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# Mobilization of F-Actin and Clathrin during Redistribution of *Chlamydia trachomatis* to an Intracellular Site in Eucaryotic Cells

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**Immunofluorescence was used to examine the distribution of *Chlamydia trachomatis* serovars L2 and E, F-actin, and clathrin in infected McCoy and HeLa cells. After incubation at 4°C, *C. trachomatis* serovar L2 was randomly distributed on the McCoy cell surface. After a temperature shift to 37°C, chlamydiae redistributed, within 30 min, to one local aggregate in the central or perinuclear region of individual cells. About 90% of these aggregated chlamydiae were intracellularly localized, but some remained randomly distributed on the cell surface. Similar results were obtained with HeLa cells and *C. trachomatis* serovar E, except that the redistribution was slower in HeLa cells than in McCoy cells and fewer cells infected with serovar E exhibited a local aggregate than those infected with serovar L2. Cytochalasin D inhibited more than 90% of this local aggregation. Instead, in cytochalasin D-treated cells, the entry of chlamydiae was inhibited and the organisms became localized on the cell surface in a peripheral local aggregate that distributed in a manner similar to that of phalloidin-stained actin. In a double immunofluorescence assay, F-actin and clathrin aggregated correspondingly in time and position with central or perinuclear aggregation of chlamydiae. These results indicate that polymerized actin and clathrin participate in a rapid redistribution of chlamydiae to an intracellular aggregate.**

The intracellular environment of eucaryotes is a prerequisite for the replication of chlamydiae. Although in vitro systems often have to be manipulated for efficient chlamydial attachment to the host cell surface to occur (1), the rate of ingestion of chlamydiae by nonprofessional phagocytes is 10 to 100 times greater than of *Escherichia coli* and polystyrene latex beads (3). This parasite-specified phagocytosis suggests that specific surface structures on chlamydiae facilitate the entry process. Once internalized, *Chlamydia psittaci* has the ability to escape from phagosome-lysosome fusion and, thereby, potential host defense mechanisms (6, 7).

Cells infected with *Chlamydia trachomatis* usually contain only one chlamydial inclusion, even under high multiplicities of infection, in contrast to the multiple inclusions found in *C. psittaci*-infected cells (2). Ridderhof and Barnes (17) have recently shown that *C. trachomatis*-containing inclusions fuse with each other in a microfilament-dependent process. Thus, a fundamental difference in the intracellular fate of these two *Chlamydia* species may exist.

Although the steps involved in the infectious process have been described, the molecular mechanisms are still incompletely known. The mechanism of entry into host cells is one controversial issue. Chlamydial entry has been shown to be resistant to cytochalasin B (9) but sensitive to transglutaminase inhibitors (19). Furthermore, internalizing *C. psittaci* and *C. trachomatis* organisms are associated with coated pits (10, 11). These experiments argue for a receptor-mediated endocytosislike uptake mechanism. On the other hand, the experiments by Ward and Murray (23) indicate a classical phagocytosislike mechanism by their failure to identify coated pits, by reduced uptake in the presence of cytochalasin D, and by no effect of transglutaminase inhibitors.

A study by Prain and Pearce (15) concluded that chlamydiae can enter cells both by pinocytic and phagocytic mechanisms and that the inoculation mode dictates which mechanism will dominate; centrifugation favors phagocytosis and no centrifugation favors pinocytosis.

In this work we have studied the early events following attachment of elementary bodies to the host cell surface. With immunofluorescence techniques, our results show that *C. trachomatis* serovars L2 and E redistribute and aggregate to a distinct intracellular region in McCoy and HeLa cells. By treatment with cytochalasin D, phalloidin staining, and indirect immunofluorescence for clathrin, it was found that F-actin and clathrin were involved in this redistribution.

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## MATERIALS AND METHODS

**Microorganisms.** *C. trachomatis* serovars L2 and E were used throughout this study. Stock organisms were propagated in 75-cm$^2$ plastic flasks, in McCoy cells for serovar L2 and in HeLa cells for serovar E, essentially as described previously (18). Organisms (serovars L2 and E) were harvested after 48 and 72 h, respectively. Infected cells from several flasks were combined, and chlamydiae were released from host cells by disruption for 15 min in a sonic cleaning bath (Mettler Electronics Corp., Anaheim, Calif.). The lysate was centrifuged for 10 min at 900 × *g* at 4°C in an SE-12 rotor of a superspeed RC2-B centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.) to sediment nuclei and other large host-cell debris. The supernatant from the lysate was centrifuged twice for 30 min at 12,000 × *g* and 4°C to sediment the chlamydiae. The sediment was suspended in sucrose-phos-

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phate buffer (2-SP) (8) plus 10% (vol/vol) fetal bovine serum, titrated by inclusion formation, and stored at −70°C until use (18). Determination of the number of elementary bodies (EBs) was performed as described previously (16), with some modifications. Coverslips were coated with 1% poly-L-lysine (Sigma Chemical Co., St. Louis, Mo.) in distilled water for 5 min and dried at 60°C for 1 h. Different dilutions of *C. trachomatis* organisms were added, the coverslips were centrifuged at 3,000 × *g* at 35°C for 45 min and rinsed twice in phosphate-buffered saline (PBS) (pH 7.3), and adherent organisms were stained with a fluorescein isothiocyanate (FITC)-labeled monoclonal antibody to the major outer membrane protein (MOMP) (Syva Microtrak, Palo Alto, Calif.). The number of adherent EBs were microscopically counted (Carl Zeiss, Oberkochen, Germany), and the values were transformed into numbers of EBs per milliliter. The fraction of inoculated chlamydiae binding to the coverslip had previously been determined by using $^{14}$C-labeled *C. trachomatis* organisms (18).

