells were fixed and made permeable as described above. Cells were then treated simultaneously for 30 min with a staining solution made up in solution B containing 1/20-diluted fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin (Dianova) and 1/50-diluted rhodamine-labeled phalloidin.

Transmission electron microscopy. Infected cells were first fixed with a solution containing 3.6% glutaraldehyde and 0.5% ruthenium red in cacodylate buffer (0.1 M cacodylate, 0.09 M sucrose, 0.01 M MgCl₂, 0.01 M CaCl₂, pH 7.0) for 2 h on ice. After cells were washed with cacodylate buffer containing 0.15% ruthenium red, they were fixed for a further 3 h at room temperature with cacodylate buffer–3.6% glutaraldehyde–1% aqueous osmium tetroxide, washed with cacodylate buffer, and scraped off the culture flask. After centrifugation ($2,000 \times g$, 2 min), the infected cells were embedded in 2% agar, dehydrated with acetone, and then embedded in Spurr resin (32). Sections were examined with a Zeiss electron microscope EM 10B at an acceleration voltage of 80 kV at calibrated magnifications.

Scanning electron microscopy. Infected cells grown on coverslips were fixed for 1 h on ice with a mixture of 3% glutaraldehyde and 5% formaldehyde in cacodylate buffer. They were washed with the same buffer, fixed with 1% osmium tetroxide for 1 h on ice, and dehydrated with a graded series of acetone. Critical-point drying was carried out with CO₂. The samples were then covered with a 10-nm gold film before examination in a Zeiss DSM 940 scanning electron microscope.

FIG. 1. Scanning electron micrographs showing the formation of protrusions in Henle and HeLa cells infected with *S. flexneri* for 1 to 4 h. Uninfected Henle (A) and HeLa (B) cells. Different stages in the formation of the protrusions in Henle cells (C to I). Presumable infection of adjacent Henle (I) and HeLa (J) cells by the protrusions. Location of bacteria within protrusion (arrow). Bars: 1.0 μm (A and B); 0.5 μm (C to F); 2.0 μm (G and H); 5.0 μm (I and J).



