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Entry of *Shigella flexneri* into HeLa Cells: Evidence for Directed Phagocytosis Involving Actin Polymerization and Myosin Accumulation

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The enteroinvasive bacterium *Shigella flexneri* expresses a plasmid-mediated capacity to penetrate into nonphagocytic cells. By using 7-nitrobenz-2-oxa-1,3-diazole–phallacidin (NBD-phallacidin), a fluorescent dye which specifically stains microfilaments, we observed condensations of filamentous actin underneath the plasma membrane of HeLa cells which interacted with the invasive isolate M90T. As demonstrated by indirect immunofluorescence with the antimyosin monoclonal antibody CC-212, myosin accumulated at the same sites. The entry process could be synchronized by using strain SC301, a pIL22 transformant of M90T. pIL22, a recombinant plasmid encoding the *Escherichia coli* afimbrial adhesin AFA I, rendered shigellae highly adherent to HeLa cells. Using such a system, we demonstrated that the occurrence of bacterial penetration and the appearance of structures brightly stained by NBD-phallacidin were simultaneous events. Such microfilamentous structures resulted from de novo polymerization of the monomeric actin pool in a DNase I inhibition assay, as shown by measurement of the monomeric versus total actin content of infected HeLa cells. These data provide direct evidence that the penetration of *S. flexneri* into HeLa cells occurs through a mechanism similar to phagocytosis by professional phagocytes.

Shigellae are enteric gram-negative bacilli which cause a dysenteric syndrome in humans by invasion of the colonic mucosa. This invasion process encompasses entry into intestinal epithelial cells (20, 34), intracellular multiplication (26, 30), and killing of host cells (6, 34). Subsequent inflammatory lesions within the lamina propria of intestinal villi are responsible for abscesses and ulcerations (34) which are characteristic of the dysenteric syndrome. The crucial step of *Shigella flexneri* entry into epithelial cells is controlled by a 220-kilobase plasmid (21, 28, 29). Invasion can be studied in vitro by examining the infection of epithelial cells in culture (20). Invasive shigellae penetrate into these cells without any leakage of cellular components (12). This suggests that the uptake of these bacteria is achieved through a mechanism similar to one of the classical routes of endocytosis, i.e., phagocytosis or receptor-mediated endocytosis.

Phagocytosis has generally been studied by using professional phagocytes such as macrophages and polymorphonuclear leukocytes, which perform particle uptake with great efficiency. During the initial step of phagocytosis, particles bind to the plasma membrane surface through the so-called zipper mechanism (10). They enter into the cell and are enclosed within a plasma membrane-derived phagosomal membrane. The overall process is known to require metabolic energy (16). However, the events which link binding and phagocytosis remain as yet unknown. Transient actin assembly, with augmentation of the filamentous/monomeric actin ratio of the cell, occurs during the early stage of phagocytosis in neutrophils (31, 33). Polymerization of actin is localized underneath the plasma membrane in contact with the particle to be ingested (5, 33), and a dense network of actin filaments appears in this region (5, 24, 27), along with the accumulation of myosin and actin-binding proteins (32, 35). Such microfilamentous reactions resolve once the ingested particle is completely enclosed within a membrane-bound vacuole (31). On the other hand, little information is available on the mechanisms by which nonphagocytic cells such as HeLa cells uptake large particles. Although the triggering of particle ingestion by fibroblasts seems to require a very high degree of interaction between opposing surfaces (8), invasive shigellae do not demonstrate efficient adhesion to epithelial cells in culture.

Receptor-mediated endocytosis is a ubiquitous mechanism among eucaryotic cells which permits internalization of macromolecules and small particles such as viruses. This pathway requires specialized plasma membrane domains called coated pits, which are transformed into coated vesicles (9). Clathrin, a 180-kilodalton protein, is the major component of this coat (25).

As for several other invasive bacteria, only indirect evidence has been obtained concerning the mechanism of shigella penetration into epithelial cells. Cytochalasin B, which is a potent inhibitor of phagocytosis, blocks the penetration of shigellae into cells (13), suggesting that microfilaments are involved in invasion. However, cytochalasin B may demonstrate other activities, such as the inhibition of glucose transport (17). In addition, in a few instances, transmission electron microscopic observations have revealed associations of coated membrane domains with penetrating shigellae (11). Receptor-mediated endocytosis has also been implicated in the penetration of chlamydiae (14).