**Preparation of monolayers.** McCoy and HeLa cells were grown and maintained in minimal essential medium (Flow Laboratories, Irvine, Scotland) supplemented with 10% fetal bovine serum, 2 mM glutamine, and 10 mg of gentamicin per liter (MEM). Fresh monolayers were prepared by seeding 1 ml of $2 \times 10^5$ cells per ml on glass coverslips (Knittel, Braunschweig, Germany) contained in flat-bottom plastic tubes (15 by 40 mm) (Grainer, Nürtyngen, Germany). Cells were incubated for 24 h at 37°C in an atmosphere of 5% $CO_2$ to obtain subconfluent monolayers. Cells were tested for mycoplasma contamination periodically by staining with 4-6-diamine-2-phenyl-indole dihydrochloride (Boehringer, Mannheim, Germany).

**Inoculation with *C. trachomatis*.** Subconfluent McCoy and HeLa cell monolayers were infected with 70 μl of a multiplicity of infection (MOI) of about 300 EBs of *C. trachomatis* serovar L2 per cell and an MOI of about 1,000 EBs of *C. trachomatis* serovar E per cell in 2-SP plus 10% fetal bovine serum. During the subsequent 3.0-h adsorption period at 4°C, the culture tubes were gently shaken 15 times per min on a rocking device (Rockomat; Tecnomara AG, Zürich, Switzerland) to ensure even dispersion of EBs in the inoculum. After decanting the inoculum, infected cells were washed three times at 4°C with PBS (pH 7.3) to remove nonadherent chlamydiae. Fresh MEM heated to 37°C was added, and the infected cells were incubated at 37°C in a water bath for the indicated times. Infected cells were fixed in methanol for 10 min and stained for 30 min at 37°C with an FITC-labeled anti-*C. trachomatis* monoclonal antibody to MOMP with Evans blue counterstain (Syva). Coverslips were then washed twice in PBS (pH 7.3), and 100 or 200 cells per coverslip were counted for randomly distributed or aggregated *C. trachomatis* organisms at ×1,000 magnification in a fluorescence microscope (Zeiss) (excitation at 450 to 490 nm and emission at 520 nm). Randomly distributed chlamydiae were defined as visible individual EBs associated with most of the host cell area. Aggregated chlamydiae were defined as a confluent host-cell-associated green fluorescence without visible individual EBs. As a control, uninfected cells were treated and stained identically and showed neither randomly distributed nor aggregated fluorescence. Infected cells stained with the monoclonal antibody without fixation tended to detach from the coverslip. Therefore, for unfixed preparations, coverslips were coated with 1% poly-L-lysine before seeding with host cells and infection with *C. trachomatis*. This substantially diminished detachment. These preparations were processed as fixed preparations, and cells with randomly distributed and aggregated EBs were determined. Pretreatment with poly-L-lysine did not affect the distribution of cell-associated chlamydiae. The viability of infected cells was evaluated by staining with 0.2% trypan blue (Sigma) for 5 min.

**Indirect immunofluorescence staining of clathrin.** *C. trachomatis* serovar L2-infected or uninfected HeLa cells were fixed in acetone at −20°C overnight, washed three times in PBS (pH 7.3), and incubated with 4% normal donkey serum (Jackson Immunoresearch Laboratories, West Grove, Pa.; code no. 017-000-001) for 30 min at room temperature in a moist chamber. Excess normal donkey serum was removed; goat polyclonal antibody with specificity for the heavy chain of clathrin (Sigma; product no. C 8034), diluted 1:40 in PBS (pH 7.3), was added; and the cells were incubated for 45 min at room temperature. The monolayers were rinsed three times in PBS for 3 min. Cells were overlaid with biotin-SP-conjugated donkey anti-goat immunoglobulin G (IgG) (heavy plus light chains) (Jackson Immunoresearch; code no. 705-065-131), diluted 1:50,000 in distilled water, and the monolayers were incubated for 30 min at 37°C and washed as above. Texas Red-conjugated streptavidin (Jackson Immunoresearch; code no. 016-070-084), diluted 1:1,024 in distilled water, was added. After incubation for 30 min at 37°C and rinsing, monolayers were overlaid with mouse IgG3 monoclonal antibody to chlamydial MOMP (Washington Research Foundation, Seattle, Wash.; no. KG-5) (diluted 1:2,048 in PBS [pH 7.3]), incubated for 30 min at 37°C, and then washed as described above. FITC-conjugated donkey anti-mouse IgG (heavy plus light chains) (Jackson Immunoresearch; code no. 715-095-141), diluted 1:60 in distilled water, was added, and cells were incubated for 30 min at 37°C and washed three times in PBS for 3 min, once in distilled water, and once in absolute ethanol for 5 min before observation in a fluorescence microscope alternating between FITC- and rhodamine isothiocyanate (RITC)-filter combinations with excitation at 450 to 490 nm and 546 nm, respectively, and emission at 520 nm and 590 nm, respectively. Cells were evaluated for random or aggregated fluorescence patterns. Where indicated, cells were also separately stained for *C. trachomatis* and clathrin and evaluated as described above to rule out the possibility that aggregation of clathrin at EB aggregation was an artifact due to inefficacy of wavelength selection filters. Control experiments revealed that anti-MOMP antibodies did not react with clathrin and anticlathrin antibodies did not react with chlamydiae. Furthermore, conjugated secondary antibodies did not unspecifically react with host cells or chlamydiae, except that Texas Red-conjugated streptavidin faintly stained host cells red. However, this did not interfere with the evaluation.

