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Cytoskeletal Requirements in *Chlamydia trachomatis* Infection of Host Cells

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Infection of genital epithelial cells by the closely related sexually transmitted pathogens *Chlamydia trachomatis* serovars E and L2 results in different clinical disease manifestations. Following entry into target host cells, individual vesicles containing chlamydiae fuse with one another to form one large inclusion. At the cellular level, the only obvious difference between these serovars is the time until inclusion maturation, which is 48 h for the invasive serovar L2 and 72 h for serovar E. To begin to define the intracellular events of these pathogens, the effect of cytoskeletal disruption on early endosome fusion and inclusion development in epithelial (HEC-1B) and fibroblast (McCoy) cells was analyzed by fluorescence microscopy. Disruption of microfilaments with cytochalasin D markedly reduced serovar E, but not serovar L2, infection of both cell lines. Conversely, microfilament as well as microtubule disruption, with colchicine or nocodazole, had no effect on serovar E inclusion development but resulted in the formation of multiple serovar L2 inclusions per cell during early and mid-development. Later in serovar L2 inclusion development (>36 h postinfection), vesicles containing chlamydiae fused to form one large inclusion in the absence of an intact cytoskeleton. These results imply that (i) *C. trachomatis* serovar E may utilize a different pathway for uptake and development from serovar L2; (ii) these differences are consistent in both epithelial cells and fibroblasts; and (iii) the cytoskeleton plays a unique role in the infection of host cells by these two genital pathogens.

Chlamydia trachomatis strains consist of serological variants responsible primarily for eye disease and genital tract infections. The strains are all antigenically related but are distinguished by their immunologic reactions into various serovars. Serovars A, B, Ba, and C cause hyperendemic blinding trachoma. The lymphogranuloma venereum (LGV) biovar (serovars L1, L2, and L3) and the *C. trachomatis* biovar (serovars D to K) are responsible for sexually transmitted infections. The former is epidemic in parts of Asia, Africa, and South America, whereas the latter is responsible for epidemic disease in the United States and Europe, causing an estimated 4.5 million infections annually (63). These obligate intracellular bacteria infect the columnar epithelial cells of the genital mucosae, causing an initial urethritis or cervicitis. In the absence of antibiotic exposure, the *C. trachomatis* serovars, which are luminal pathogens, spread canalicularly in an ascending fashion to infect the epididymus or the endometrium and fallopian tubes. In contrast, the LGV serovars are more invasive pathogens which spread through the submucosa to infect the inguinal lymph nodes and may eventually reach the bloodstream.

At the cellular level, chlamydial pathogenesis begins with the entry of infectious elementary bodies (EB) into the genital epithelia. Once inside the eukaryotic host cell, EB remain within membrane-bound vesicles which fuse with one another early after entry to form one large vesicle, even under high multiplicities of infection (4), but presumably evade fusion with lysosomes (12–14, 64). The metabolically inert EB then transform into metabolically active, noninfectious forms, called reticulate bodies (RB). The RB divide by binary fission to fill the vesicle containing chlamydiae. Once this vesicle becomes large enough to detect with a light microscope, 16 to 24 h after infection, it is then called an inclusion (50). Late in the developmental cycle, which is approximately 48 h for LGV and 72 h for serovar E, an unknown signal triggers the condensation of RB into EB. The infectious EB progeny are then released from the host cell to infect neighboring cells and perpetuate the infectious cycle.

Since chlamydiae are ubiquitous pathogens which infect a variety of cells in culture, many investigators have used in vitro model systems to examine the entry process. From these studies, evidence exists for the entry of *C. trachomatis* serovar E into host cells by receptor-mediated endocytosis via clathrin-coated pits (53, 62), a mechanism which is believed to be microfilament (MF) independent. The requirement of a functional actin network for endocytosis in the yeast *Saccharomyces cerevisiae* however, has recently been discovered (27) and found to be similar to the endocytosis seen at the apical membrane in polarized mammalian cells. Other investigations suggest that the actin cytoskeleton is required for all apical endocytosis, whether it be by adsorptive or fluid-phase pinocytosis (17), and that proteins in the coat of clathrin-coated vesicles bind to actin filaments (26). Hence, endocytosis may involve MF.

Ward and colleagues first demonstrated a need for MF (59) and calcium (60) in chlamydial uptake. These investigators showed that the uptake of *C. trachomatis* serovar L2 into HeLa cells was suppressed 50% by the disruption of MF. This finding was subsequently confirmed by Reynolds and Pearce (46) for the uptake of serovar L2 into McCoy cells and added to the growing hypothesis that chlamydiae utilize both phagocytic (MF-dependent) and pinocytic (MF-independent) mechanisms for entry. Recently, a study showed data which suggest that bacteria utilize different invasion mechanisms depending upon the presence of different surface receptors on various cell types (37). Ridderhof and Barnes (47) demonstrated that MF permitted the fusion of serovar E-containing vesicles with serovar F-containing vesicles in multiply infected HeLa cells.

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While the studies of Majeed (32, 33) colocalized clathrin and F-actin to the site of entry of serovars E and L2 into HeLa and McCoy cells, these investigators seemed to believe that the roles of intracellular calcium and MF were more important for the aggregation of vesicles containing chalmydiae following chlamydial entry. More recently, the calcium-dependent fusion of vesicles containing chlamydiae has been shown to involve annexins (31). The identification of proteins in the chlamydial inclusion membrane may soon shed light on the mechanisms that regulate vesicle aggregation and fusion during chlamydial infections.

Currently, the role of the microtubule (MT) lattice in the primary stages of genital chlamydial infection is also unclear. MT are ubiquitous elements of the cytoskeleton and are involved in many cellular functions, including (i) the maintenance of cell shape (51) and cellular organelle organization (25); (ii) vesicular trafficking (18, 20), including endosomes and lysosomes (35) as well as Golgi vesicles (57); and the fusion of apical to basolateral late endosomes (1, 6); (iii) mitochondrial transport (29); (iv) apical protein transport and apical secretion of certain lysosomal proteins (11, 42); and (v) basolateral-to-apical transcytosis of the polymeric immunoglobulin receptor (23) as well as basolateral laminin secretion (5). Several investigations have also found that endocytosis and receptor recycling are slowed down in the presence of MT-disrupting drugs (24, 30, 38). Only a few studies have examined the effects of MT disruption on chlamydial infection.

The MT-disrupting drugs vincristine and vinblastine did not appear to affect serovar L2 attachment to HeLa cells but did have an effect on internalization (59) as well as coalescence and localization of vesicles containing chlamydiae in the perinuclear region (36). Since vesicles containing EB previously exposed to UV irradiation could correctly localize to the nuclear hof, while vesicles containing heat-treated EB could not, McBride and Wilde (36) speculated that a protein in the chlamydial inclusion membrane may interact with the host cell cytoskeleton. From these studies and the fact that, to date, no drug disrupting receptor-mediated endocytosis, MT, or MF completely abolishes chlamydial infectivity, it seems most likely that chlamydiae utilize several different routes of uptake in order to invade a wide variety of eukaryotic cells. Regardless of the entry mechanism, once inside the host cell, the multiple *C. trachomatis*-containing membrane-bound vesicles are known to fuse with one another to form one large, mature inclusion (4, 8).