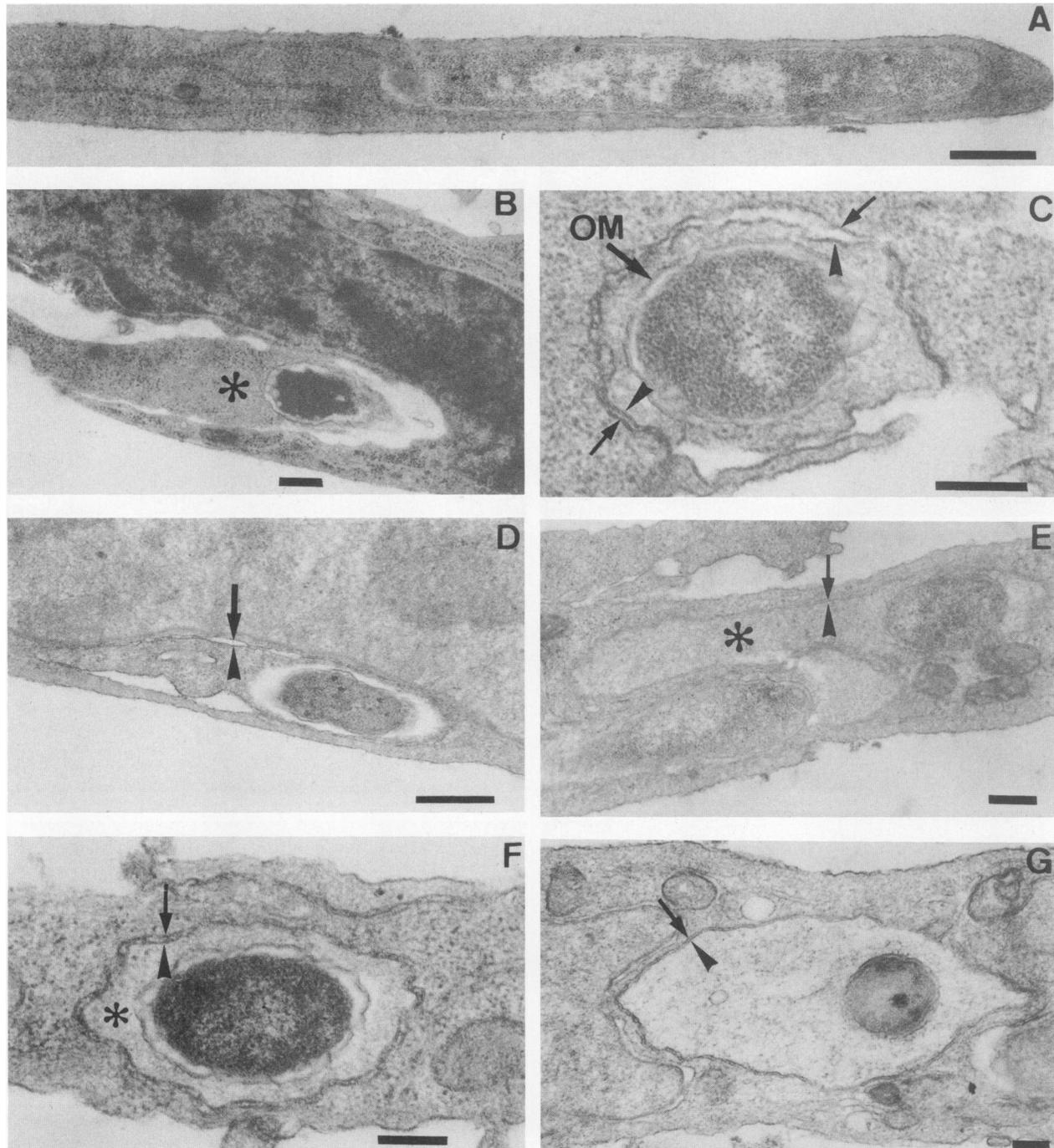


FIG. 2. Ultrathin sections of Henle cells infected with *S. flexneri*. Longitudinal section through a protrusion demonstrating the location of a bacterium at the tip of the protrusion (A). Protrusions penetrating the cytoplasm of adjacent noninfected cells: longitudinal section (B); cross section (C). Engulfed protrusion; the membrane of the protrusion (arrowhead) and the plasma membrane of the adjacent cell (arrow) are visible (D). Protrusions located in the cytoplasm of the newly infected Henle cell (E and F); a double membrane is detectable consisting of the plasma membrane of the infected cell (arrows) and the protrusion membrane (arrowheads). In the longitudinal section (E), a long tail of spongy material (asterisk) most probably representing actin (see Fig. 3A) is located behind the bacterium. Ongoing destruction of phagosomal contents surrounding the bacterium in Henle cells after 4 h of infection (G and H). Note that the double membrane is still visible. Five hours after infection, the double membrane is partially dissolved (little arrows in I). Subsequently, the bacteria are free in the cytoplasm (J). Bars: 0.5 μm (A, B, D, E, and J); 0.2 μm (D and F to I).