**Phalloidin staining of F-actin.** Phalloidin specifically labels only the actin cytoskeletal structures made of filamentous actin and not the monomeric pool of actin (24). *C. trachomatis* serovar L2-infected or uninfected HeLa and McCoy cells were fixed in 2% (vol/vol) formaldehyde in PBS (pH 7.3) for 20 min and in acetone for 15 min (4). F-actin was then labeled with 156 ng of tetramethyl-RITC (TRITC)-labeled phalloidin (Sigma), containing 100 mg of lysolecithin per liter, for 30 min and rinsed twice in PBS (pH 7.3). HeLa cell monolayers were then incubated with the anti-MOMP antibody described above for the staining of clathrin and treated identically to the clathrin preparations, except that the rinsing in distilled water and the postfixation in ethanol were omitted. McCoy cells were labeled with the anti-MOMP antibody (Washington Research Foundation) or by a combi-

nation of FITC-labeled IgG1 monoclonal antibodies with specificity for MOMP and lipopolysaccharide (LPS), respectively (bioMérieux, Lyon, France). Cells were incubated for 30 min at 37°C and then rinsed three times in PBS. Cells were examined microscopically, alternating between FITC- and RITC-filter combinations, and evaluated for random and aggregated fluorescence patterns. As for clathrin staining, cells were also separately stained for F-actin and chlamydiae to rule out inefficacy of wavelength selection filters. Controls showed that TRITC-phalloidin did not label chlamydiae and that anti-MOMP or anti-LPS antibodies did not label F-actin.

**Cytochalasin D treatment.** McCoy cells were seeded on poly-L-lysine-pretreated coverslips and inoculated with *C. trachomatis* serovar L2. The cells were treated with cytochalasin D (1, 2, or 4 mg/liter), initially dissolved in dimethyl sulfoxide, during the last 30 min of the 3.5-h adsorption period at 4°C. The inoculum was then decanted and the monolayer was rinsed twice at 4°C with MEM containing cytochalasin D. Fresh MEM heated to 37°C with cytochalasin D was added, and the cells were incubated for 30 or 60 min at 37°C in a water bath. Fixed and unfixed cells were stained with the FITC-labeled monoclonal anti-MOMP antibody (Syva) and examined for randomly distributed and aggregated chlamydiae. In cytochalasin D-treated preparations, cells were separately evaluated for a peripheral local aggregate of EBs. Duplicate monolayers were washed three times with fresh MEM lacking cytochalasin D and incubated for a further 30 min before fixation and staining to study the reversible effect of the drug. Where indicated, HeLa cells were inoculated with *C. trachomatis* serovar L2, treated with 4 mg of cytochalasin D per liter as described above, and double fluorescence stained with TRITC-phalloidin and monoclonal anti-MOMP antibody (Washington Research Foundation) before being examined by use of a fluorescence microscope. Control experiments showed that the concentration of dimethyl sulfoxide used did not affect cell morphology and viability as assessed by trypan blue exclusion.

**Statistics.** Student's $t$ test was used to compare differences between percentages.

## RESULTS

When *C. trachomatis* serovar L2 was incubated with McCoy cells for 3 h at 4°C, the bacteria were diffusely distributed on the cell surface. By raising the temperature to 37°C, the EBs began to redistribute and aggregate to one region of individual cells (Fig. 1A to C). The number of cells with an aggregate increased with increasing incubation time at 37°C (Fig. 2). Substantially fewer infected and unfixed cells than fixed cells showed a local aggregation of EBs. However, when these former cells were fixed and relabeled with the anti-MOMP monoclonal antibody, the number of cells with a local aggregate increased to a value similar to that in initially fixed cells. This indicates that the majority of aggregated EBs were internalized. Furthermore, the local aggregate was distributed in different focal planes, suggesting that it had a three-dimensional distribution. Individual EBs could be identified in the aggregation in unfixed cells but not in fixed cells. Thus, EBs appeared more densely packed when cells were fixed. Only one local aggregate was observed per cell. This aggregate was localized to the center or to the perinuclear region of the cell. Diffusely distributed EBs were also seen in cells with a local aggregate. Thus, all EBs that attached to a single cell did not redistribute to one spatial location. When cells were incubated at 37°C for 30 or 60 min without preincubation at 4°C, only 19.7 and 54.3%, respectively, showed a local aggregate. This was significantly less than the corresponding figures after preincubation ($P < 0.005$ and $P < 0.05$, respectively) (Fig. 2).