We examined by fluorescence microscopy the occurrence of filamentous actin and myosin accumulation during the penetration of *S. flexneri* into HeLa cells. These studies were performed with a wild-type invasive strain and its plasmidless avirulent derivative. To synchronize the entry process, we also constructed a pair of invasive and noninvasive strains which had conferred adherence properties upon HeLa cells. By using these strains we were able to

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TABLE 1. Characteristics and plasmid content of *S. flexneri* 5 strains

S. flexneri strain (reference)	Relevant characteristics and plasmid content[a]	HeLa cells	
		Adhesion	Invasion
M90T (21)	Km^r; pWR110	−	+
BS176 (30)	Derivative of M90T cured of pWR110	−	−
SC301 (this study)	Km^r and Amp^r; transformant of M90T pWR110 + pIL22	+	+
SC300 (this study)	Amp^r; transformant of BS176 pIL22	+	−

[a] Km^r, Kanamycin resistance; Amp^r, ampicillin resistance. pWR110 is a Tn*5*-labeled derivative of the *S. flexneri* 5 virulence plasmid pWR100 (29). pIL22 is a pBR322 recombinant plasmid containing a 6.7-kilobase insert of uropathogenic *E. coli* K552 chromosomic DNA. pIL22 encodes for afimbrial adhesin AFA I (19).

determine the precise kinetics of penetration and to measure the filamentous-actin content in HeLa cells during the first steps of interaction with bacteria. We report here that transient actin polymerization and myosin accumulation occur underneath the plasma membrane at the entry site of virulent shigellae.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains and their relevant characteristics are listed in Table 1. Rapid plasmid detection was performed as previously described (15). Cells competent for transformation with pIL22 were prepared as previously described (7).

Medium and growth conditions. Bacteria were routinely grown in tryptic soy broth (Difco Laboratories, Detroit, Mich.). The antibiotics kanamycin and ampicillin were used at final concentrations of 50 and 100 μg/ml, respectively.

Infection of HeLa cells with wild-type strains. HeLa cells were grown routinely in Eagle minimal essential medium (MEM; GIBCO Laboratories, Grand Island, N.Y.) with 10% fetal calf serum (GIBCO). Bacteria were harvested in the exponential phase, washed in phosphate-buffered saline (PBS; NaCl [8.767 g/liter], $Na_2HPO_4 \cdot 2H_2O$ [2.250 g/liter], $NaH_2PO_4 \cdot H_2O$ [0.257 g/liter]; pH 7.4), and suspended at the appropriate density in MEM. Nonconfluent HeLa cell monolayers (4.5×10^4 cells per cm^2) grown in 35-mm plastic tissue culture dishes (Becton Dickinson Labware, Oxnard, Calif.) or on glass cover slips (22 by 22 mm) were washed in MEM and overlaid with bacterial suspensions. The first stage of contact between bacteria and cells was achieved by centrifugation (2,200 × *g*, 10 min) at 4°C; these are nonpermissive conditions for bacterial penetration. Plates were then rapidly transferred onto the surface of a water bath at 37°C as indicated below. Infection was stopped by three washes in PBS at 20°C followed by fixation either with formaldehyde for fluorescence-specific staining (see below) or with methanol for Giemsa staining.

Infection of HeLa cells with adherent strains. HeLa cells were infected with adherent strains as described above for wild-type strains, except that the first stage of contact was achieved by a 4-min incubation at 4°C without centrifugation. Plates were then washed twice with MEM at 4°C, refilled with MEM at 37°C, and transferred onto the surface of a water bath at 37°C. Infection was stopped as described above.

Double fluorescence staining of F actin and bacteria. Fixation and cell permeabilization procedures were performed as described previously (36) with minor modifications. After three washes in PBS, the infected monolayers were fixed with 3% paraformaldehyde (Aldrich-Chemie, Steinheim, Federal Republic of Germany)–0.1 μM $CaCl_2$–0.1 μM $MgCl_2$ in PBS for 20 min. Cover slips were then washed three times in PBS, treated for 10 min with 50 mM NH_4Cl in PBS to quench the remaining formaldehyde, and washed three more times in PBS. Permeabilization of the plasma membrane of cells for internal staining was achieved by treating the cover slips with 0.1% Triton X-100 in PBS for 4 min.

Bacteria were labeled by indirect immunofluorescence. After being washed in 0.2% gelatin in PBS (PBSG), cover slips were incubated for 20 min in a 1/120 dilution of a rabbit antiserum to the lipopolysaccharide (LPS) of *S. flexneri* serotype 5. After three washes in PBSG, incubation in a 1/80 dilution of goat anti-rabbit rhodamine-conjugated immunoglobulin G (heavy and light chain specific; Miles Laboratories, Naperville, Ill.) for 20 min stained the bacteria with an intense red fluorescence (excitation at 545 nm and emission 720 nm).

Cover slips were then treated with a 10-U/ml solution of 7-nitrobenz-2-oxa-1,3-diazole-phallacidin (NBD-phallacidin; Molecular Probes, Inc., Junction City, Oreg.) in PBS for 20 min to specifically stain filamentous actin (F actin) with a yellow-green fluorescence (2). Cover slips were washed three times in PBS and mounted in glycerol and Moviol 4-88 (Behring Diagnostics, La Jolla, Calif.) as described previously (23). NBD-phallacidin uses the same microscopic fluorescence filters as fluorescein derivatives (excitation at 490 nm and emission at 525 nm).