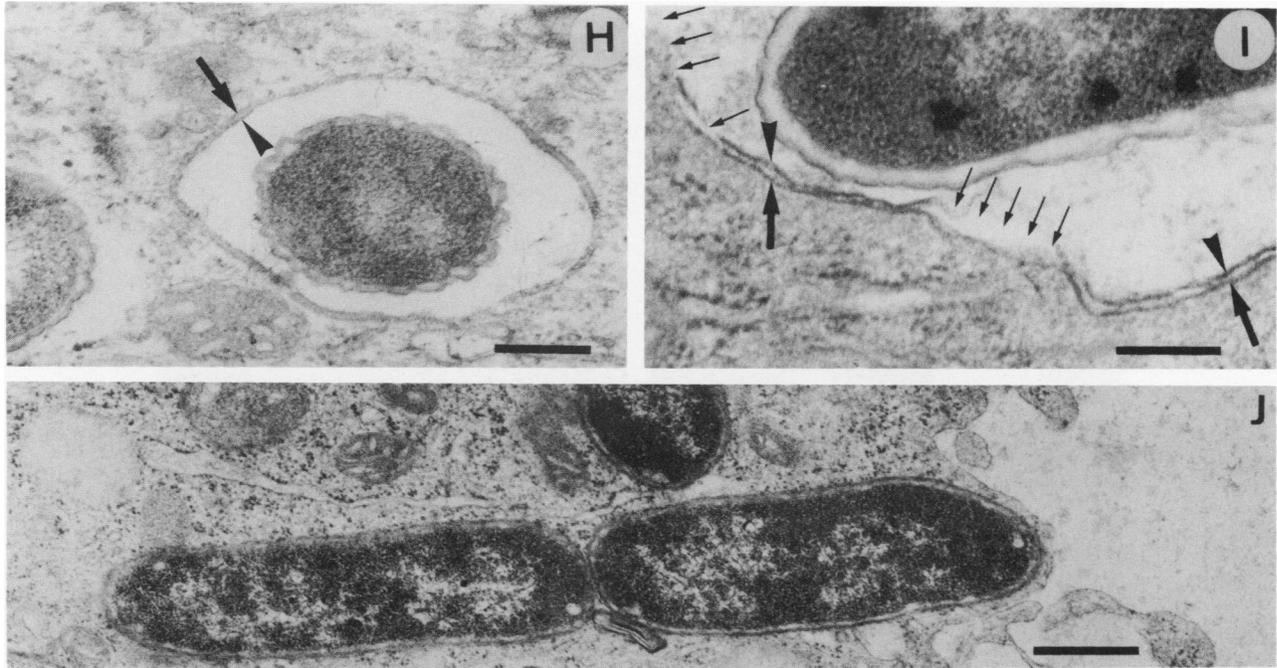


FIG. 2—Continued.

RESULTS

Electron microscopic analysis of the intercellular spread of shigellae in Henle and HeLa tissue culture cells. Approximately 45 min after infection with *S. flexneri*, Henle and HeLa cells with protrusions on their surfaces were seen by light microscopy (data not shown). To characterize the *S. flexneri*-induced protrusions in more detail, cells infected for a period of 1 to 4 h were examined by scanning electron microscopy. As can be seen in Fig. 1, both infected and uninfected Henle and HeLa cells had numerous normal microvilli with characteristic diameters of approximately 0.10 μm . Infected cells also displayed numerous specialized protrusions having larger diameters of 0.50 to 0.60 μm . The difference in diameter is especially obvious with infected cells which produce both normal and *S. flexneri*-induced protrusions (Fig. 1E to J). Some of the latter protrusions formed intercellular connections and appeared to be harboring shigellae (Fig. 1F, I, and J). They were variable in length (up to 18 μm) but rather constant in diameter in regions containing bacterial cells (Fig. 1D, F, I, and J).

A transmission electron micrograph of a protrusion containing a bacterium within the tip, present on a Henle cell 3 h after infection, is shown in Fig. 2A. Figure 2B and C shows *S. flexneri* cells within protrusions penetrating neighboring cells through highly invaginated plasma membranes. Figure 2C shows a cross section of a protrusion partially surrounded by the plasma membrane of a newly penetrated cell during its internalization. Figure 2D exhibits a protrusion clearly enclosed within double host cell membranes, implying that the protrusions arising from the primary infected cells have been engulfed and completely surrounded by the plasma membranes of the penetrated cell. A less densely stained "spongy material" in close contact with one polar end of a bacterium is seen in Fig. 2E; this material, which stains with phalloidin, is probably the heavy deposit of F-actin seen in Fig. 3A. A newly formed phagosome con-