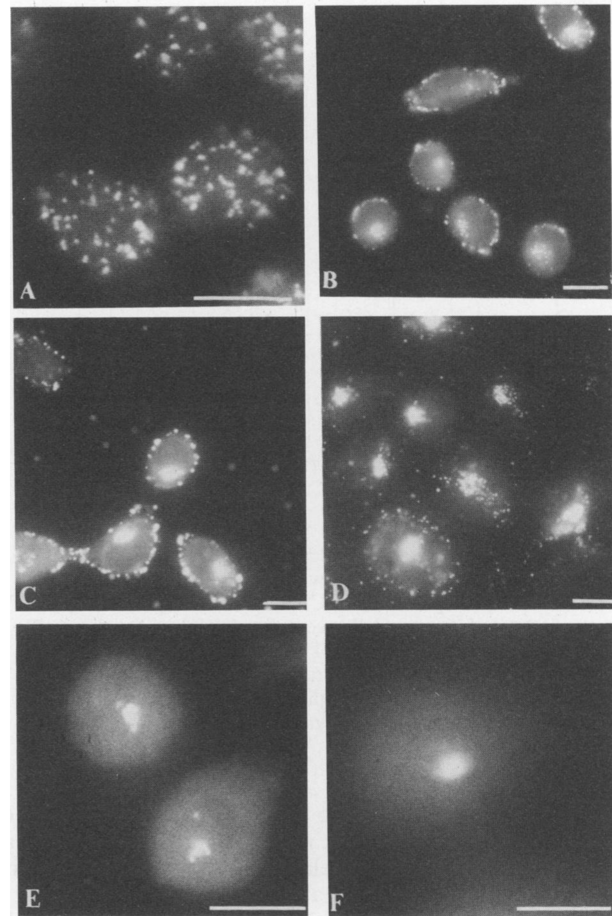


FIG. 1. Fluorescence photomicrographs showing aggregation of *C. trachomatis* in infected host cells. Serovar L2 was incubated with McCoy cells for 3 h at 4°C (A), for 3 h at 4°C plus 30 min at 37°C (B), and for 3 h at 4°C plus 60 min at 37°C (C) and with HeLa cells for 3 h at 4°C plus 3 h at 37°C (D), fixed in methanol, and stained with a monoclonal antibody to MOMP (Syva). Serovar E was incubated with McCoy cells for 3 h at 4°C plus 60 min at 37°C (E) and with HeLa cells for 3 h at 4°C plus 3 h at 37°C (F), fixed, and stained as described above. The MOIs for serovars L2 and E were 300 and 1,000 EBs per cell, respectively. Bars, 15 μm.

All of the above results were obtained with an MOI of 300 EBs per cell. About 95% of these cells were viable after incubation with *C. trachomatis* serovar L2 as assessed by trypan blue exclusion. When the MOI was reduced to 150, 75, and 37 EBs per cell, similar percentages of cells with a local aggregate were observed, as had been found for an MOI of 300 EBs per cell (78.5, 80.0, and 77.0%, respectively), when the cells had been incubated for 3 h at 4°C plus 60 min at 37°C. By varying the preincubation time at 4°C in 30-min increments from 30 min to 3 h, the percentages of cells with redistributed EBs did not significantly change; 87 and 90.5% of cells showed a local aggregate when preincubated for 30 min and 3 h, respectively, at 4°C plus 60 min at 37°C. However, as expected, the number of EBs per cell increased with increasing incubation time. Similar experiments were also performed with *C. trachomatis* serovar E

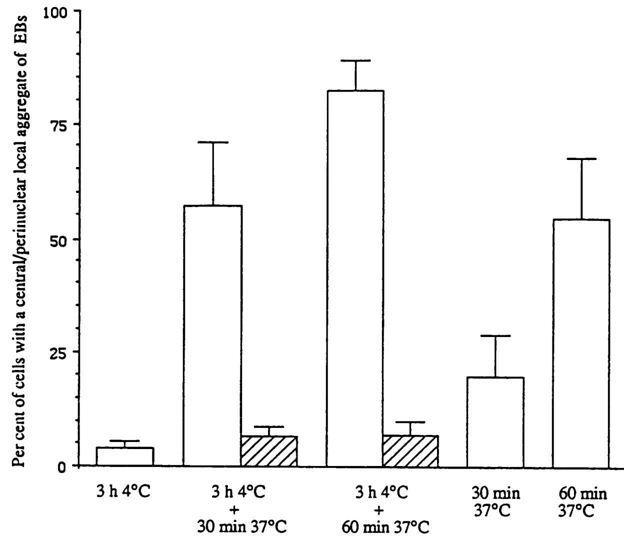


FIG. 2. Percent cells with local aggregation of *C. trachomatis* serovar L2 in McCoy cells. EBs were incubated with McCoy cells at an MOI of 300 EBs per cell at 4°C, at 4 and 37°C, or directly at 37°C for the indicated times. Cells were fixed (□) or not fixed (▨) in methanol and stained with an FITC-labeled monoclonal antibody to MOMP (Syva). Cells with a central or perinuclear local aggregate of EBs were determined microscopically. Values represent means ± standard deviations of two to eight experiments.

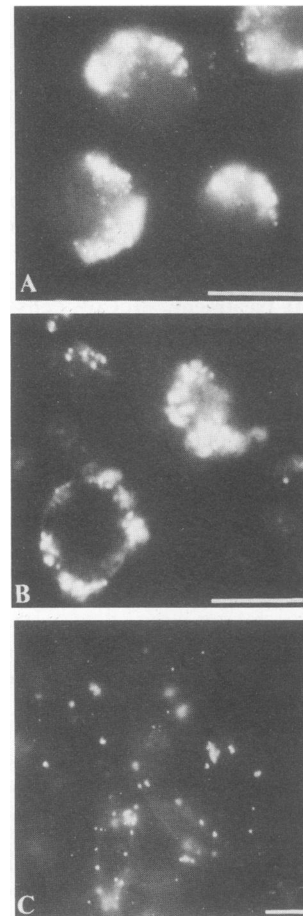


FIG. 4. Effect of cytochalasin D on redistribution of *C. trachomatis*. McCoy cells were treated with 4 mg of cytochalasin D per liter and infected with serovar L2 at an MOI of 300 EBs per cell for 3.5 h at 4°C plus 60 min at 37°C as described in Materials and Methods. Cells were fixed (A) or not fixed (B) in methanol and stained with a monoclonal antibody to MOMP (Syva). Cells identically infected but not treated with cytochalasin D and not fixed before staining with the monoclonal antibody are shown in panel C. For comparison with untreated, fixed cells, see Fig. 1C. Bars, 15 μm.

