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Entry of Genital *Chlamydia trachomatis* into Polarized Human Epithelial Cells

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To study the initial invasion process(es) of genital chlamydiae, a model system consisting of hormonally maintained primary cultures of human endometrial gland epithelial cells (HEGEC), grown in a polarized orientation on collagen-coated filters, was utilized. After *Chlamydia trachomatis* inoculation of the apical surface of polarized HEGEC, chlamydiae were readily visualized, by transmission electron microscopy, in coated pits and coated vesicles. This was true for HEGEC maintained in physiologic concentrations of estrogen (proliferative phase) and of estrogen plus progesterone (secretory phase), despite the finding that association of chlamydiae with secretory-phase HEGEC is significantly reduced ($P = 0.025$; A. S. Maslow, C. H. Davis, J. Choong, and P. B. Wyrick, Am. J. Obstet. Gynecol. 159:1006–1014, 1988). In contrast, chlamydiae were rarely observed in the clathrin-associated structures if the HEGEC were cultured on plastic surfaces. The same pattern of coated pit versus noncoated pit entry was reproducible in HeLa cells. The quantity of coated pits associated with isolated membrane sheets derived from HeLa cells, grown on poly-L-lysine-coated cover slips in medium containing the female hormones, was not significantly different as monitored by radiolabeling studies and by laser scanning microscopy. These data suggest that culture conditions which mimic in vivo cellular organization may enhance entry into coated pits for some obligate intracellular pathogens.

Simply stated, our understanding of how *Chlamydia* spp. enter tissue culture cells is in a state of confusion. Several of the proposed pathways are summarized in Table 1. There are a number of explanations which may account for the various perspectives in different laboratories regarding the method(s) of entry of chlamydiae into nonprofessionally phagocytic cells. We shall focus on two areas, terminology and technology.

There are essentially four defined mechanisms for entry of particles into eucaryotic cells (for recent reviews, see references 2, 9, and 30): (i) fluid-phase pinocytosis; (ii) nonspecific adsorptive pinocytosis or high-affinity adsorptive, nonreceptor-mediated endocytosis; (iii) phagocytosis; and (iv) specific absorptive pinocytosis or receptor-mediated endocytosis. As more information emerges on the functional role of clathrin, it appears that the latter endocytic pathway may be subdivided into clathrin-mediated internalization and noncoated-membrane uptake. The difference may hinge on the role of clathrin in facilitating the concentration of receptors in coated pits and increasing the efficiency of their delivery to the endosomal sorting pathway (4) as opposed to the entry of toxins (26, 32), for which no facilitated uptake would be expected.

Phagocytosis and endocytosis have been primarily implicated in the entry of chlamydiae into nonprofessionally phagocytic target cells. Part of the confusion is whether there are variations in these definitions, i.e., can an epithelial cell, which is devoid of Fc receptors, phagocytize? Are bacteria in vivo always opsonized? Are microfilaments engaged in the entry of bacteria into epithelial cells? A recent study by Clerc and Sansonetti (6) is an example in point. They show very convincingly that entry of *Shigella flexneri* into HeLa cells both is parasite directed and involves actin polymerization and myosin accumulation at the sites of penetration, namely, parasite-directed phagocytosis in an epitheliallike cell. Obviously, as more biochemical and cytoskeletal data are generated in relevant model systems along with appropriately constructed bacterial mutants (8, 15, 28), we shall be able to understand how bacteria enter target host cells, which is the important part; then perhaps we can agree on terminology.

The purpose of this study was to examine the entry of a genital strain of *Chlamydia trachomatis* into primary cultures of human endometrial gland epithelial cells (HEGEC). By utilizing a model system more relevant to the natural infectious process in vivo, we hoped to have a more clear understanding of the initial chlamydial invasion process(es). In addition, the data generated from these studies have provided pertinent information, especially of a technological nature, for resolving some of the controversy surrounding the entry of chlamydiae in laboratory-adapted HeLa cell lines.