The roles of MF and MT in *C. trachomatis* genital tract infections are still unclear but appear to be important in chlamydial vesicle fusion, migration, and inclusion development. We hypothesize that MT function in the transport of the mature inclusion to the appropriate membrane, apical or basolateral, for escape from the host cell. Since serovars E and L2 differ in their in vivo sequelae, efficiency of infection, length of developmental cycle, and possibly the host cell receptor used for entry, we focused on comparing their use of the host cell cytoskeleton. Previous investigations concerning the role of the cytoskeleton in chlamydial infections quantitated the amount of binding or entry of radiolabeled EB to drug-exposed fibroblasts without examining the morphological changes in inclusion formation which occurred upon cytoskeletal damage in infected cells. The purpose of these studies was to examine the aggregation and formation of chlamydial inclusions within fibroblasts and epithelial cells devoid of polymerized MF or MT as a result of exposure to cytoskeleton-disrupting drugs.

MATERIALS AND METHODS

Cells and medium. The human endometrial epithelial cell line HEC-1B (HTB-113; American Type Culture Collection, Rockville, Md.) and the mouse fibroblast cell line McCoy (ATCC CRL 1696) were used in these experiments. Eukaryotic cells were grown on 12-mm-diameter glass coverslips coated with poly-L-lysine or in a polarized fashion on collagen-coated polycarbonate filters as described previously (63) in Dulbecco's minimal essential medium (DMEM-H) containing Earle's salts, 584 mg of L-glutamine per liter, 4,500 mg of glucose per liter, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and supplemented with 10% (vol/vol) fetal calf serum (HyClone).

Chlamydial strains and growth conditions. The chlamydial strains used were *C. trachomatis* E/UW-5/CX (serovar E), a human urogenital isolate, and *C. trachomatis* biovar LGV L2/434/Bu (serovar L2), obtained from C. C. Kuo and S. P. Wang, University of Washington, Seattle. Chlamydial stocks were harvested from McCoy cells grown on Cytodex 3 beads (Sigma) as described previously (55). Eukaryotic cells were inoculated at 35°C with purified EB stock, diluted in 0.02 M phosphate buffer containing 0.2 M sucrose and 0.73 mg of glutamine per ml (2SPG), to yield a 90 to 100% infection of the monolayer. To ensure an even infection of the monolayer, a small inoculum volume (50 μl) was used and the tissue culture plates were tapped by hand at 15-min intervals during the 2-h adsorption period. DMEM-H was then added, and cells were incubated in an atmosphere of 5% CO_2 at 35°C.

Disruption of MF. Cytochalasin D (CD; 0.5 mg/ml in dimethyl sulfoxide [DMSO]; Sigma) was diluted in DMEM-H to the desired concentration. Host cells, grown to subconfluency on glass coverslips, were pretreated with 0.5, 1.0, 2.5, or 4.0 μg of CD per ml for 1 h at 35°C before infection with chlamydiae. CD was maintained within the medium during all subsequent incubations.

Disruption of MT. Host cell MT were depolymerized with 10 μM colchicine (Sigma) in DMEM-H for 30 min at 4°C and then for 3.5 h at 35°C by the procedure of Gilbert et al. (16). Cells were then washed with fresh DMEM-H and 2SPG before inoculation with chlamydiae. Colchicine was not present in the medium during or after chlamydial infection.

MT disruption was alternatively obtained with exposure to 33 μM nocodazole (Sigma) for 30 min at 4°C followed by a 2.5-h incubation at 35°C before chlamydial inoculation. Nocodazole was maintained at 33 μM during the 2-h chlamydial adsorption and within the medium for subsequent incubation times.

Fluorescence staining and examination. Following cold methanol fixation, chlamydial inclusions were stained with fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies specific to the major outer membrane protein of *C. trachomatis*, which results in yellow-green inclusions and Evans blue-counter-stained red eukaryotic cells (Syva; Syva Microtrak, San Jose, Calif.).

MF were visualized by staining F-actin with BODIPY fluorescein (FL)-phallacidin (Molecular Probes). Eukaryotic cells on coverslips were fixed for 10 min with 3.7% formaldehyde in phosphate-buffered saline (PBS), washed, and dehydrated in acetone. Coverslips were air dried and incubated in FL-phallacidin for 20 min at 25°C. Samples were washed and mounted in a 1:1 solution of PBS-glycerol.

To stain for MT, methanol-fixed eukaryotic cells were incubated for 1 h at 37°C with a primary mouse monoclonal anti-β-tubulin antibody (Sigma), diluted 1:200 in PBS containing 1% immunoglobulin G-free bovine serum albumin (BSA; Sigma). Primary antibody was detected with a FITC-conjugated goat anti-mouse secondary antibody (Sigma), diluted 1:200 in PBS. Samples were dried and mounted in polyvinyl alcohol.

BODIPY FL C_5-DMB-ceramide (Molecular Probes) was used to stain the Golgi apparatus. This dye associates with cholesterol in the membrane bilayer of this organelle (41) and fluoresces green in dilute amounts or red when concentrated. Eukaryotic cells grown on coverslips were washed in serum-free DMEM-H and incubated for 15 min at 35°C with 2.5 μM DMB-ceramide in serum-free medium containing 0.34 mg of fatty acid-free BSA (DF-BSA; Sigma) per ml. Coverslips were then washed and incubated in serum-free medium containing 0.34 mg of DF-BSA per ml for 40 min at 35°C.

Eukaryotic cell viability was assessed by incubating the cells in a mixture of 0.1 μg of FL-diacetate (Sigma) per ml and 0.1 μg of propidium iodide (Sigma) per ml in PBS. Live cells take up FL-diacetate, while the red propidium iodide enters only dead cells. The cells were incubated at 35°C for 10 min, washed in PBS, mounted, and examined immediately. All fluorescent stains were examined by epifluorescence microscopy at a magnification of ×400 with a Zeiss Axiovert inverted microscope (Carl Zeiss Inc., Thornwood, N.Y.) equipped with a mercury bulb and fluorescence filter sets. Confocal fluorescence microscopy of polarized, Syva-stained HEC-1B cells infected with serovar L2 was performed with a Zeiss LSM 10 microscope.

Determination of chlamydial infectivity. To determine the effects of CD on *C. trachomatis* inclusion development, we examined the infection of host monolayers grown on glass coverslips from three separate experiments. Each time point and concentration of CD examined represents an average of cells counted from four replicate coverslips. Approximately 400 Syva-stained cells from four separate microscopic fields were examined per coverslip. In the early stages of infection (4 to 16 h postinfection [p.i.]), the number of vesicles containing chlamydiae per cell was counted and the average was determined. At mid- to late stages of infection (24 to 48 h p.i.), the number of inclusions per cell and the percent infectivity were determined. Percent infectivity was determined as the

number of infected cells divided by the total number of cells times 100. Values were adjusted so that untreated control cells were 100% infected.