and HeLa cells (Fig. 1D to F). The redistribution of *C. trachomatis* serovar L2 EBs was slower in HeLa cells than in McCoy cells. Of HeLa cells, 59% showed a local aggregate after 3 h of incubation at 37°C compared with 94% in McCoy cells ($P < 0.05$) (Fig. 3). Furthermore, in HeLa cells the local aggregate was larger than in McCoy cells. During prolonged incubation, the size of the local aggregate in individual cells gradually increased.

Fewer cells infected with *C. trachomatis* serovar E exhibited a local aggregate than those infected with serovar L2, despite an inoculum of serovar E 3.3 times larger than that of serovar L2. Of McCoy cells infected with *C. trachomatis* serovar L2 for 3 h at 37°C, 94% ± 5.7% exhibited a local aggregate of EBs compared with 29.3% ± 13.0% in serovar E-infected cells ($P < 0.005$). The corresponding figures for HeLa cells were 59.0% ± 5.7% and 13.3% ± 4.3%, respectively ($P < 0.005$).

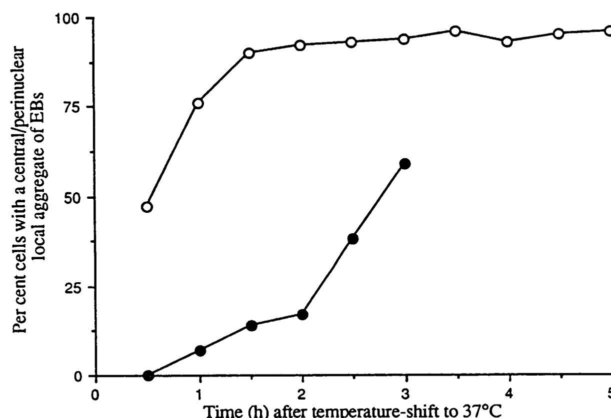


FIG. 3. Percent cells with local aggregation of *C. trachomatis* serovar L2 in McCoy and HeLa cells. Monolayers were incubated with EBs at an MOI of 300 EBs per cell for 3 h at 4°C plus 30 min to 5 h at 37°C, fixed in methanol, and stained with an FITC-labeled monoclonal antibody to MOMP (Syva). Cells with a central or perinuclear local aggregate of EBs were determined microscopically. Symbols: ○, McCoy cells; ●, HeLa cells. Values represent the means of two experiments.

**Cytochalasin D treatment.** When *C. trachomatis* serovar L2-infected McCoy cells were incubated with cytochalasin D, the redistribution to local aggregates was substantially inhibited and altered (Fig. 4). In cells treated with 4 mg of cytochalasin D per liter, significantly fewer cells exhibited a central or perinuclear local aggregate compared with untreated cells (Table 1). On the other hand, cytochalasin B in concentrations up to 8 mg/liter had no effect on this local aggregation. However, cytochalasin D-treated cells showed a peripheral local aggregate at the cell border. Such peripheral local aggregation was not seen in untreated cells. When the incubation time with cytochalasin D at 37°C was in-

TABLE 1. Effect of 4 mg of cytochasalin D (CD) per liter on local aggregation (LA) of *C. trachomatis* serovar L2 in McCoy cells[a]

| Treatment of cells | % Cells with:[b] | | | | | |
|---|---|---|---|---|---|---|
| | Central or perinuclear LA | | Peripheral LA | | No LA | |
| | 30 min, 37°C | 60 min, 37°C | 30 min, 37°C | 60 min, 37°C | 30 min, 37°C | 60 min, 37°C |
| No CD; fixation | 67.0 ± 16.3 | 79.5 ± 14.4 | 0 | 0 | 33.5 ± 16.3 | 20.5 ± 14.4 |
| CD added (4 mg/liter); fixation | 2.8 ± 1.6 | 5.1 ± 2.8 | 22.3 ± 10.3 | 65.3 ± 14.7 | 74.9 ± 9.8 | 29.6 ± 13.0 |
| CD added (4 mg/liter); no fixation | ND[c] | 3.0 ± 1.4 | ND | 65.5 ± 7.8[d] | ND | 31.5 ± 9.2 |

[a] EBs were incubated with McCoy cells at an MOI of 300 EBs per cell for 3 h at 4°C plus 30 or 60 min at 37°C. Cells were fixed or not fixed in methanol and stained with monoclonal antibody to MOMP (Syva). Cells with central or perinuclear, peripheral, or no LA were determined microscopically.

[b] Mean ± standard deviation of two to eight experiments.

[c] ND, not determined.