taining a bacterium is seen in Fig. 2F; the *Shigella* cell is enclosed in the protrusion membrane along with actin filaments of the donor cell that, in turn, is surrounded with membrane from the recipient cell. The double membrane consisting of the protrusion membrane and that of the penetrated cell is clearly visible. Figure 2G shows a phagosome undergoing dissolution or breakdown of the less compact phagosomal contents. Completely digested phagosomal contents surrounding shigellae and partially digested protrusion membranes are shown in Fig. 2H and G, respectively. After 5 h of infection, bacteria escape into the cytoplasm (Fig. 2J) after lysing the double membrane (Fig. 2I) during the infectious process, as shown by Sansonetti et al. (28).

Fluorescence microscopy of infected cells with reagents specific for F-actin and vinculin. Fluorescence labeling of infected monolayers with either phalloidin or MAb to vinculin was carried out to analyze the nature of *Shigella*-induced membrane protrusions. Intense staining of the protrusions with both phalloidin and MAb to vinculin is demonstrated in Fig. 3A, C, and E. Bacteria within protrusions appeared brightly labelled with MAb to vinculin, indicating that they were heavily covered with the protein (Fig. 3C and E). The brightest staining with phalloidin occurred at the end of the bacteria distal to the tip of the protrusion, suggesting heavy polymerization of F-actin behind the advancing bacteria (Fig. 3A). This is consistent with the findings of Bernardini et al. (1). Vinculin MAb however, although concentrated around the bacteria, was also uniformly distributed throughout the protrusion (Fig. 3C and E). Interestingly, protrusions containing two (Fig. 3A and B) bacterial cells were occasionally observed.

The distribution of actin cytoskeleton in uninfected Henle cells after staining with rhodamine-phalloidin is shown in Fig. 4A. After infection, the labeling pattern appeared to be slightly different from that of uninfected cells (Fig. 4A and C): the accumulation of actin slightly increased at the focus