Double fluorescence staining of myosin and F actin. For double-fluorescence stainings, infected cells were rapidly washed in PHEM buffer (10 mM EGTA, 1 mM $MgCl_2$, 60 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], 23 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid]; pH 6.9) and permeabilized for 1 min with 5% Triton X-100 in PHEM. Fixation was achieved by treatment in acetone for 3 min at −20°C.

Specific staining for myosin was performed by indirect immunofluorescence. Cells were first incubated in an undiluted culture supernatant of a mouse hybridoma cell line secreting the CC-212 monoclonal antibody. CC-212 is specific for myosin from smooth muscle and from nonmuscular cells (18). After being washed as described above, the cells were incubated in a 1/40 dilution of rhodamine-conjugated goat anti-mouse immunoglobulin G (Sigma Chemical Co., St. Louis, Mo.). Cells were subsequently stained with NBD-phallacidin as described above.

Assay for time course penetration of adherent strains. To correlate the penetration process with the modifications of microfilaments induced by invasive *S. flexneri*, an assay was designed to monitor the penetration of the bacteria over a short period. In this assay, the infected HeLa cell monolayers were radioimmunolabeled by using anti-LPS antibodies and ^{125}I-labeled protein A. In nonpermeabilized cells, such components would label extracellular bacteria but not intracellular bacteria. HeLa cell monolayers in 12-well tissue culture dishes (Costar, Cambridge, Mass.) were infected as described above. At different incubation times at 37°C, infected cells were washed in PBS and fixed for 20 min with 3% paraformaldehyde–0.1 μM $CaCl_2$–0.1 μM $MgCl_2$ in PBS. After three washes in PBS, cells were incubated with a 1/120 dilution of anti-LPS antiserum for 20 min, washed three times in PBSG, and then washed three times in PBS. Cells were then incubated for 1 h with 0.03 μCi of ^{125}I-protein A per ml in PBSG. After four washes in PBSG and four washes