MATERIALS AND METHODS

Growth, purification, and titration of chlamydiae. A human urogenital isolate of *C. trachomatis* E/UW-5/CX, obtained from C. C. Kuo and S. P. Wang, University of Washington, Seattle, was grown in McCoy cells, purified, and titrated as previously described (22). Another human biovar of *C. trachomatis*, biovar lymphogranuloma venereum (LGV) L2/434/Bu, was prepared by the same methods.

Human epithelial cell cultures. Isolation of HEGEC and their cultivation on plastic surfaces has been documented in detail by Moorman et al. (22). Two major modifications of the HEGEC culture technique involved the maintenance of cultures in hormone-supplemented medium in accordance with the menstrual cycle of the patient at the time of tissue collection (19) and the use of polycarbonate Transwell filters

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TABLE 1. Reported modes of entry of chlamydiae in tissue culture cell lines

Terminology and reason	Strain and cells	Reference
Parasite-specified phagocytosis More efficient entry than latex beads or *Escherichia coli*	*C. psittaci* 6BC, L cells LGV, HeLa cells	Byrne and Moulder (5)
Microfilament-independent phagocytosis Cytochalasin B did not inhibit entry	*C. psittaci* 6BC, L cells	Gregory et al. (10)
Receptor-mediated endocytosis Methylamine and monodansylcadaverine did inhibit entry	*C. trachomatis* (serovar E), McCoy	Soderlund and Kihlstrom (29)
Microfilament-dependent phagocytosis Cytochalasin D did inhibit entry Amantadine and monodanyslcadaverine did not inhibit entry No coated pits visualized by TEM	LGV, HeLa cells	Ward and Murray (33)
Receptor-mediated endocytosis Ultrastructural; coated pits, coated vesicles, curls	*C. psittaci* Cal10, L cells *C. trachomatis* (serovar E), McCoy	Hodinka and Wyrick (14) Hodinka et al. (13)
Phagocytosis and pinocytosis Ultrastructural Pinocytosis-no centrifugation Phagocytosis-centrifugation	*C. psittaci* GPIC, McCoy	Pearce (23)
Endocytosis Ultrastructural Pointed tip contact EB outer membrane rearrangement Multiple anchor sites	*Chlamydia* TWAR, HeLa	Kuo et al. (18)

(3.0-μm pore size, 6.5-mm diameter; Costar, Cambridge, Mass.) to obtain polarized monolayers. The circular filter is attached to the bottom of a plastic support, which is suspended from the top of the well of a 24-well cluster plate (Costar). This construction raises the filter 1 mm above the bottom of the plastic tissue culture well. The filters were precoated with 50 μl of a mixture of collagen types IV and X from human placenta (Sigma Chemical Co., St. Louis, Mo.) at a concentration of 0.15 mg/ml in sterile deionized water and allowed to dry at room temperature under a UV lamp inside a laminar flow hood. Before the addition of the filter inserts, each well of a 24-well culture plate received 600 μl of Eagle minimum essential medium (Eagle MEM) with D-valine substituted for L-valine (GIBCO Laboratories, Grand Island, N.Y.) containing 12% fetal bovine serum (Hyclone), 10 mM glutamine, 100 μg of streptomycin per ml, 100 μg of kanamycin per ml, 40 μg of insulin per ml, and 17β-estradiol (10^{-10} mol/liter) (Sigma) or 17β-estradiol and 10 ng of 4-pregnene-3,20-dione (Sigma) per ml. Each filter chamber was seeded with 20 to 30 glands in 100 μl of the appropriate medium. Transparent Transwell-COL (Costar) filters were set up at the same time to monitor cell growth and monolayer confluency by phase-contrast microscopy. Outgrowth of the epithelial cells from the endometrial glands results in a whirl-like pattern such that there are always gaps in the monolayer. This precludes obtaining an electrical potential that would approach 1,000 Ω/cm^2. Before chlamydial infection of the endometrial epithelial cell monolayers, the medium was changed to Dulbecco modified Eagle MEM (with L-valine) which contained the same additives and appropriate hormone supplements as did Eagle MEM.