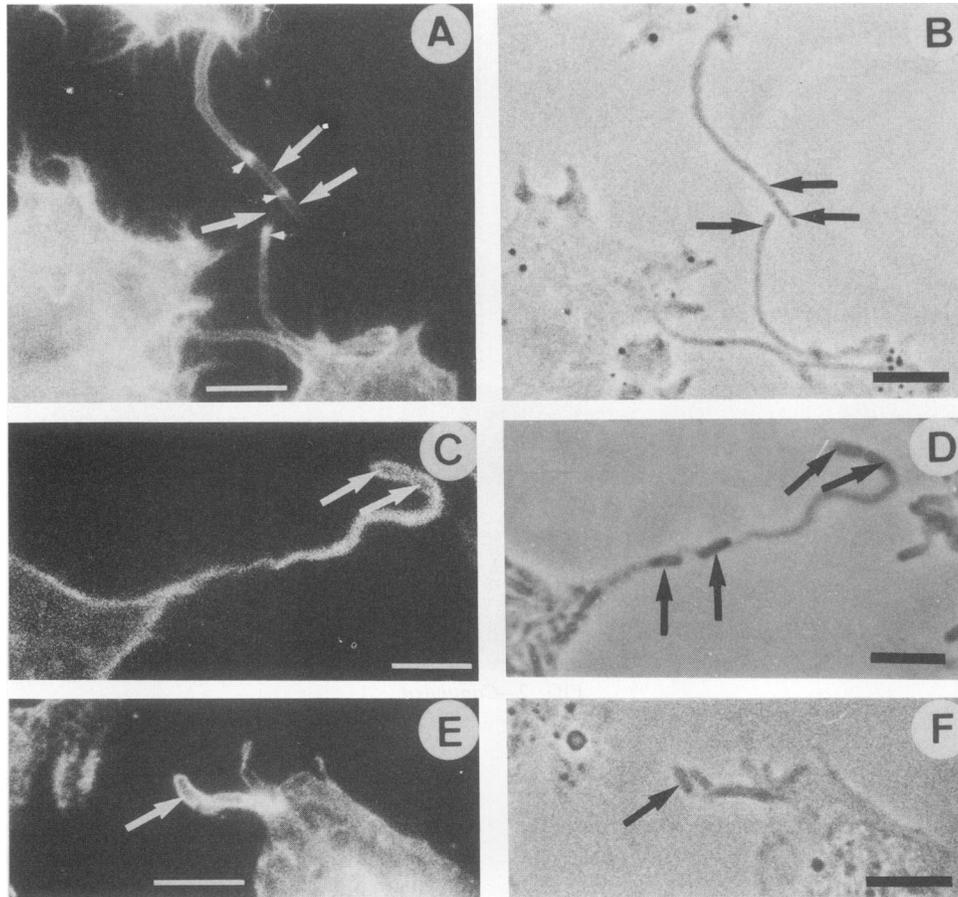


FIG. 3. Distribution of actin and vinculin in protrusions induced in *S. flexneri*-infected Henle (A to D) and HeLa cells (E and F). Three hours after infection, cells were stained with rhodamine-labeled phalloidin (A) or with vinculin MAb followed by fluorescein-labeled secondary antibody (C and E). The corresponding phase-contrast micrographs (B, D, and F) illustrate the location of bacteria within protrusions (arrows). Note the presence of more than one bacterium within a protrusion (A to D). Arrowheads (A) indicate brightly phalloidin-labeled regions at one polar end of the bacteria, thus identifying heavy accumulation of actin in that region. Uniform distribution of vinculin surrounding the bacteria and along the protrusion indicates that the association of vinculin with the protrusions (C and E) is different from that of actin (A). Bars, 2.5 μm .

of the infection, where the bacteria accumulate in the cytoplasm of the infected Henle cell (Fig. 4C, arrows). However, the reorganization of cytoplasmic actin was not as profound as that seen for vinculin.

In noninfected cells, vinculin was predominantly localized at the cell periphery at sites which anchor the cells to the glass surface (Fig. 4D). In infected cells, however, a profound rearrangement of vinculin architecture occurred, with a substantial reduction in the number of vinculin-containing plaques at the cell periphery and a concentration of the protein mainly around the clusters of bacterial aggregates in the cytoplasm (Fig. 4F, arrows). The reorganization of vinculin pattern was accompanied by a slight alteration in the morphology of Henle and HeLa cells (Fig. 1A, B, I, and J and 4E and F): as the infection progressed, the adhesion plaques disappeared gradually from the cell periphery, and at 5 to 6 h post-infection, the majority of heavily infected cells had become rounded and detached from the glass surface.

Infected cells were also incubated with fluorescein-labeled anti-LPS antibodies to determine whether or not the bacteria in protrusions were completely enclosed by the host cell

membrane. Bacteria within protrusions of nonpermeabilized cells were not labeled with antibodies to LPS, indicating effective exclusion of antibody by the host cell membrane. However, LPS antibodies labeled bacteria in lysing host cells and those which had escaped into the extracellular environment (data not shown), demonstrating the validity of the detection system.

DISCUSSION

Earlier studies with cinematographical techniques (24), electron microscopy (25), and immunofluorescence microscopy (1) revealed fibrillar structures protruding from the surface of *S. flexneri*-infected host cells and containing bacteria. It was postulated that these protrusions may participate in the cell-to-cell spread of bacteria within the monolayers (1, 24, 25). We have confirmed and extended these studies by demonstrating penetration of recipient cells by the protrusions, the subsequent transfer of bacteria into penetrated cells, the lysis of the protrusion and phagosome membranes, and the release of the infecting bacteria into the cytoplasm of the new host cell. Though these features