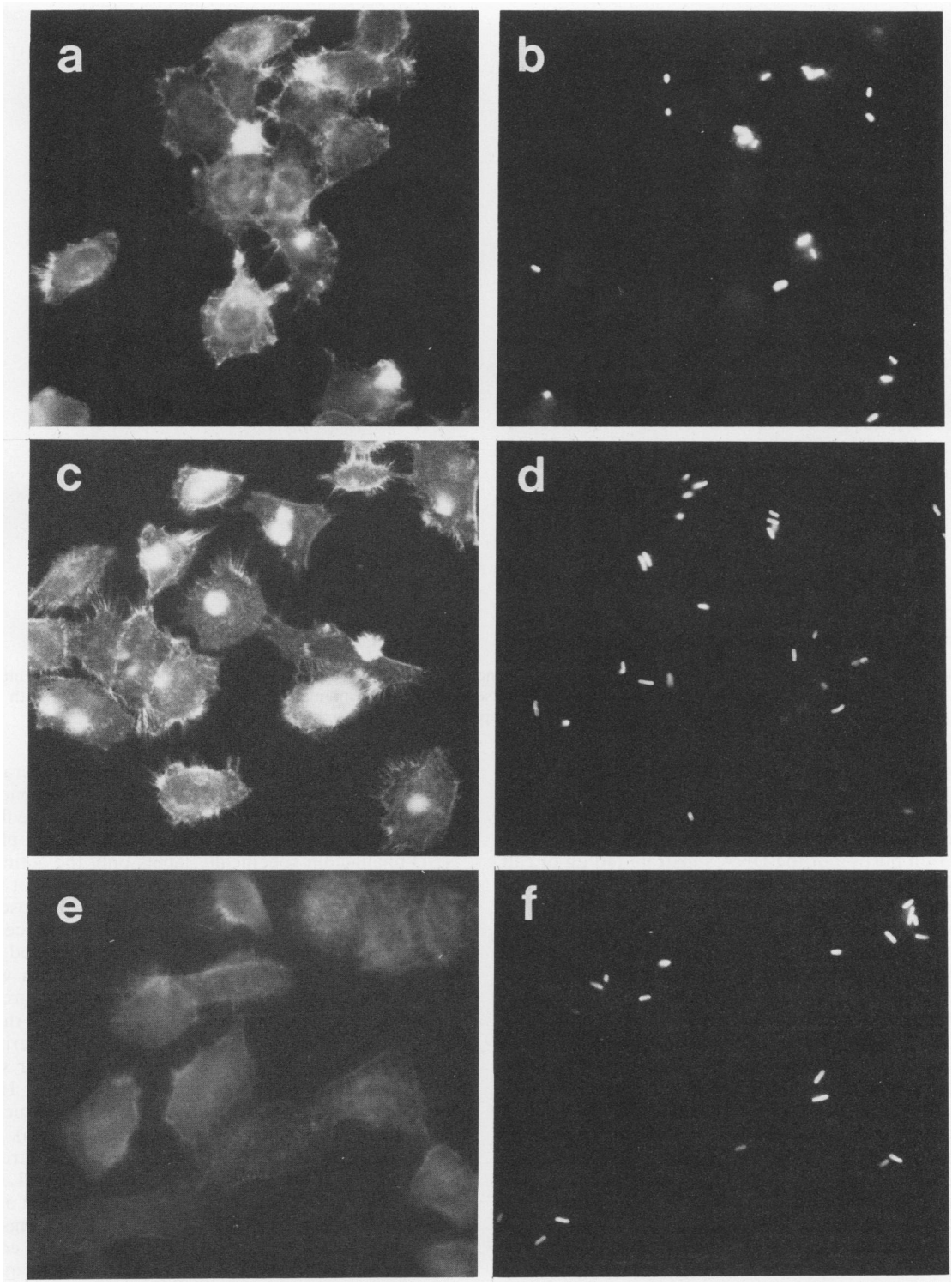

FIG. 1. NBD-phallacidin (a, c, and e) and anti-LPS (b, d, and f) double fluorescence labeling of infected HeLa cells. The procedures for the infection of HeLa cells in culture with M90T (a and b), SC301 (c and d), and SC300 (e and f) and for fluorescence stainings were as described in Materials and Methods. The bacterial concentrations used in these experiments were 2×10^8/ml (a and b) and 6×10^7/ml (c to f). The infection process at 37°C was stopped after 12 min (a and b) or 7 min (c to f).

in PBS, monolayers were solubilized in 0.5 M KOH for 12 h at 37°C and radioactivity was counted in an ACS II apparatus (Amersham, Buckinghamshire, England).

Measurement of the monomeric actin pool in infected cells. Selective assays for monomeric (G) and filamentous (F) actins were performed as previously described (3, 4) and are only summarized here. The selective measurement of G actin is based on the concentration-dependent inhibition of

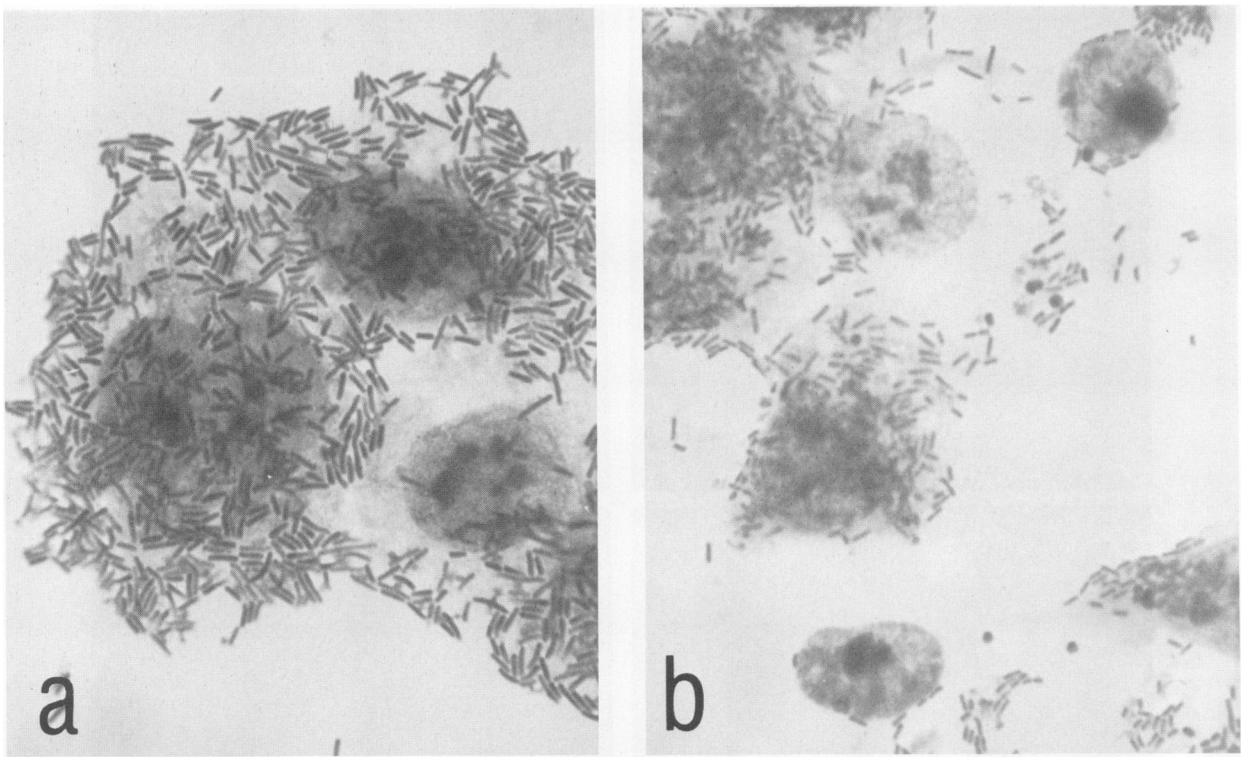

FIG. 2. Infected HeLa cells fixed in methanol and Giemsa stained. The procedure for the infection of HeLa cells in culture was as described in Materials and Methods. The bacterial concentration used in this experiment was 2×10^{10}/ml. (a) Infection with SC300 was stopped before incubation at 37°C. (b) Infection with SC301 was stopped after 2 h of incubation at 37°C.