For those experiments which involved HeLa cells, filters were collagen coated as described above and seeded with approximately 10^5 cells per insert. Dulbecco modified Eagle MEM with appropriate supplements was used exclusively.

Chlamydial attachment. Confluent monolayers on collagen-coated filters or subconfluent monolayers on plastic surfaces were inoculated with 50 or 100 μl, respectively, of a purified preparation of *C. trachomatis* serovar E elementary bodies (EB) suspended in sucrose-phosphate-glutamine buffer and diluted in Dulbecco modified Eagle MEM to multiplicities of infection (MOI) ranging from 500:1 to 2,000:1 particles per cell. During the subsequent 2-h adsorption period at 35°C, the culture plates were shaken intermittently by hand. The infected monolayers were washed three times with phosphate-buffered saline to remove the nonadherent chlamydiae and then processed for fluorescence and transmission electron microscopy (TEM).

For quantitation of chlamydiae bound by HeLa cells or HeLa plasma membrane sheets (see below), 100 μl of purified EB, at a MOI of 500:1, was added to cover slip cultures in 24-well dishes and incubated at 35°C for the 2-h adsorption period. After removal of the nonadherent chlamydiae by washing, infected whole cells were fixed with methanol and stained with fluorescein isothiocyanate (FITC)-labeled anti-*C. trachomatis* monoclonal antibody diluted in Evans blue counterstain (Syva Microtrak, Palo Alto, Calif.). The number of fluorescent chlamydiae associated per HeLa cell on a total of 50 HeLa cells was counted, and the mean was plotted. Formaldehyde (3%)-fixed isolated HeLa cell membrane sheets were doubly stained with the FITC-labeled anti-*C. trachomatis* monoclonal antibody and R-phycoerythrin-streptavidin (Molecular Probes, Eugene, Oreg.)-amplified, biotin-conjugated second-affinity antibody (Sigma) directed against monoclonal antibody to the clathrin heavy chain (see below). When viewed in the fluorescence

microscope at 490 nm, the green chlamydiae are easily visualized against the orange highlighted membrane sheets (see Fig. 6B). The mean number of chlamydiae per membrane sheet in a total of 50 sheets was plotted.

According to our previous studies on the kinetics of binding of *C. trachomatis* serovar E EB to McCoy cells at 37°C, only 10% of the input chlamydial population had bound by 2 h (13). Thus at MOIs of 500:1 and 2,000:1, we could predict a binding of approximately 50 and 200 particles, respectively. Assuming that one of every three particles is infectious, there would be about 70 inclusion-forming units per cell for TEM studies and approximately 20 inclusion-forming units per cell for fluorescence microscope studies, both of which are well below immediate cytotoxicity for *C. trachomatis*.

TEM. Monolayers of chlamydial-infected HEGEC or HeLa cells were washed twice in phosphate-buffered saline after the 2-h adsorption or uptake period and processed for TEM by a slight modification of the procedure of Hodinka et al. (13). Briefly, the infected monolayers were fixed in situ with 2% glutaraldehyde–0.5% paraformaldehyde in 0.1 M cacodylate buffer for 45 min at 37°C. The filters were cut from their plastic supports and enrobed by dipping in 2% molten Noble agar. Cells grown on plastic were scraped with a rubber policeman and pelleted in 2% agar. After two washes with 0.1 M sodium cacodylate buffer, the samples were postfixed with 1% osmium tetroxide prepared in cacodylate buffer for 1 h at room temperature and washed again with 0.1 M sodium cacodylate buffer. After two brief washes with 70% ethanol, the samples were exposed to 5% uranyl acetate in 70% ethanol for 10 min to enhance contrast, followed by dehydration in 95 and 100% ethanol and finally propylene oxide. Infiltration and curing in Epon-Araldite were as described by Hodinka et al. (13). Gold thin sections were cut with a diamond knife on a ultramicrotome (Reichart Scientific Instruments, Southbridge, Mass.), counterstained with uranyl acetate followed by lead citrate, and examined in a Philips 201 electron microscope operating at 60 kV.