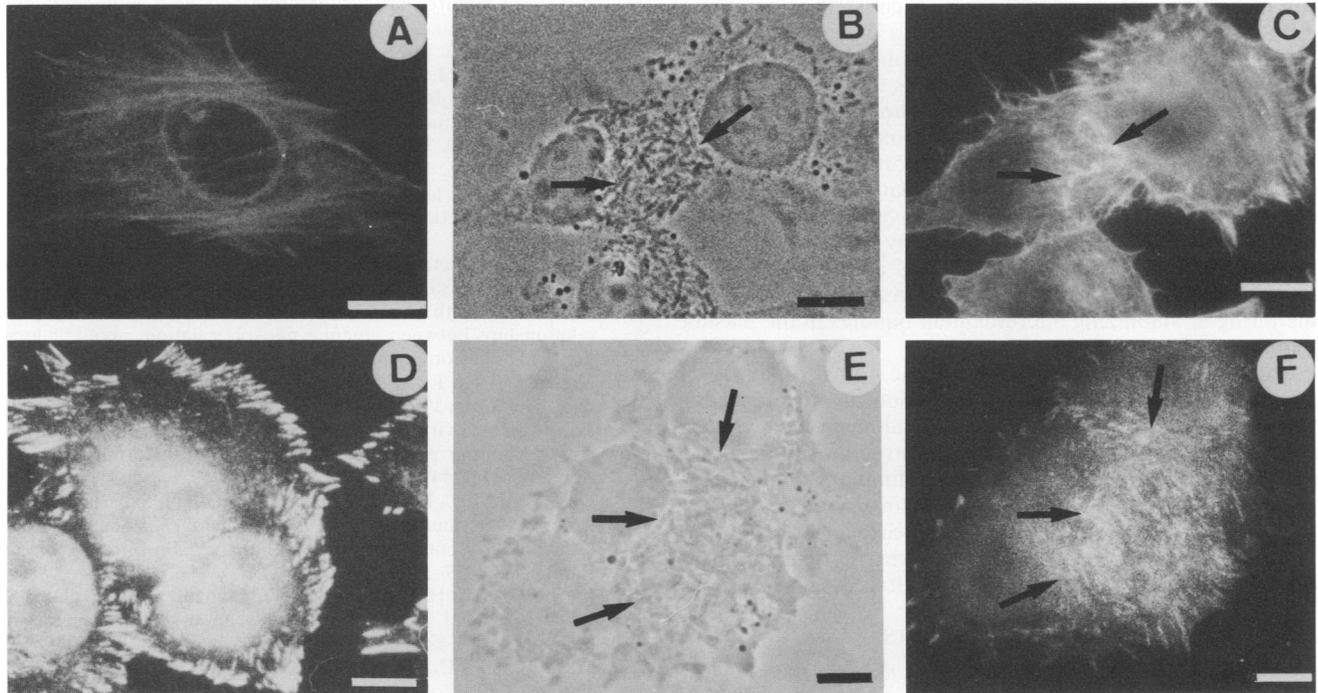


FIG. 4. Distribution of actin and vinculin in noninfected and infected Henle cells. Cells were stained with rhodamine-labeled phalloidin (A and C) or with vinculin MAb followed by fluorescein-labeled secondary antibody (D and F). Distribution of actin in a noninfected Henle cell (A) or in *S. flexneri*-infected cells (C) 4 h after infection; the corresponding phase-contrast micrograph (B) illustrates the distribution of bacteria (arrows). After infection, bright spots can be seen in the infected cell (arrows), demonstrating the accumulation of actin around the bacteria. In noninfected cells, vinculin is mainly located at the cell periphery in focal adhesion plaques (D); in *S. flexneri*-infected cells 4 h after infection, most of the vinculin appears to be concentrated around the bacteria (arrows in F) when compared with the corresponding phase-contrast micrograph (E). Bars, 2 μ m.

parallel those reported for *L. monocytogenes* (6, 22, 37), the presence of multiple shigellae within protrusions has not been reported yet for *Listeria* infections. In this study, we have also confirmed the inaccessibility of *Shigella*-specific antibodies to bacteria within these protrusions, which may not be unimportant for the spread of epithelial cell pathogens within target tissues.