beef pancreas DNase I activity by G actin but not by F actin. The total amount of actin in a sample was determined after depolymerization of the F actin with guanidine hydrochloride. The conditions for the DNase activity assay were as follows. A 3-ml portion of the DNA substrate (40 μg of calf thymus DNA type I per ml [Sigma], 4 mM $MgSO_4$, 1.8 mM $CaCl_2$, 0.1 mM Tris hydrochloride; pH 7.5) was added to 12 μl of enzyme solution (0.1 mg of beef pancreas DNase I per ml [Sigma], 0.2 mM $CaCl_2$, 0.01 mM phenylmethylsulfonyl fluoride [PMFS; Sigma], 50 mM Tris hydrochloride; pH 7.5), and hyperchromicity was measured at 260 nm at 25°C.

Nonconfluent HeLa cell monolayers (4.5×10^4 cells per cm^2) grown in 60-mm plastic tissue culture dishes (Becton Dickinson) were infected with adherent strains as described above. At the times indicated the plates were removed from the 37°C water bath and rapidly washed twice in PBS at 20°C. Cells were then scraped into a 150-μl portion of lysis buffer (1 mM $MgCl_2$, 1 mM GTP, 1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′,-tetraacetic acid], 0.2 mM dithiothreitol, 0.01 mM PMFS, 1 M sucrose, 0.5% Triton X-100, 100 mM PIPES; pH 6.75). The inhibition activity of cell extracts was then determined. To measure the total amount of actin, a portion of the extract was added to an equal volume of depolymerization buffer (1 mM ATP, 1 mM $CaCl_2$, 1 M sodium acetate, 1.5 M guanidine hydrochloride, 20 mM Tris hydrochloride; pH 7.5), and the inhibition activity of this mixture was determined. Results were expressed as the monomeric/total actin ratio.

RESULTS

Concentration of F actin underneath the plasma membrane areas interacting with shigellae. To study the distribution of microfilaments within HeLa cells during the interaction with shigellae, infected monolayers were fluorescence stained with NBD-phallacidin. This molecule is an active fluorescent derivative of the actin-binding mushroom toxin phallacidin. NBD-phallacidin specifically labels only the actin cytoskeletal structures made of filamentous actin and not the monomeric pool of actin (2). In resting cells, fluorescence was mainly observed at the periphery of the cell and in microspikes. Stress fibers could also be observed. A typical aspect of HeLa cells after 12 min of infection with strain M90T is shown in Fig. 1a. The brightest green-yellow staining could be observed underneath 20% of the bacteria (Fig. 1a and b). Variation in focus characterized these structures as irregular bowls lying on the upper side of the cell and always associated with a bacterium. HeLa cells infected with BS176 exhibited microfilament structures similar to those of noninfected cells (data not shown). However, it was not possible to obtain a true negative control showing the avirulent strain BS176 in contact with HeLa cells because these bacteria established no significant interaction with HeLa cells and were eliminated by washings.

In an attempt to circumvent this problem, we constructed two derivatives of M90T and BS176 by transformation with recombinant plasmid pIL22, which encodes the human-specific afimbrial adhesin AFA I adhesin of uropathogenic *Escherichia coli* (19). Both strains SC301 and SC300 (Fig. 2a) exhibited very efficient adhesion to HeLa cells at 4°C. After 2 h at 37°C, strain SC300 remained uniformly associated with unaltered cells (data not shown), whereas most HeLa cells infected with SC301 had been lysed (Fig. 2b). Using gentamicin as previously described (30) to quantitate intracellular bacteria, we observed that SC301 penetrated HeLa cells 1,000-fold more efficiently than M90T when no