Quantitation of coated pits. Unstripped and stripped HeLa cell membrane sheets, maximized for coated pit formation, were prepared by the procedure of Moore et al. (21). Briefly, HeLa cells on poly-L-lysine-coated cover slips were grown to subconfluency in medium with or without added hormones. The cultures were then placed at 4°C for 1 h to maximize coated pit formation at the membrane. After being sonicated for 1 s and extensively washed with buffer containing several protease inhibitors, the resulting adherent membrane sheets were probed with various antibodies. As a control, triplicate cover slips of membrane sheets were exposed to high pH buffer under conditions known to reduce the number of coated pits associated with the membrane (stripped).

For radioisotopic labeling, the Formalin-fixed membrane sheets in poly-L-lysine-coated Removawells (Dynatech Laboratories, Inc., Chantilly, Va.), were exposed to monoclonal anticlathrin heavy-chain antibody (immunoglobulin M, clone CHC 5.9; ICN Immunobiologicals, Lisle, Ill.) followed by biotin-conjugated goat anti-mouse immunoglobulin M (Sigma) and ^{125}I-labeled streptavidin, 50 μl per well of a concentration of 1 μCi/ml (Amersham Corp., Arlington Heights, Ill.), and counted for 1 min in a Minaxi γ autogamma counter 5000 series (Packard Instrument Co., Inc., Sterling, Va.).

For laser scanning microscopy, the unstripped and stripped membrane sheets on poly-L-lysine-coated cover slips in 1-dram (3.7-ml) vials were stained with the same anticlathrin heavy-chain monoclonal (immunoglobulin M) antibody, followed by second-affinity biotinylated antibody and FITC-labeled streptavidin. A Zeiss confocal microscope with argon laser (488 nm) and long pass filter (525 nm full width half maximum) was used. Appropriate brightness and contrast settings were established on test membranes and remained fixed throughout image acquisition. Digital images of membrane sheets were obtained by using three passes of the laser over previously unexposed areas. Focus was achieved on the first two passes and image capture was achieved on the third pass. Images were transferred to an IP-512 image processing system (Imaging Technology, Woburn, Mass.) in which average pixel values for the center two-thirds of each image were calculated.

Immunoblot analysis. Membranes were isolated from HeLa cells cultured in 150-cm^2 flasks in phenol red. After Dounce homogenization and Percoll density gradient centrifugation at 30,000 × *g* for 30 min, membranes were washed two times and pelleted at 100,000 × *g* for 1 h. Membranes were resolved on a 6 to 18% polyacrylamide gel and transferred to nitrocellulose as previously described (13). Membranes were isolated from McCoy cells in the same manner for use as a control for the presence of clathrin.

Clathrin was detected with either polyclonal or monoclonal anticlathrin heavy-chain antibody followed by biotinylated secondary antibody as described previously, except that alkaline phosphatase-conjugated streptavidin was used.

Statistics. The significance of the differences for (i) quantitation of clathrin basket assembly on plasma membrane sheets derived from HeLa cells exposed to different hormonal conditions and for (ii) attachment of chlamydiae to HeLa membrane sheets or whole HeLa cells grown in medium with and without various hormones was determined by analysis of variance. The hormonal conditions were also compared with other factors present within each experiment. Results for which P was ≤ 0.05 were defined as statistically significant.