[d] Individual EBs could be identified in local aggregates.

creased, cells with a peripheral local aggregate also increased, with a corresponding reduction in cells with no local aggregation (Table 1). Cells treated with cytochalasin D, but labeled with monoclonal antibody without prior fixation, had a similar number of peripheral local aggregates as treated, fixed cells (Table 1). In unfixed cells, individual EBs were visible in the aggregate. This indicates that the peripherally aggregated EBs were on the cell surface.

In a separate series of experiments, the dose-dependent effect of cytochalasin D was evaluated. With a postincubation period of 60 min at 37°C, 19.5, 62.0, and 74.0% of cells exhibited a peripheral local aggregate in the presence of 1, 2, and 4 mg of cytochalasin D per liter, respectively. At the same time, the percentages of cells with a central or perinuclear local aggregate decreased to 40.5, 10.0, and 3.0%, respectively. No corresponding dose-dependent effect was seen during a 30-min postincubation period at 37°C, obviously because most EBs had not redistributed at this time but were still diffusely distributed on the cells. When infected cytochalasin D-treated cells were rinsed three times in MEM and incubated for a further 30 min at 37°C without cytochalasin D, the number of cells with a central or perinuclear local aggregate returned to the same value as that found in untreated cells, concomitant with a complete disappearance of cells with a peripheral local aggregate, showing that the effect of cytochalasin D was completely reversible.

**Staining of F-actin and clathrin.** McCoy and HeLa cells infected with *C. trachomatis* serovar L2 were stained for F-actin by TRITC-labeled phalloidin after permeabilization with lysolecithin and then by indirect immunofluorescence for EBs by using a monoclonal antibody to MOMP or a combination of two monoclonal antibodies with specificity for MOMP and LPS, respectively. Some cells were also separately stained for F-actin and MOMP, respectively. The results showed that F-actin distributed to a central or perinuclear local aggregate in time and position like redistributed EBs did (Fig. 5). In McCoy cells, 81.2% showed a local aggregation of EBs and 70.8% showed a local aggregation of F-actin, compared with 28.5% aggregation of F-actin in uninfected cells (Table 2). Concomitantly, the percentage of cells with visible stress fibers was almost three times lower in infected cells. Variation of focus suggested that local aggregation of F-actin was localized deeper within the cell cytoplasm than stress fibers. The aggregation of EBs and F-actin and the formation of stress fibers were temperature dependent. Similar results were also obtained with HeLa cells, with some notable exceptions. Local aggregation of EBs was, as expected from a previous experiment, lower in HeLa cells than in McCoy cells. Furthermore, the percent-

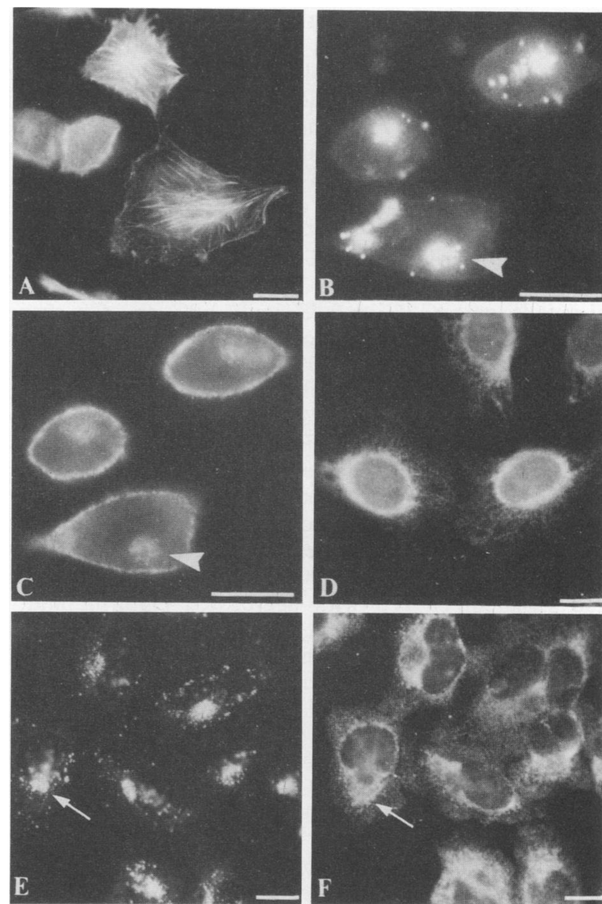


FIG. 5. Cells stained for *C. trachomatis*, F-actin, and clathrin. (A) Uninfected McCoy cells stained with TRITC-phalloidin showing the presence of stress fibers. (B and C) McCoy cells infected with *C. trachomatis* serovar L2, at an MOI of 300 EBs per cell, for 3 h at 4°C plus 60 min at 37°C, and double fluorescence labeled with a monoclonal antibody to MOMP (Washington Research Foundation) (B) and with TRITC-phalloidin (C). (D) Uninfected HeLa cells stained with antibodies to clathrin showing a punctate fluorescence pattern. (E and F) HeLa cells infected for 3 h at 4°C plus 3 h at 37°C and double fluorescence labeled with a monoclonal antibody to MOMP (Washington Research Foundation) (E) and with antibodies to clathrin (F). Aggregation of EBs and F-actin are indicated by arrowheads. Aggregation of EBs and clathrin are indicated by arrows. Bars, 15 μm.