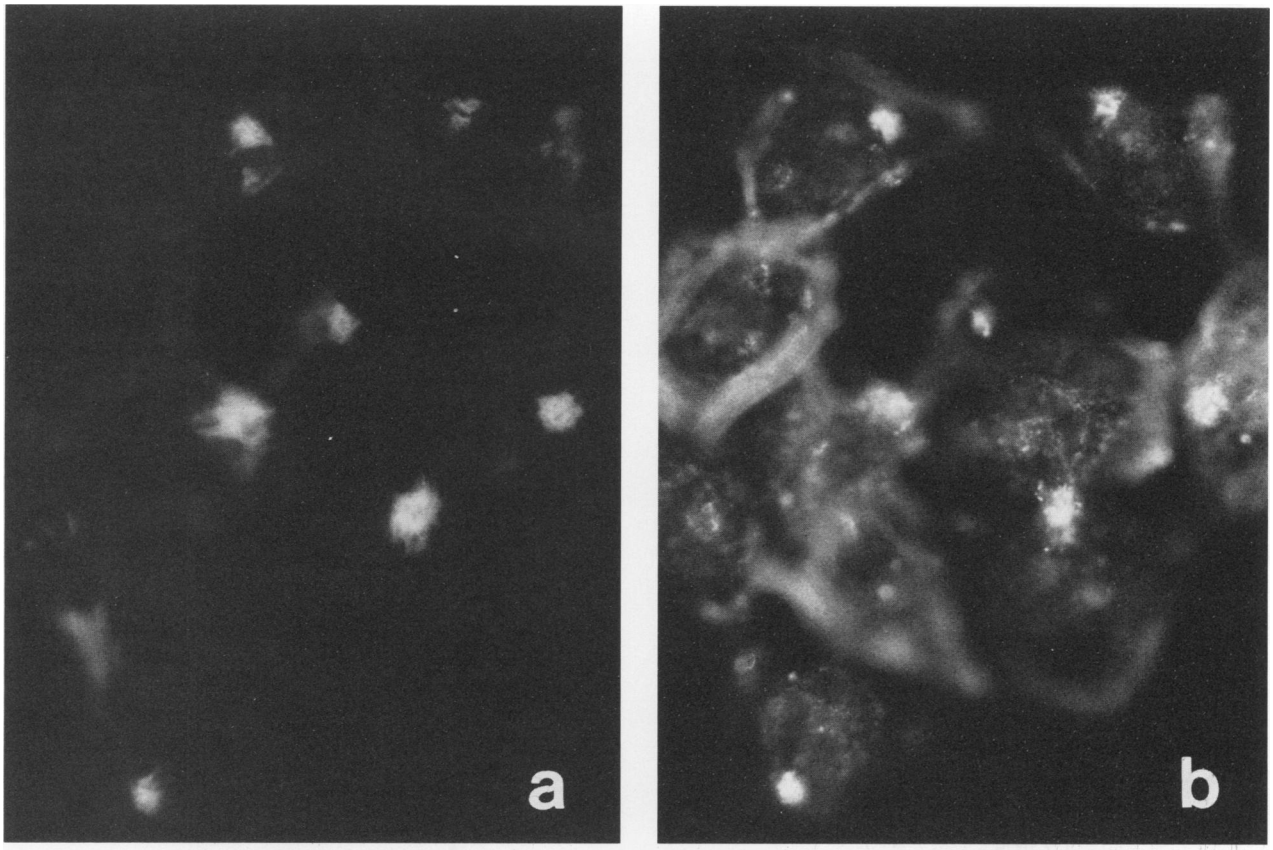

FIG. 3. Double fluorescence labeling with NBD-phallacidin (a) and CC-212 antibody (b) of SC301-infected HeLa cells. The bacterial concentration used in this experiment was 2×10^8/ml, and the infection process at 37°C was stopped after 7 min.

centrifugation was performed during the infection procedure (data not shown). No intracellular bacteria could be detected after infection with SC300.

Typical aspects of HeLa cell monolayers infected for 7 min at 37°C with these strains are shown in Fig. 1c to f. In the case of SC301, 50% of the bacteria were associated with an intense accumulation of F actin having a shape similar to that obtained with M90T. However, in SC301-infected HeLa cells the F-actin condensations were randomly distributed, whereas in M90T-infected HeLa cells they were predominantly localized at the edges of the cells. With both strains, this phenomenon was completely inhibited by 0.5 μg of cytochalasin D per ml in MEM at 37°C (data not shown). On the other hand, HeLa cells infected with SC300 demonstrated an F-actin pattern similar to that of noninfected monolayers, even underneath adherent bacteria (Fig. 1e and f).

Accumulation of myosin. Noninfected HeLa cells labeled by indirect immunofluorescence using the antimyosin monoclonal antibody CC-212 demonstrated a punctated pattern as already described (18). The brightest NBD-phallacidin fluorescence of F actin in areas of contact with SC301 bacteria demonstrated total overlap with the accumulation of myosin after staining with CC-212 (Fig. 3). Such myosin accumulations also appeared to be punctated. Similar results were obtained with strain M90T (data not shown). Sites of myosin accumulation were also observed when infected HeLa cells were stained for myosin only. Thus the myosin accumulation could not have been an artifact due to the inefficacy of wave-length selection filters.

Time course for bacterial penetration and condensation of F actin. To determine whether the redistribution of microfilaments was involved in the penetration of virulent shigellae, we examined the kinetics of both phenomena and the effect of bacterial concentrations. The use of strain SC301 permitted the adjustment of the number of interacting bacteria per cell and the synchronization of the penetration process by a rapid shift in the temperature from 4 to 37°C. The ingestion of extracellular bacteria was completed by 18 min at 37°C, and F-actin redistribution occurred during the same period with a maximum at 6 min (Fig. 4). The absolute rate of bacterial penetration (number of ingested bacteria per cell per minute) and the number of sites of F actin accumulation were both dependent on the initial number of bacteria at the surface of the cell. Surprisingly, only about 50% of the bacteria penetrated into HeLa cells under these conditions. This was not due to the loss of the invasive phenotype during subculture because all of the clones were invasive after plating on agar.