Transmission electron microscopy. Serovar L2-infected HEC-1B cells grown on polycarbonate filters were prefixed for 1 h at 25°C with 2% glutaraldehyde–0.01% paraformaldehyde in 0.1 M sodium cacodylate buffer. Polycarbonate filters supporting polarized HEC-1B cells infected with serovar L2 were processed for embedding in Epon-Araldite resin for examination by contrast transmission electron microscopy as described previously (62). Thin sections were cut on a Reichert Ultracut S microtome (Leica) and poststained with 5% aqueous uranyl acetate and 6% lead citrate before viewing on a Zeiss 900 microscope operating at 50 kV.

RESULTS

MF disruption. Uninfected McCoy cells were first exposed to various concentrations of the MF-disrupting agent CD and then stained with FL-phallacidin to determine if any polymerized actin remained. After a 1-h incubation in 0.5 μg of CD per ml, a few stress fibers were visible, but no filamentous actin was visible in McCoy cells exposed to 1.0, 2.5, and 4.0 μg of CD per ml. HEC-1B cells, incubated with all CD concentrations specified above and stained with FL-phallacidin, did not contain any visible actin filaments. The viability of HEC-1B and McCoy cells was examined with fluorescent dyes after 24- and 48-h incubations in the presence of 4 μg of CD per ml. Approximately 10% of the HEC-1B cells were not viable after 24 h and 20% were not viable after 48 h, whereas only 5% of the McCoy cells were dead at 24 and 48 h in the presence of 4 μg of CD per ml.

When subconfluent monolayers of HEC-1B and McCoy cells were exposed to the range of CD concentrations (0.5, 1.0, 2.5, and 4.0 μg/ml) for 1 h and then infected with serovar E EB, a reduction in the number of serovar E-containing vesicles within the infected cells was seen at 4 and 8 h p.i. compared with mock (DMSO)-treated and untreated cells. This concentration-dependent reduction in the percent infection of both HEC-1B and McCoy cells was maintained through 24 (Fig. 1), 32, and 42 (Fig. 2) h p.i. The serovar E inclusions that did form, however, had a morphology similar to those in DMSO-treated and untreated cells (Fig. 2), in which all of the serovar E-containing vesicles fused to form one inclusion. Even at the highest concentration of CD, the infectivity of host cells by serovar E was not completely abolished.

In contrast, serovar L2-infected monolayers of HEC-1B or McCoy cells exposed to the various concentrations of CD for 1 h did not show a reduction in the number of L2-containing vesicles within the cells at 4 or 8 h p.i. compared with DMSO- and untreated cells. Similarly, there was no reduction in the percentage of CD-exposed HEC-1B or McCoy cells that were infected by serovar L2 at 24 or 32 h p.i. (Fig. 3) compared with DMSO-treated and untreated cells.

Serovar L2-containing vesicles normally aggregate and fuse rapidly after entering the cytoplasm of the host cell and by 8 to 12 h p.i., depending on the number of EB that continue to enter the cell during this time, have fused to form one inclusion surrounded by a single vesicular membrane. In both cell types exposed to CD, serovar L2-containing vesicles did not aggregate within the host cell at 4 or 8 h p.i. and remained as separate multiple vesicles within the host cell at 24 and 32 h p.i. (Fig. 3). Figure 3 shows the inclusion morphology of the single serovar L2 inclusion within each DMSO-exposed cell compared with the multiple inclusions seen at 24 and 32 h p.i. in CD-exposed cells. At 24 h p.i., the average number of L2-containing vesicles per cell was two to four times higher than that in untreated cells (see Fig. 5). By 42 h p.i., however, serovar L2 inclusions did fuse with one another in the presence of 4 μg of CD per ml (Fig. 3). Thus, CD was an effective disrupting agent of MF in epithelial cells and fibroblasts and

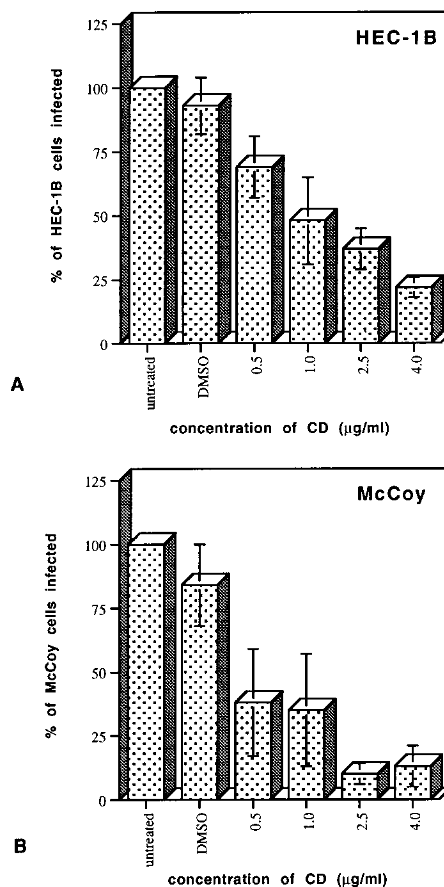

FIG. 1. Disruption of MF with CD reduces *C. trachomatis* serovar E infection of epithelial cells and fibroblasts. HEC-1B (A) or McCoy (B) cells were preexposed to DMSO (1:125) or CD at various concentrations for 1 h before infection with serovar E. Inclusions were stained with Syva at 24 h p.i., and the percent infectivity was determined. Values are means ± standard errors of the means from three experiments.

discriminated between serovar E and L2 infection and inclusion development in both eukaryotic cell types.

MT disruption. The effectiveness of MT disruption by colchicine or nocodazole in epithelial cells and fibroblasts was determined by fluorescent staining of β-tubulin. The MT in HEC-1B and McCoy cells remained depolymerized for up to 48 h after colchicine was removed from the medium. At 74 h after colchicine removal, MT started to repolymerize in HEC-1B cells. In cells exposed to nocodazole, MT started to repolymerize 4 h after the drug was removed. All HEC-1B and McCoy cells remained viable after 24 and 48 h of exposure to colchicine or nocodazole, although they were rounded and became multinucleate. FL-phallacidin staining of HEC-1B cells exposed to colchicine did not reveal any MF damage as assessed by epifluorescence microscopy.

The effect of MT disruption on the development of *C. trachomatis* was determined by examining inclusion morphology in drug-exposed cells. Colchicine and nocodazole did not affect the ability of *C. trachomatis* serovar E to infect and form mature inclusions in both HEC-1B and McCoy cells. At 24, 36 (Fig. 4), and up to 70 h p.i., the morphology of serovar E inclusions appeared the same as those in DMSO-exposed cells.