RESULTS

The plasma membrane of epithelial cells in vivo exhibits polarity, i.e., the apical surface faces the lumen and the basolateral surface faces the serosal side (27). The separation of these two domains is maintained by tight junctions and is essential for transport function. In conventional tissue culture plastic vessels, the basolateral surface of primary cultured epithelial cells is attached to the petri dish and the apical surface faces the medium. As the cells become confluent and tight junctions form, the basolateral surface becomes sealed and isolated from the culture medium. Since the basolateral surface in epithelial cells is the nutrient surface as well as the surface that receives hormonal and other signals from the host, the cells become nutrient restricted and lose their hormone responsiveness as well as other differential functional properties, such as motility of cilia. Greater differentiation of epithelial cells can be achieved by growing the primary cultures on porous surfaces (11). Figure 1A is a representative transmission electron photomicrograph of our polarized primary HEGEC grown on collagen-coated polycarbonate filters (3.0-μm pore size; Transwell [Costar]). Tight junctions, desmosomes (insert), secretory granules, and cilia (arrowheads) can be seen.

Entry of *C. trachomatis* into polarized HEGEC. Examination by TEM of HEGEC monolayers, grown on plastic surfaces, 2 h postinoculation with *C. trachomatis* revealed EB associated with the epithelial cell surface at the bases of