Recent studies have shown that interaction of bacteria with the host cell cytoskeleton is necessary for the generation of intracellular movement of both *L. monocytogenes* and *S. flexneri* (1, 6, 22, 25, 36, 37), and Kuhn et al. (18) have described an *L. monocytogenes* mutant which induced the polymerization of actin but was unable to rearrange the actin coat to generate movement. As a result, bacteria remained entrapped in an actin cloud. The actual mechanism by which actin tails generate movement is not known since the mechanism of actin nucleation in mammalian cells is not fully understood (35). We have identified the association of F-actin in the formation of protrusions within *S. flexneri*-infected monolayers and thereby confirmed the results of Bernardini et al. (1). In addition, we have shown the association of vinculin with the development of protrusions as well as a reorganization of its cytoskeletal pattern in Henle and HeLa cells following infection with *S. flexneri*. It is interesting to speculate that cytoskeletal proteins other than actin, such as vinculin, may be involved in the generation of the intracellular movement of bacteria. Such a possible involvement is, however, more difficult to assess since no nondestructive treatment is known that selectively inhibits reorganization of vinculin.

Since vinculin is associated with actin and adhesion plaques, it is to be expected that any reorganization of vinculin distribution would be accompanied by a change in the organization of actin. Though the redistribution of cytoplasmic actin was not as profound as that seen for vinculin, some reorganization of actin fibers was detected in *S. flexneri*-infected cells. Time course studies on actin-vinculin double-labeled cells indicate that *Shigella*-induced reorganization of vinculin occurs prior to that of actin cytoskeleton (unpublished observations), which is consistent with the observations of Geiger et al. (9), who suggested that focal contacts and associated vinculin serve as organizing centers for the assembly of actin-containing microfilament bundles. As a *Shigella* infection progresses, a reduction in adhesion plaques at the cell periphery is accompanied by the rounding of cells and detachment from the glass surface. It is tempting to speculate that perturbation of vinculin organization might be related to both the dissolution of actin fibers and the loss of adhesive properties of detaching cells. A strong correlation between the reduction in number of adhesion plaques and morphological alterations in Rous sarcoma virus-transformed cells has been demonstrated previously (3, 7, 16).

A disruption in the organization of vinculin and actin has also been reported for cells treated with the synthetic peptide GRGDS (33), the tumor-promoting phorbol ester (21, 29), and the phosphorylation of tyrosine residues in vinculin during neoplastic transformation of cells by Rous sarcoma virus (2, 13, 30). In cells infected with Rous sarcoma virus, a single gene product, pp60^{src} (a protein kinase that modifies cellular proteins through phosphoryla-

tion of tyrosine residues), is known to cause the alterations (14, 30). Several authors have shown a correlation among Rous sarcoma virus infection, vinculin phosphorylation, and presence of pp60^{src} in adhesion plaques (15–17, 20, 27, 31). However, it is believed that vinculin phosphorylation alone is not enough to explain the rearrangement of cytoskeletal elements observed during transformation (4, 16). Recently, Burn et al. (4) have demonstrated that vinculin contains transformation-sensitive fatty acids covalently attached to the molecule. Modification of vinculin by acylation has been suggested as the mechanism that alters the organization of vinculin within cells and that plays a regulatory role in anchoring or stabilizing microfilament bundles in the plasma membrane (4).

It is not clear whether changes in the cytoskeletal structures observed after *S. flexneri* infection are the result of *Shigella*-induced changes in cell metabolism or whether shigellae directly modify these cellular structures to facilitate their own intracellular movement. Preliminary data do not suggest that phosphorylation of vinculin occurs in *S. flexneri*-infected Henle or HeLa cells. We are investigating the nature of the alterations that provoke the reorganization of vinculin during *Shigella* infection in cultured cells.

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