In contrast, a detectable decrease in serovar L2-containing vesicle aggregation, manifested as multiple well-spread small inclusions, was identified at 4, 8, and 16 h p.i. in nocodazole- or

FIG. 2. MF disruption in HEC-1B and McCoy cells with CD does not affect *C. trachomatis* serovar E inclusion formation but does decrease infectivity. Chlamydial inclusions were stained with Syva after methanol fixation. (A and B) Serovar E inclusions 42 h p.i. in HEC-1B cells; (C and D) serovar E in McCoy cells 42 h p.i. (A and C) Cells exposed to DMSO at 1:125; (B and D) cells exposed to CD at 4 μg/ml.

colchicine-exposed HEC-1B and McCoy cells. These serovar L2 inclusions remained separated at 24 (Fig. 6) and 36 h p.i. and had the same morphology as those in CD-exposed cells. By 44 h p.i., which is late in the serovar L2 developmental cycle, inclusion fusion had occurred in the presence of colchicine or nocodazole and single serovar L2 inclusions developed in both HEC-1B and McCoy cells (Fig. 6).

Transmission electron microscopic examination of thin sections of polarized, colchicine-exposed HEC-1B cells confirmed the existence of multiple serovar L2-containing vesicles per cell

FIG. 3. MF disruption with CD in HEC-1B and McCoy cells leads to the formation of multiple *C. trachomatis* serovar L2 inclusions per cell but does not affect infectivity. Serovar L2 inclusions were methanol fixed and stained with Syva in HEC-1B (A and B) or McCoy (C to H) cells exposed to DMSO at 1:125 (A, C, E, and G) or CD at 4 μg/ml (B, D, F, and H). (A to D) 24 h p.i.; (E and F) 32 h p.i.; (G and H) 42 h p.i.

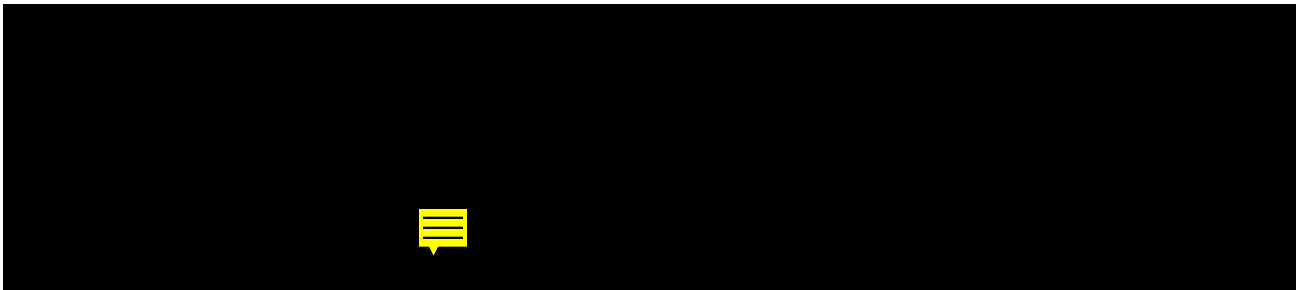

FIG. 4. MT disruption does not affect the development of *C. trachomatis* serovar E inclusions or the amount of infection of HEC-1B or McCoy cells. Serovar E-infected McCoy (A and B) or HEC-1B (C and D) cells at 24 (A and B) or 36 (C and D) h p.i. Host cells were untreated (A and C) or treated with 10 μM colchicine (B) or 33 μM nocodazole (D) for 4 h before inoculation with serovar E.

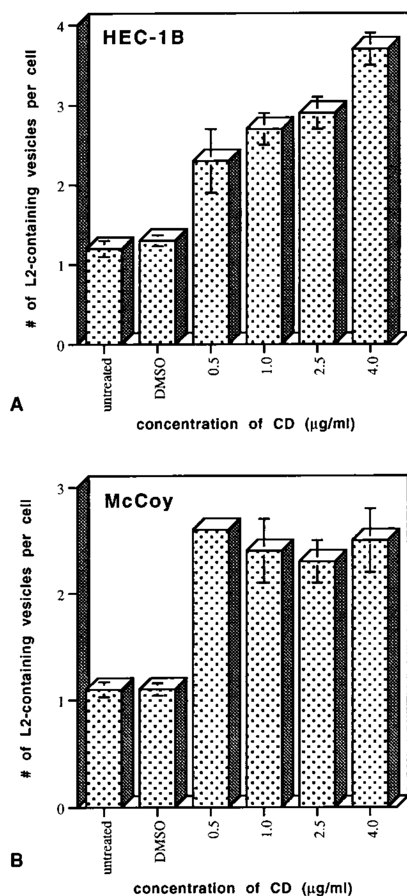

FIG. 5. Disruption of MF with CD delays *C. trachomatis* serovar L2 inclusion fusion and leads to multiple inclusions per cell. HEC-1B (A) and McCoy (B) cells were infected with serovar L2 after a 1-h incubation with DMSO (1:125) or CD at various concentrations. Inclusions were stained with Syva at 24 h p.i., and the number of inclusions per cell was determined and averaged for each coverslip examined. Values are means ± standard errors of the means from three experiments.

(Fig. 7). The electron micrographs of 24-h-p.i. serovar L2 inclusions within colchicine-exposed HEC-1B cells showed EB and RB that appeared the same as those in untreated cells. Confocal fluorescence microscopy of infected colchicine-exposed HEC-1B cells, grown polarized on polycarbonate filters, also revealed multiple serovar L2-containing vesicles at 24 h p.i. (data not shown).

When nocodazole, a reversible disrupter of MT, was removed from the medium of HEC-1B cells at 2 or 4 h after serovar L2 infection, inclusion fusion proceeded normally. However, if nocodazole was removed at 8 or 12 h after infection, multiple L2 inclusions per cell were seen. When nocodazole was added to the medium as late as 12 h p.i., multiple serovar L2 inclusions were still observed in HEC-1B cells. These data show that MT disruption discriminates between serovar E and L2 inclusion development but does not affect the ability of chlamydia to infect host cells.

Chlamydial progeny viability. To determine the infectivity titer of the progeny, *C. trachomatis* serovar L2 was grown within cytoskeleton-disrupted HEC-1B cells, isolated at 42 h p.i., and inoculated onto untreated HEC-1B cells. These untreated HEC-1B cells were examined 24 h later to determine the percentage which developed mature inclusions. Serovar L2, isolated from HEC-1B cells exposed to 4 μg of CD per ml, infected 50% of the untreated host monolayer, compared with 100% infection by serovar L2 isolated from untreated control cells. In contrast, serovar L2 isolated from colchicine-exposed HEC-1B cells infected 100% of the untreated HEC-1B monolayer, as did the progeny from untreated HEC-1B cells. Thus, *C. trachomatis* serovar L2 is capable of producing infectious progeny within cells lacking an intact cytoskeleton.