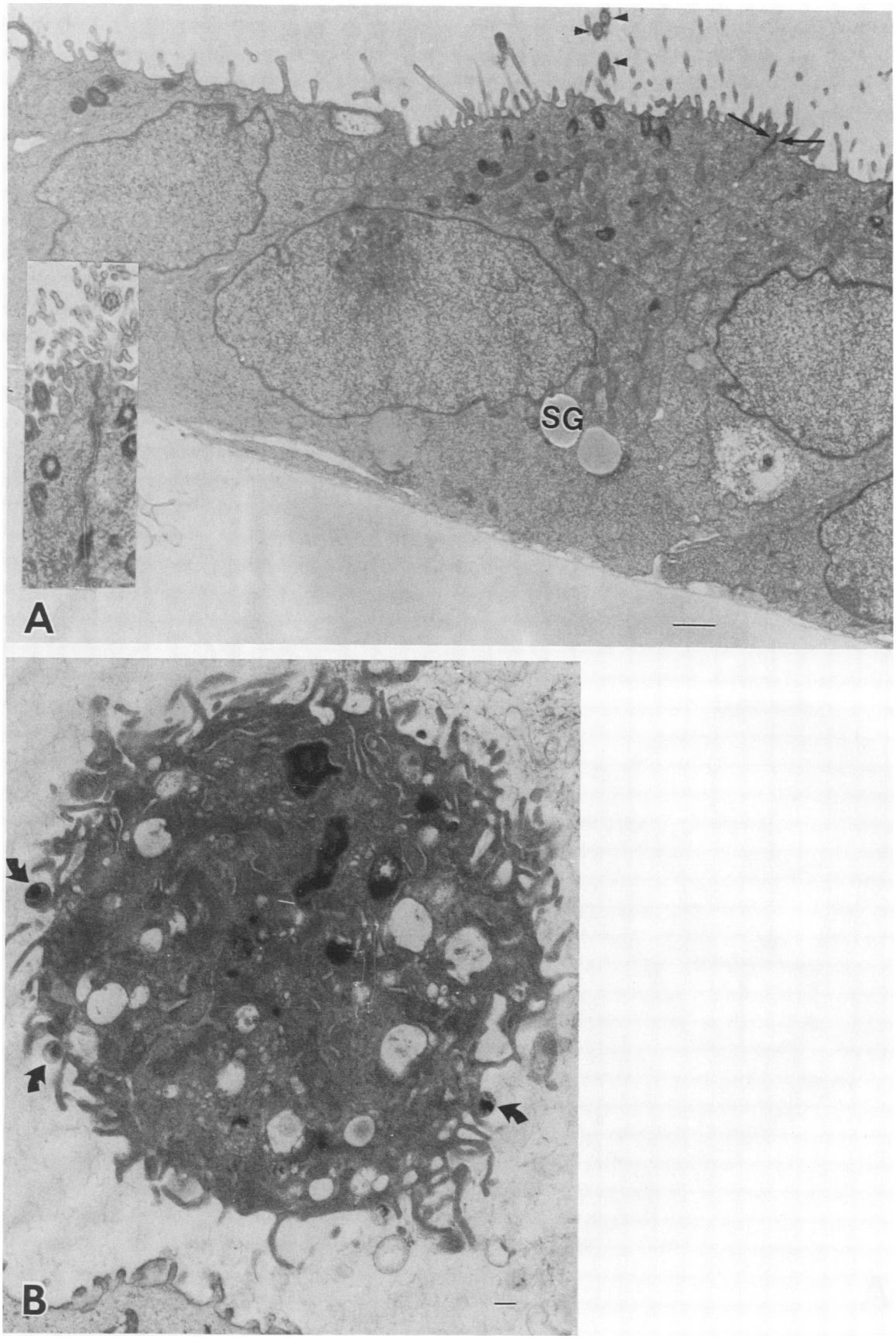

FIG. 1. Transmission electron photomicrograph representing the ultrastructure of HEGEC. (A) HEGEC grown in a polarized orientation on a collagen-coated polycarbonate filter. Tight junctions (arrows), desmosomes (insert), secretory granules (SG), and cilia (arrowheads) can be seen. (B) HEGEC grown on a plastic surface. At 2 h postinoculation with *C. trachomatis* EB (arrows) associated with microvilli and at the epithelial cell surface were seen. Bar = 0.1 μm.