TABLE 2. Percent McCoy or HeLa cells with local aggregation (LA) of EBs, F-actin, clathrin, and visible stress fibers[a]

| Treatment | % McCoy cells[b] with: | | | % HeLa cells[c] with: | | | % HeLa cells[c] with: | |
|---|---|---|---|---|---|---|---|---|
| | LA of F-actin | LA of EBs | Stress fibers | LA of F-actin | LA of EBs | Stress fibers | LA of clathrin | LA of EBs |
| Uninfected cells; 3 h at 4°C | 19.5 ± 0.7 | NA[d] | 10.5 ± 0.7 | ND[e] | NA | ND | ND | NA |
| Uninfected cells; 4°C + 37°C[f] | 28.5 ± 10.7 | NA | 77.5 ± 16.3 | 44.0 ± 2.8 | NA | ND | 8.0 ± 4.2 | NA |
| Infected cells; 3 h at 4°C | 20.0 ± 2.8 | 4.0 ± 1.4[g] | 11.0 ± 7.1 | 4.5 ± 0.7 | 5.0 ± 1.4[g] | ND | 7.0 ± 1.4 | 5.0 ± 1.4[g] |
| Infected cells; 4°C + 37°C[f] | 70.8 ± 16.4 | 81.2 ± 9.5 | 27.0 ± 5.7 | 63.0 ± 2.8 | 55.0 ± 7.1 | ND | 51.0 ± 4.2 | 55.0 ± 1.4 |

[a] Cells were infected with *C. trachomatis* serovar L2 at an MOI of 300 EBs per cell and incubated as indicated. After incubation, cells were fixed (see Materials and Methods) and labeled with monoclonal antibodies to *C. trachomatis* plus FITC-labeled anti-mouse antibodies and/or with TRITC-labeled phalloidin or stained for clathrin by indirect immunofluorescence. Values represent mean ± standard deviation of 2 to 11 experiments.

[b] EBs were stained with a combination of two monoclonal antibodies with specificity for MOMP and LPS, respectively (bioMérieux).

[c] EBs were stained with a monoclonal antibody with specificity for MOMP (Washington Research Foundation).

[d] NA, not applicable.

[e] ND, not determined.

[f] McCoy cells were incubated for 3 h at 4°C plus 60 min at 37°C and HeLa cells were incubated for 3 h at 4°C plus 3 h at 37°C (see Materials and Methods).

[g] Individual EBs could be identified in local aggregates.

age of cells with local F-actin aggregation was higher in uninfected HeLa cells than in McCoy cells and closer to the value found in infected cells (Table 2). We calculated in how many cells the local distribution of F-actin colocalized with local aggregation of EBs and vice versa. By alternating between FITC- and RITC-filter combinations in double-stained cells, 97.0% ± 2.9% of McCoy cells with a local aggregation of EBs showed distribution of F-actin to the same cell region. Conversely, 92.0% ± 2.9% of McCoy cells with a local aggregation of F-actin showed aggregation of EBs to the same cell region. The corresponding figures for HeLa cells were 83.0% ± 2.3% and 63.0% ± 1.4%, respectively. When infected HeLa cells treated with cytochalasin D were double stained, 38.0% showed a peripheral local aggregation of F-actin in a stellatelike punctate disorganized pattern. At the same time, 35.0% of treated cells had a peripheral local aggregate of EBs.

Clathrin also aggregated like EBs (Fig. 5). About the same number of cells showed a local aggregation of clathrin as they did of EBs (Table 2). The redistribution of clathrin was increased six to seven times in infected cells and was temperature dependent. In 90.0% ± 2.8% of cells, aggregated EBs colocalized with aggregated clathrin, and, in 90.5% ± 2.1% of cells, aggregated clathrin colocalized with aggregated EBs. However, there were differences in character and appearance of the colocalization observed for F-actin compared with that for clathrin. F-actin always overlapped the localization of aggregated EBs, whereas clathrin surrounded the aggregated EBs (compare Fig. 5B and C with Fig. 5E and F). By variation of focus, clathrin could sometimes be observed overlapping aggregated chlamydiae.

Control experiments showed no immunological cross-reactions of conjugates for EBs, clathrin, and F-actin and no unspecific staining. Redistribution of chlamydiae, F-actin, and clathrin were performed both in double- and single-stained preparations with identical results. Thus, the overlap between FITC- and RITC-filter combinations did not explain our results.

## DISCUSSION

After incubation at 4°C, *C. trachomatis* organisms are randomly distributed on the McCoy cell surface. After a temperature shift to 37°C, the majority of EBs redistribute, within 30 min, to one local aggregate per cell in the central or perinuclear region. This aggregation occurs at sites where chlamydiae are inaccessible to antibodies, i.e., intracellularly. Cytochalasin D inhibits more than 90% of this aggregation, and phalloidin, with affinity for F-actin, and clathrin localize to the same region of the cell as aggregated EBs. Our results suggest that, shortly after internalization, *C. trachomatis* organisms redistribute into an aggregate in the central or perinuclear region of infected cells and that F-actin and clathrin are intimately associated with this aggregate.