Measurement of actin polymerization during the penetration process. The DNase I inhibition assay (3, 4) was used to measure the ratio of G actin versus total actin (G/F+G) in infected HeLa cells. These experiments could only be performed with adhesive strains which allowed us to synchronize penetration events. Noninfected HeLa cells demonstrated a G/F+G ratio of 0.46, whereas HeLa cells with 12 adherent bacteria per cell demonstrated, after the adhesion step at 4°C, a G/F+G ratio of 0.49. Thus, bacteria only slightly interfered in the DNase I inhibition assay. The G/F+G ratio of HeLa cells infected with strain SC300 was

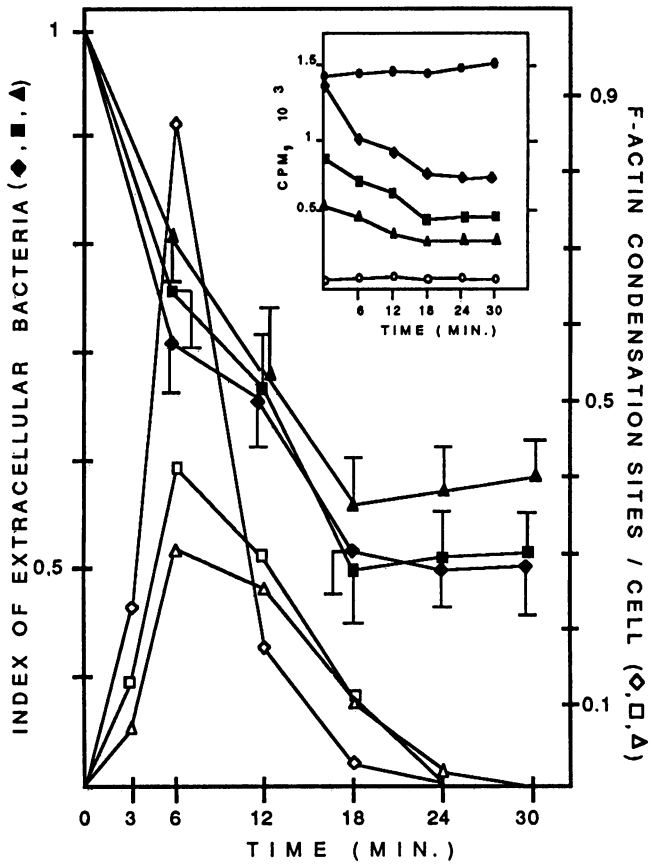

FIG. 4. Time course data for the penetration of SC301 into HeLa cells and for the appearance of F-actin condensation sites. HeLa cells grown in 12-well tissue culture dishes and on glass cover slips (22 by 22 mm) were infected with SC301. The bacterial concentrations used in these experiments were 2×10^8/ml (◇ and ◆). 10^8/ml (□ and ■), and 6×10^7/ml (△ and ▲), which resulted in average numbers of 5.5, 3.5, and 1.5 adherent bacteria per cell, respectively. Cover slips were processed for F-actin labeling with NBD-phallacidin, and 200 cells on each cover slip were examined by fluorescence microscopy for F-actin condensation sites (◇, □, and △). Infected HeLa cells in 12-well tissue culture dishes were processed to measure the number of extracellular bacteria (◆, ■, and ▲). Results were expressed for each bacterial concentration as follows: radioactivity at each time point/radioactivity at time zero. Each point represents the mean ± the standard deviation of four wells. (Inset) Same experiment as in the main figure. Results are directly expressed as radioactivity counting (◆, ■, and ▲). The following control experiments were added: noninfected HeLa cells (○) and HeLa cells infected with noninvasive strain SC300 (●).

not significantly altered during the experiment (Fig. 5). On the contrary, HeLa cells infected with SC301 demonstrated an important increase in the pool of filamentous actin, with a G/F+G ratio of 0.37 after 6 min at 37°C. This ratio returned to a base-line value after 12 min.

DISCUSSION

Shigellae, yersiniae, and salmonellae, as well as rickettsiae and chlamydiae (for a review, see reference 22) are all able to penetrate nonphagocytic cells. The cellular mechanisms responsible for this penetration have usually been studied by electron microscopy, with some researchers visualizing associations between coated pits and invading microorganisms, or by using pharmacological inhibitors such as cytochalasins to establish the involvement of microfilamentous functions. However, electron microscopy is a poorly quantitative method, and utilization of pharmacological inhibitors provides only indirect evidence.

The results presented here support the conclusion that the penetration of invasive *S. flexneri* into HeLa cells in culture occurs through a mechanism similar to macrophage and polymorphonuclear leukocyte phagocytosis. This mechanism involves localized and transient actin polymerization, as well as the accumulation of myosin at the site of entry of the bacteria.