Localization of the Golgi. Because vesicles departing from the Golgi are known to traffic along MT (57), we were concerned that the depolymerization of MT within host cells may inhibit the flow of vital nutrients from the Golgi to the growing inclusion and thus result in the observed decrease in serovar L2 inclusion fusion. To detect the fusion of Golgi vesicles with the chlamydial inclusion membrane, the sphingolipids of the *trans*-Golgi apparatus in infected HEC-1B cells were localized with C_5-DMB-ceramide. At 24 h after serovar L2 infection of HEC-1B or McCoy cells, the Golgi label was identified as punctate dots surrounding the chlamydial inclusion (Fig. 8). Host cells exposed to colchicine exhibited a more diffuse staining pattern, but the ceramide was still seen ringing the serovar L2 inclusions. The same staining pattern occurred when serovar E was examined within HEC-1B cells at 35 h p.i. in the presence or absence of colchicine. No C_5-DMB-ceramide was identified within the chlamydial inclusion membrane or within the chlamydial inclusion.

DISCUSSION

Concerning the role of the cytoskeleton in chlamydial development subsequent to internalization, we devoted our attention early to the aggregation and fusion of chlamydiae-containing vesicles and later to inclusion formation and maturation into one vesicle. The findings reported here indicate a clear difference in the utilization of the host cell cytoskeleton by the more invasive LGV biovar than by the genital *C. trachomatis* biovar (Table 1). In LGV-infected cells, both MF and MT disruption interfered with the fusion of multiple EB-containing vesicles to form one large mature inclusion. The ability of serovar L2 to infect cells lacking an intact cytoskeleton, however, was not affected. In serovar E-infected cells, MF and MT disruption did not affect vesicle aggregation or inclusion formation. However, a dose-dependent decrease in the percentage of cells infected by serovar E was seen in cells lacking intact MF. This finding suggests that serovar E requires an intact MF network for the efficient infection of host cells. From these results, we do not know whether serovar E utilizes MF for entry into host cells and/or for vesicle aggregation and fusion immediately after entry. Serovar E-containing vesicles may require aggregation and fusion directly after entry in order to survive within the host cell. If this is a MF-dependent process, it would explain the low percentage of CD-exposed cells that were infected by serovar E.

The need for MF in chlamydial entry is more understandable for host cells grown in vitro, since eukaryotic cells have been shown to pick up EB as they migrate across the substratum (9). In vivo, the naturally polarized cells of the genital epithelial mucosae are presumably less motile. The nonmotile, metabolically inert EB bind initially to the tips of microvilli en route to the cell surface, where they are seen to enter into coated pits at the base of microvilli (21, 22, 62). Disruption of the MF in these microvilli may decrease serovar E infectivity. Alternatively, the host cell receptor to which serovar E binds may require MF to move to clathrin-coated pits in the apical membrane prior to endocytosis.

The capability of serovar L2 to infect cells without an intact cytoskeleton suggests that this pathogen binds to a receptor

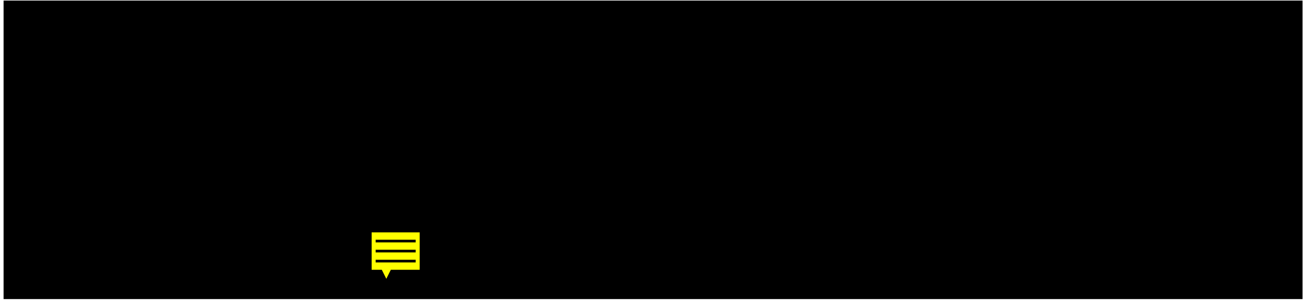

FIG. 6. MT depolymerization delays the formation of one *C. trachomatis* serovar L2 inclusion per cell until later times in the developmental cycle. HEC-1B cells infected with serovar L2 were untreated (A and D) or treated with 10 μM colchicine (B and E) or 33 μM nocodazole (C and F). Serovar L2 inclusions were stained with Syva at 24 (A to C) or 44 (D to F) h p.i.

that does not associate with the host cell cytoskeleton for uptake. Serovar L2 may utilize a ubiquitous cell surface receptor which is endocytosed via an MF-independent pathway besides that involving clathrin. It has recently been reported that molecules endocytosed without clathrin are delivered to the same endocytic compartment as the transferrin receptor which is internalized through clathrin-coated pits (19). Therefore, serovar L2 may enter target cells by several pathways, from both apical and basolateral surfaces, and still congregate in a common endosomal vesicle.

The various routes of uptake and development of the two serovars into host cells may reflect inherent differences in their in vivo sequelae. Serovar E is a noninvasive pathogen which presumably enters and exits from the apical surface of host cells, while serovar L2 invades tissue underlying the epithelium and may enter and exit through both the apical and basolateral surfaces. From these data, we have devised a model for the modes of *C. trachomatis* serovar E and L2 infection and spread in an epithelial monolayer (Fig. 9). We hypothesize that serovar L2 enters host cells from both the apical and basolateral surfaces without the help of the cytoskeleton. After entry, serovar L2-containing vesicles utilize MF and MT to aggregate and fuse to form one mature inclusion. Upon maturation, the serovar L2 inclusion may transport along MT to the basolateral domain, where it leaves the host cell to infect cells under the epithelial layer. Our model of serovar E infection starts with entry at the apical surface with the help of MF. Serovar E-containing vesicles then aggregate and fuse without the use of the cytoskeleton. Because of this lack of association with MT, the mature serovar E inclusion remains in the apical domain, where it eventually escapes from the host cell to infect the apical surfaces of neighboring cells.