In cells infected at 37°C without preincubation at 4°C, which allows organisms to be internalized as they attach, identical aggregates are also seen, but in fewer cells than is the case with temperature-shifted cultures. In both types of cultures, the numbers of cells with a local aggregate increase with time. In cells with a local aggregate of EBs, organisms are also seen diffusely distributed. At least some of these are on the cell surface since they are observed in both fixed and unfixed preparations. Thus, some *C. trachomatis* organisms are not internalized even by actively endocytosing McCoy cells and are not incorporated in local aggregates. The number of cells with a local aggregate is independent of MOIs ranging from 300 to 37 EBs per cell and of duration of incubation time at 4°C. However, as expected, the number of EBs per cell and the size of the local aggregate decrease with decreases in MOI and incubation time. A similar aggregation of EBs is also seen in HeLa cells and when *C. trachomatis* serovar L2 is substituted by serovar E. However, the aggregation is slower in HeLa cells than in McCoy cells, which is in accordance with our previous finding that McCoy cells more readily ingest chlamydiae than do HeLa cells (20). Fewer cells infected with *C. trachomatis* serovar E exhibit a local aggregate than cells infected with serovar L2. This is true even when the inoculum size of serovar E is 27 times higher than that of L2. However, we consider that this does not provide direct evidence that serovars E and L2 behave differently in this respect but may rather be explained by the higher infectivity of L2 (13).

Inhibition of formation of the central or perinuclear local aggregate and induction of a peripheral cell surface aggregate of *C. trachomatis* L2 by cytochalasin D but not by cytochalasin B are in accordance with previous studies showing that cytochalasin D inhibits internalization of chlamydiae (15, 23). It has been shown that cytochalasin B and D induce capping of molecules including viral proteins (14, 21). In light of these results, our experiments strongly suggest that cytochalasin D inhibits entry of *C. trachomatis* serovar L2 by disruption of the microfilament and induces redistribution on the host cell surface of chlamydiae to sites where no signal

for endocytosis is induced. Local aggregation of EBs is probably related to the fusion of *C. trachomatis*-containing inclusions observed by Ridderhof and Barnes (17). They also reported that this fusion is microfilament dependent.

There is a concomitant redistribution of chlamydiae, F-actin, and clathrin. Our results indicate a transformation of stress fibers into a local aggregate of F-actin that, to a high extent, colocalizes with redistributed chlamydiae. However, immunofluorescence studies do not allow a resolution of F-actin associated with individual EBs. However, the substantial increase in local aggregation of F-actin in infected McCoy cells incubated at 37°C compared with that in uninfected or infected cells incubated at 4°C strongly suggests that the redistribution of F-actin is induced by chlamydiae. In HeLa cells, the results are more difficult to interpret because of the comparatively high percentage of uninfected cells with a confluent phalloidin-stained region that is difficult to discriminate from local aggregation of F-actin. This is also reflected by the lower percentage of colocalization of EBs and F-actin in HeLa cells compared with that in McCoy cells.

F-actin remains intimately associated with EBs for at least 3 h after induction of endocytosis, which is consistent with a function for F-actin in the fusion of chlamydiae-containing inclusions during the developmental cycle (17). However, our studies do not directly show endosomal fusion but rather that chlamydiae are rapidly redistributed to one region of infected cells, where fusion subsequently may occur. This is further strengthened by the rapid translocation to an intracellular site of cell-surface-attached chlamydiae in cells released from cytochalasin D inhibition. Campbell et al. (4) reported that chlamydial infection had little effect on the distribution of microfilaments. However, their results were recorded later in the developmental cycle than ours and they did not provide quantitative data. It is possible that the decrease we observed in the number of cells with stress fibers later returns to normal.

Clathrin redistributes and colocalizes with *C. trachomatis* within 3 h after induction of endocytosis in HeLa cells. The different appearance of F-actin from that of clathrin in relation to aggregated chlamydiae suggests that actin is more intimately associated with the aggregate. This may imply different functions for actin and clathrin in the redistribution and aggregation of chlamydiae. Electron microscopic studies have shown that *C. trachomatis* organisms are internalized into host cells in coated pits and that the coat material remains associated with endosomes containing chlamydiae for at least 3 h at 37°C (10). According to Prain and Pearce (15), chlamydiae are taken up by host cells by both cytochalasin D-sensitive and -resistant pathways. For a *C. trachomatis* LGV strain, the cytochalasin D-resistant pathway can be further subdivided into uptake into coated and uncoated membranes (16). Furthermore, the number of chlamydiae associated with coated pits is dependent on the culture conditions of the host cells. Cells grown in a polarized orientation exhibit more chlamydiae in clathrin-associated structures than cells cultured on plastic surfaces (25). With polarized cells it might be possible to follow the redistribution of clathrin to the local aggregate of chlamydiae. Our results show that, despite at least three proposed different uptake pathways, chlamydiae rapidly redistribute to an intracellular site and become associated with F-actin and clathrin in the majority of cells.

Cell-surface-attached enteropathogenic and enterohemorrhagic *E. coli* are associated with polymerized actin, despite the fact that they are not internalized by the host cell (12). This indicates that a transition from G-actin to F-actin may occur at the site of attachment without generation of a signal for endocytosis. *Shigella flexneri* invading nonprofessional phagocytes is also surrounded by both F-actin and clathrin (5). In shigella, actin and stress fibers seem to participate in both internalization and intracellular translocation of bacteria devoid of a vacuolar membrane (22); this may therefore be different from the situation in chlamydiae, which are surrounded by a host-cell-derived membrane. Previous studies have shown that both clathrin and microfilament mediate internalization of chlamydiae by host cells, albeit to different degrees depending on, e.g., experimental conditions (10, 11, 16, 19).

This study shows that both clathrin and F-actin remain associated with intracellular *C. trachomatis* organisms during a rapid redistribution immediately after induction of endocytosis. We hypothesize that clathrin and microfilament are involved in intracellular translocation of chlamydiae and may affect their interaction with other subcellular components.

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