Wild-type strains of *S. flexneri* demonstrated a plasmid-encoded property to locally concentrate membrane-associated microfilaments in HeLa cells as shown by double fluorescent labeling with NBD-phallacidin and an anti-LPS immune serum. This capacity did not appear to be the result of a plasmid-encoded adhesion property that would cause M90T but not BS176 to bind to HeLa cells since SC300, which adhered efficiently to HeLa cells, did not induce any cytoskeletal reaction. This finding suggested that the accumulation of microfilaments subsequent to contact of the plasma membrane with invasive shigellae required some specific, plasmid-encoded signal from the bacterium. In addition to providing this critical evidence, the construction of adherent strains allowed us to synchronize the penetration events. Adhesive strain SC301 demonstrated all of the virulence-associated characteristics of shigellae: penetra-

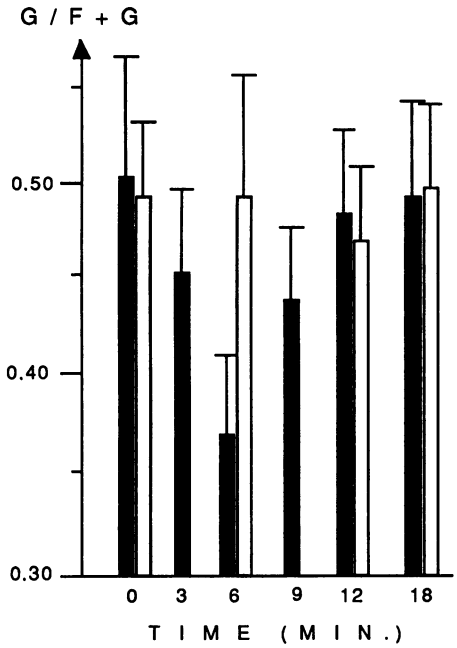

FIG. 5. Measurement of actin polymerization within HeLa cells infected with SC301 and SC300. The procedure for infection of HeLa cells with strain SC301 (■) or SC300 (□) was as described in Materials and Methods. The bacterial concentration used in these experiments was 10^9/ml, thus resulting in an average number of 12 adherent bacteria per cell. At different times after the temperature was shifted to 37°C, a plate was removed and assayed for G actin and total actin (F+G). Each bar represents the mean value of the G/F+G ratios obtained in six different determinations of G and F+G. Standard deviations have been computed from the 36 possible ratios between G and F+G. Two other experiments gave similar results.

tion, intracellular multiplication, and killing of host cells (in this case, HeLa cells). Using SC301, we observed that the appearance of F-actin condensation sites was dependent upon the number of interacting bacteria and occurred at the same time as bacterial penetration. We also observed that only about 50% of the bacteria could be ingested by HeLa cells within the 30 min of the experiment. After 18 min at 37°C, the ingestion process was completed. This apparent arrest in bacterial entry could reflect either the presence of nonpermissive cells for bacterial penetration, possibly due to cell cycle heterogeneity, or nonpermissive areas on the plasma membrane of each cell. A requirement for particular plasma membrane domains was also strongly suggested when we used strain M90T, which penetrated only at the edges of the cells. On the other hand, the rapid shut-off of cellular metabolism by intracellular bacteria (6) could account for the early inhibition of their capacity to further ingest bacteria.

By using the DNase I inhibition assay on HeLa cells heavily infected with SC301, we demonstrated that filamentous actin condensation was the result of de novo polymerization from the pool of monomeric actin. No significant alterations of the monomeric/filamentous actin ratio were detected in HeLa cells infected with M90T (data not shown). Insufficient synchronization of the entry process even after centrifugation certainly accounted for such a failure. In HeLa cells infected with SC301, the polymerization reaction involved as much as 26% of the pool of monomeric actin. After the entry process was completed, the G/F+G actin ratio returned rapidly to its base-line value. This suggested that actin polymerization only occurred during the entry process and that intracellular bacteria did not alter the microfilament system of the cells during the experiment. Sites of actin polymerization were also enriched for myosin as demonstrated with CC-212 labeling of HeLa cells infected with invasive *S. flexneri*. Since actin polymerization associated with myosin accumulation is only required for the phagocytic process, this enrichment clearly suggested that the entry of invasive shigellae into HeLa cells occurred through directed phagocytosis and not through receptor-mediated endocytosis. However, receptor-mediated endocytosis is not the unique function of clathrin-coated membranes, and the possibility remains that clathrin is involved in some secondary mechanism relative to the entry process. For instance, the enrichment of nascent phagosomal membranes with large, flat clathrin patches in macrophages ingesting large particles has been described. These coated areas may account for the recycling of the plasma membrane (1).

In addition to demonstrating that invasive *S. flexneri* penetrates HeLa cells by inducing a local and transient polymerization of actin along with myosin accumulation, we have introduced here new tools for studying the mechanisms by which shigellae induce phagocytosis in HeLa cells. We are currently investigating both the molecular basis for this bacterial signal and the possible second messenger involved in actin polymerization within HeLa cells.

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