It has been proposed that the MT network functions as an anchoring point for the attachment of actin filaments (49). If MF integrity depends upon the MT network, MT disruption would lead to MF disruption. If this is so, serovar L2 may require only MF for inclusion fusion and MT disruption may indirectly lead to the formation of multiple inclusions per cell. Several reports have suggested that vesicles and organelles move bidirectionally on MT, detach, and then move along MF in a slow and unidirectional manner (28, 61). Also, in coelomocytes, the orientation of MF-dependent cortical flow has been shown to be MT dependent (10). From our results, we cannot determine whether serovar L2-containing vesicles directly use MT for inclusion fusion. Our examination of colchicine-exposed cells did not reveal any damage to MF. At the level of fluorescence microscopy, however, it may not be pos-

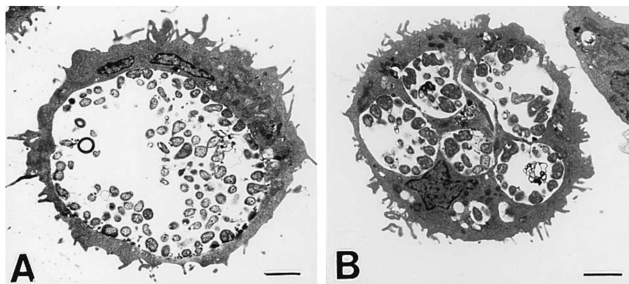

FIG. 7. Transmission electron microscopy reveals multiple *C. trachomatis* serovar L2 inclusions in polarized HEC-1B cells lacking polymerized MT. HEC-1B cells were untreated (A) or preexposed to 10 μM colchicine (B), infected with serovar L2, and processed for Epon-Araldite embedding at 24 h p.i. Bar = 1.7 μm.

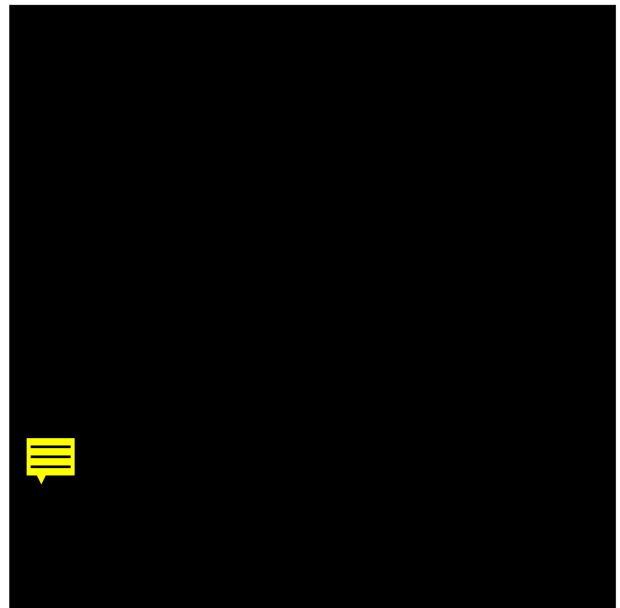

FIG. 8. The chlamydial inclusion and inclusion membrane do not contain sphingolipids of the *trans*-Golgi. The Golgi apparatus was stained with BODIPY FL C_5-DMB-ceramide in host cells infected with *C. trachomatis* serovar L2. HEC-1B (A to C) or McCoy (D to F) cells were fixed and stained 24 h p.i. Chlamydial inclusions (arrowhead) were examined with phase microscopy (A and D) and fluorescence microscopy using fluorescein filters (B and E) or rhodamine filters (C and F).

TABLE 1. Effect of host cell cytoskeletal disruption on *C. trachomatis* inclusion development

Serovar	Host cell	Inclusion morphology					
		MT disruption			MF disruption		
		Early[a]	Mid	Late	Early	Mid	Late
E	HEC-1B	Normal[b]	Normal	Normal	Normal	Normal[c]	Normal[c]
	McCoy	Normal	Normal	Normal	Less aggregated[d]	Normal[c]	Normal[c]
L2	HEC-1B	Less aggregated	Multiple[e]	Normal	Normal	Multiple	Normal
	McCoy	Less aggregated	Multiple	Normal	Less aggregated	Multiple	Normal

[a] Stage of developmental cycle.
[b] Chlamydial inclusions fused to form one large vesicle.
[c] Inclusion morphology was normal but there was a decrease in the number of infected cells.
[d] Endosomes containing chlamydiae were more dispersed in the host cell cytoplasm than in untreated, infected cells.
[e] Contained more than one inclusion per cell.

sible to detect subtle changes in the arrangement of the cytoskeleton.

In an attempt to show directly that serovar L2 inclusions traffic along MT, we labeled chlamydiae and MT with two distinct fluorescent antibodies within HEC-1B cells. Although we could identify inclusions in close proximity to MT, we could not determine if there was direct contact between the two structures at the level of fluorescence microscopy (data not shown). Future studies using high-voltage transmission electron microscopy (7) to examine the possible interaction of inclusions and MT within infected cells will help to more clearly define the mechanisms involved in chlamydial inclusion development.

The disruption of polymerized MF or MT, within cells infected with *C. trachomatis* serovar L2, inhibits inclusion fusion only early in the developmental cycle. Nocodazole removal at 4 h p.i. did not affect serovar L2 inclusion development, but its addition, as late as 12 h p.i. to infected HEC-1B cells, resulted in multiple inclusions per cell. This finding suggests that MT play a role in serovar L2 inclusion fusion a few hours after entry and through the first half of the developmental cycle. Late in the developmental cycle, chlamydial inclusions may grow large enough to collide randomly in the cytoplasm and can then fuse without the help of microtubules. Vesicle trafficking along MT is thought to be required for large distances within the cell, as in the movement of endosomes to lysosomes, and not for short movements, as from the endoplasmic reticulum to the Golgi (25). This would explain why serovar L2-containing endosomes require MT for aggregation early after entry but do not need them once the inclusions are large enough to be in close proximity.

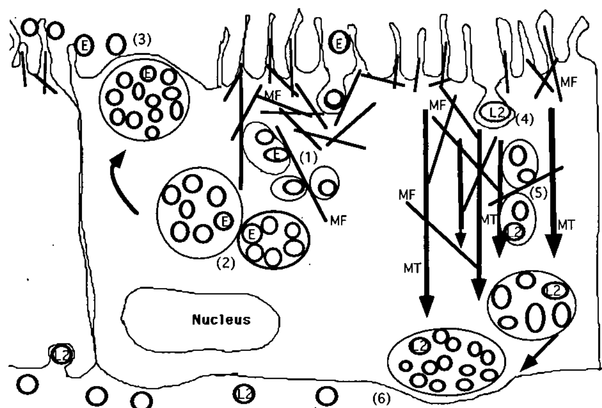

FIG. 9. A model for *C. trachomatis* infection of polarized epithelial cells. Serovar E utilizes MF during entry (1), fuses inclusions without the use of the host cell cytoskeleton (2), and is released from the apical surface (3), which allows rapid infection of the apical surfaces of neighboring cells. *C. trachomatis* serovar L2 enters cells without the use of the cytoskeleton (4) but utilizes MF and possibly MT for inclusion fusion (5). Mature serovar L2 EB are released from the basolateral cell surface to invade underlying tissues (6) and may then enter through basolateral as well as apical membranes of host cells.

It is clear that MT-depolymerizing drugs disrupt the structure of the Golgi, but it is unclear whether Golgi functions are affected. Several studies have shown that MT disruption causes a decrease in the rate of glycoprotein transport and secretion to the plasma membrane but does not affect glycoprotein synthesis and, hence, Golgi function (2, 3, 56). Other studies have shown that MT disruption has no effect on glycoprotein synthesis or transport to the cell surface (15, 54) but that the polarized expression of transported Golgi products is inhibited (45, 48). We localized Golgi vesicles within HEC-1B cells infected with chlamydiae. Golgi vesicles were found surrounding the inclusion but not incorporated into the inclusion membrane in amounts that we could detect by fluorescence microscopy. Because of technical limitations, however, we cannot rule out the possibility that Golgi vesicles are transported to and fuse with the growing inclusion to supply nutrients and membrane and that MT disruption inhibits this pathway, leading to a decrease in inclusion fusion. The appearance of Golgi vesicles ringing the inclusion may imply a need for Golgi components during chlamydial growth or may simply be the result of the growing inclusion pushing against cellular organelles in the nuclear hof.

Previously reported findings on the use of the host cell cytoskeleton by chlamydiae differ from those presented here. There are several parameters that differ between these examinations which may explain the discrepancies. (i) Experiments of chlamydial infection have been performed in many different cell lines. Not all cell types have the same amounts and distributions of filamentous actin. MT dynamics (52) and arrangement (58) have also been shown to be cell type specific. MT in the lamellae of epithelial cells are less dynamic than the majority found in fibroblasts because of a reduced rate of growth and depolymerization. Therefore, MT- and MF-disrupting drugs will affect various cells in different ways. (ii) The mode of chlamydial inoculation onto host monolayers affects the pathway of uptake. Centrifugation favors phagocytosis, while adsorption leads to pinocytic entry (43, 44). (iii) Drugs used to disrupt the cytoskeleton have different mechanisms of action. Vinblastine and vincristine disrupt the structure of preformed MT, giving rise to paracrystalline arrays (39), whereas colchicine alters the dynamic equilibrium between monomeric and polymeric tubulin (34). Several investigations of chlamydial entry into cells exposed to cytochalasin B did not find an

inhibition in infection. This may be explained by a study which revealed that cytochalasin B does not sever MF at the cell periphery as does CD (10). DMSO, at 5%, depolymerizes class I actin filaments at the plasma membrane (40, 61), and CD is routinely dissolved in DMSO. We did find a slight decrease in the infectivity of DMSO-exposed cells by serovar E (Fig. 1). (iv) Finally, temperature affects cell structure. MT are known to depolymerize at 4°C (61), and several investigators inoculate chlamydiae onto host cells at reduced temperatures.

The results from these experiments show that (i) *C. trachomatis* serovars E and L2 utilize different pathways of infection, (ii) the cytoskeleton plays a unique role in the infection of host cells by serovars E and L2, and (iii) the differences are consistent in both epithelial cells and fibroblasts. Further investigation of the involvement of the chlamydial inclusion with the host cell cytoskeleton is needed to understand the intracellular survival of this complex pathogen.

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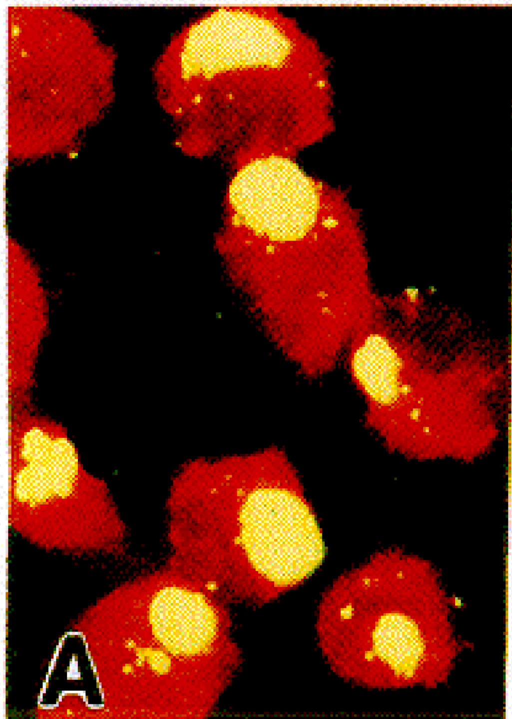
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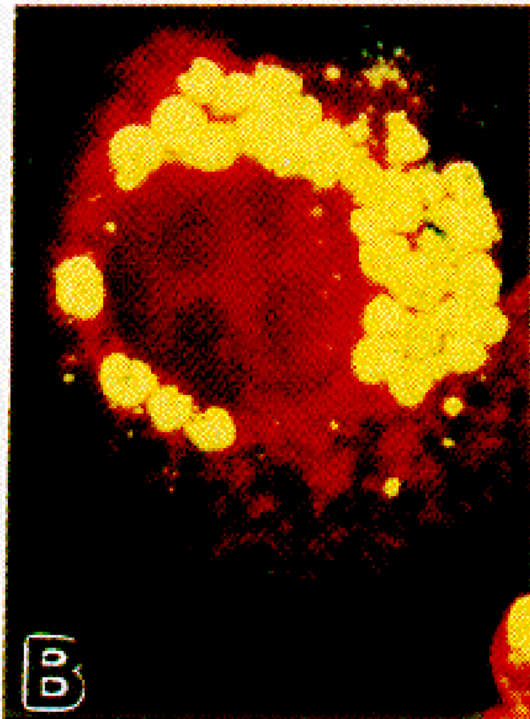
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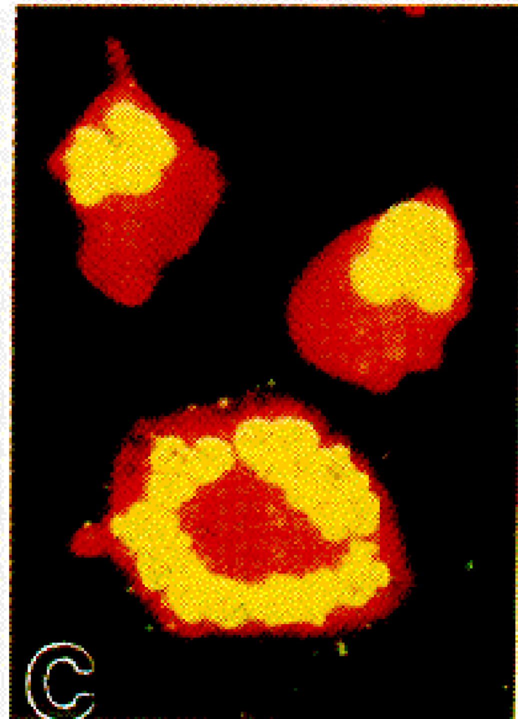
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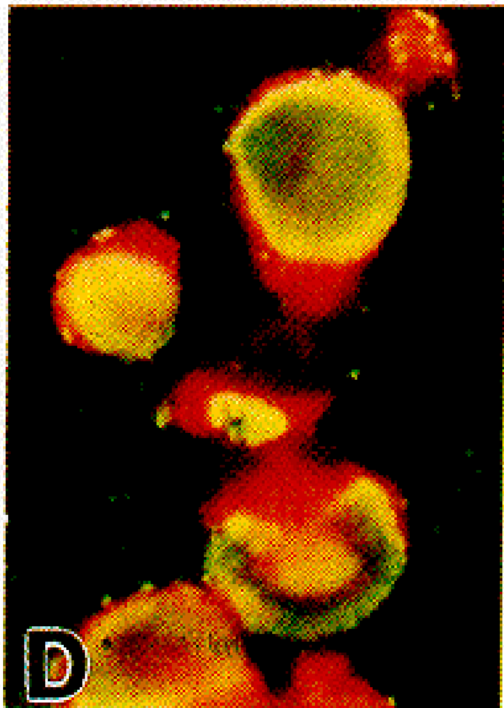
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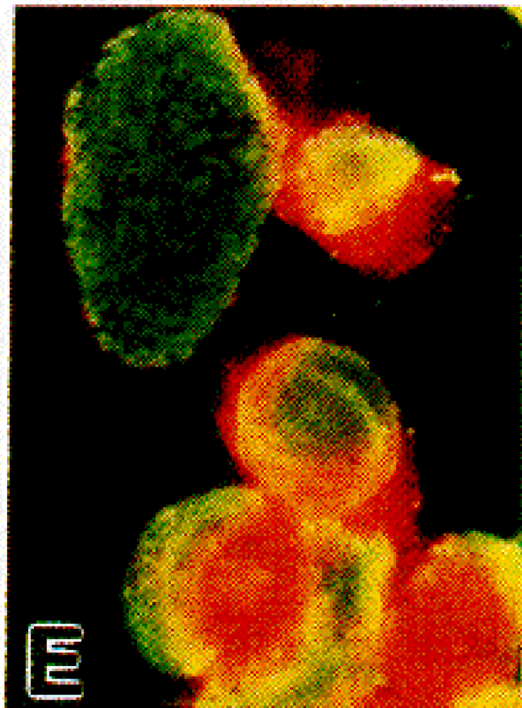
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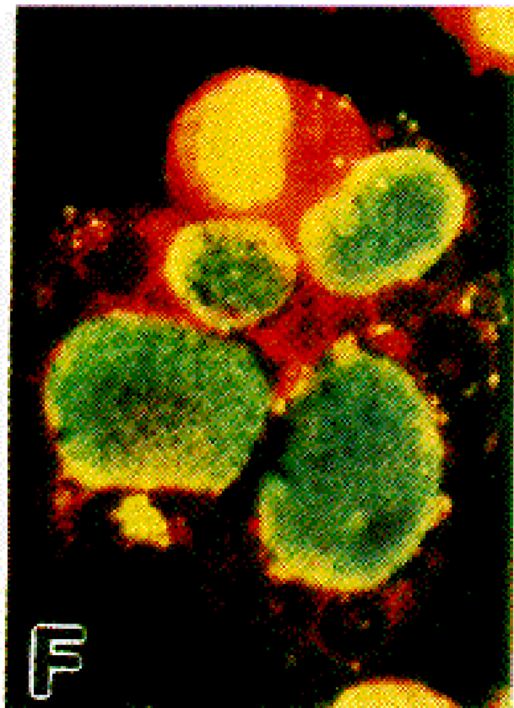
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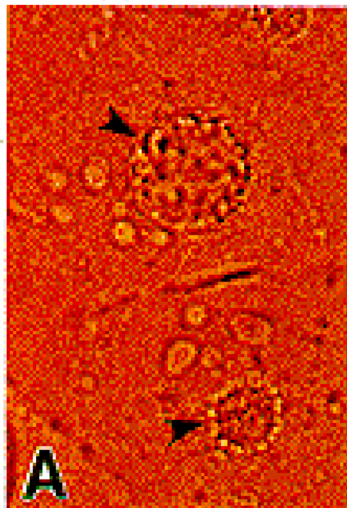
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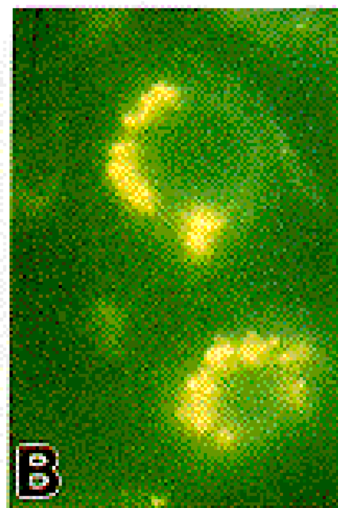
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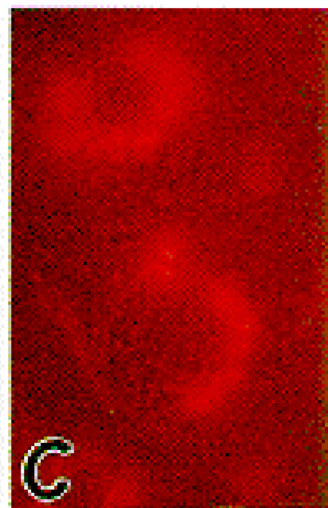
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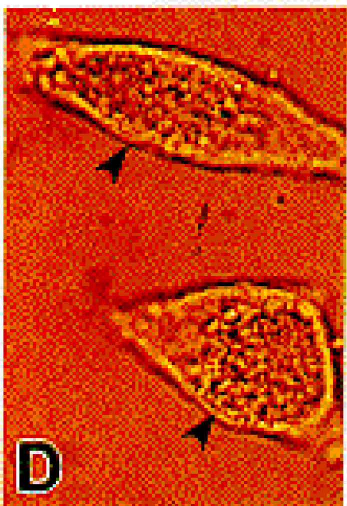
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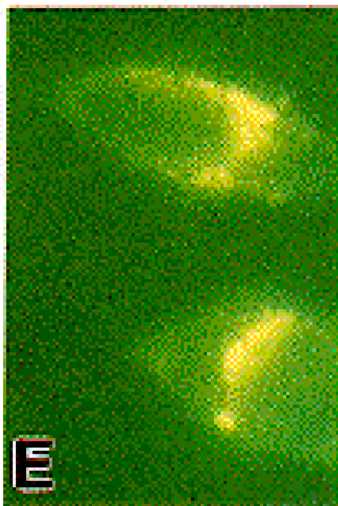
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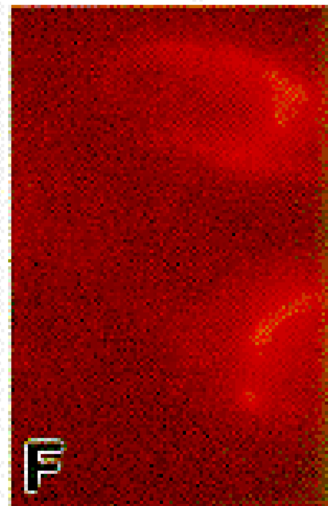
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