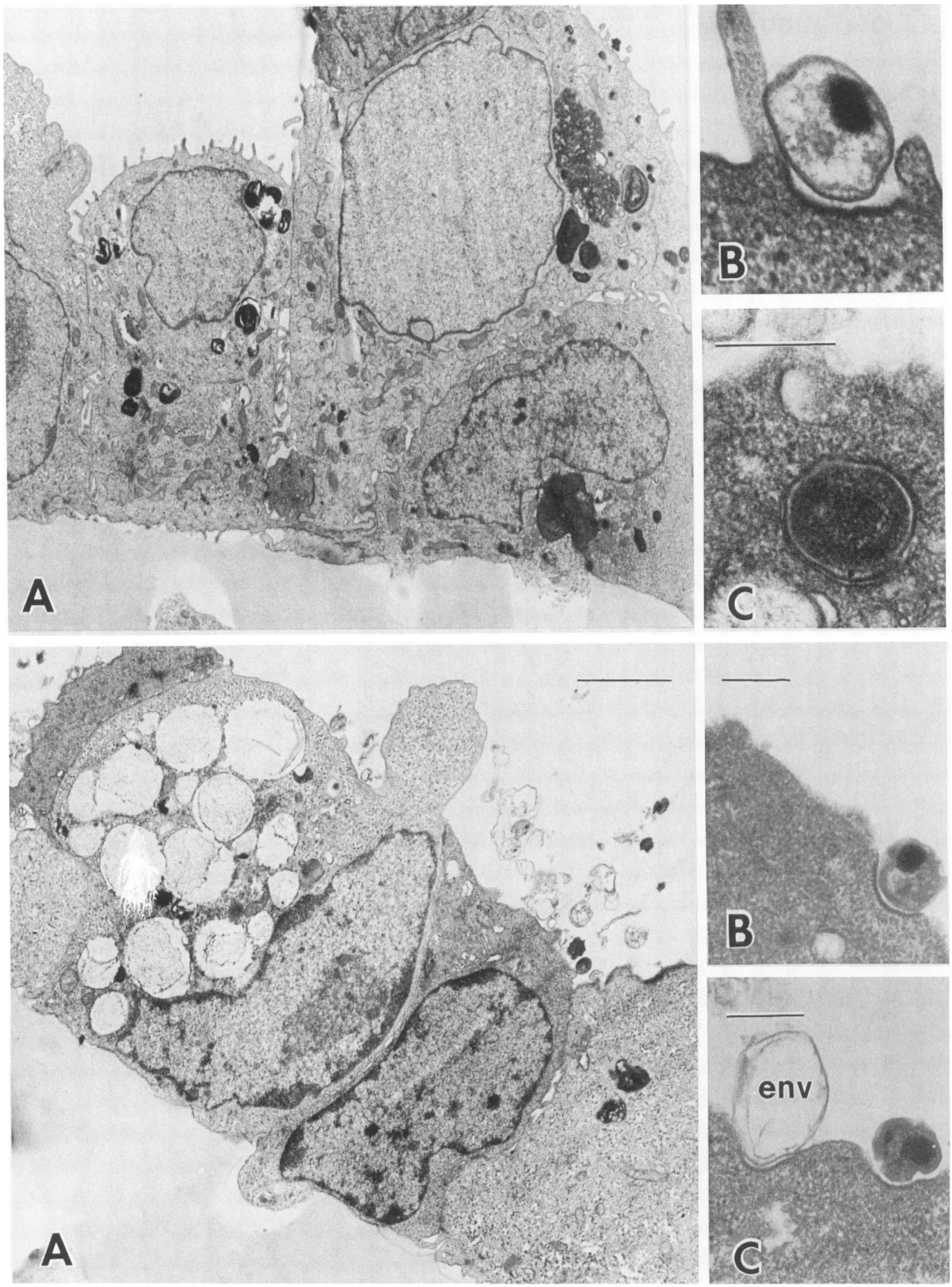

FIG. 2 and 3. Transmission electron photomicrographs of chlamydia-infected polarized HEGEC.

FIG. 2. HEGEC maintained in exogenously added estrogen. (A) Representative monolayer. Bar = 0.5 μm. EB localized in a coated pit (B) and in a coated vesicle at the apical surface (C). For panels B and C, bar = 0.1 μm.

FIG. 3. HEGEC maintained in exogenously added estrogen plus progesterone. (A) Representative monolayer. Bar = 0.5 μm. (B and C) EB and envelope (env) localized in coated pits at the apical surface. Bar = 0.1 μm.

microvilli (Fig. 1B) and internalized in vesicles. Structures resembling coated pits and coated vesicles were not visualized. However, when the HEGEC were polarized, EB inoculated on the apical side were readily visualized in coated pits and coated vesicles (Fig. 2 and 3).

Previous studies in our laboratory have demonstrated that the association of genital *C. trachomatis* with HEGEC varies considerably, depending on whether the HEGEC are in the proliferative (estrogen-dominant) or secretory (progesterone-dominant) phase (19). Rhodamine-labeled EB were adsorbed for 2 h to HEGEC, maintained in medium supplemented with physiologic amounts of either estrogen or estrogen in combination with progesterone. The number of chlamydiae bound to the epithelial cells was analyzed in the fluorescence-activated cell sorter. A summary of the percent binding of *C. trachomatis* EB to the HEGEC, exposed to the different hormonal regimens was as follows: 40 to 50% (no hormone supplement); >80% (10^{-10} M estrogen; $P = 0.005$); 50, 30, and 18% (1, 5, and 10 ng of progesterone per ml, respectively; $P = 0.025$). Figure 2A illustrates chlamydia-infected polarized HEGEC maintained in exogenously added estrogen; at a higher magnification, a representative EB in a coated pit (Fig. 2B) and a representative EB in a coated vesicle (Fig. 2C) are shown. Similarly, Fig. 3 illustrates chlamydia-infected polarized HEGEC maintained in exogenously added estrogen plus 10 ng of progesterone; at a higher magnification, representative EB and a chlamydial envelope in coated pits (Fig. 3B and C) are shown. Despite the noticeable reduction of microvilli on HEGEC in the progesterone-dominant phase, as well as the reduction in EB attached to the apical surface, there was no difficulty finding EB in coated pits.

Growth of HeLa cells in a polarized fashion. Even though HeLa cells are transformed and thus no longer subject to contact inhibition for maintaining a polarized monolayer, we decided to examine the growth of chlamydia-infected HeLa cells on the collagen-coated filters on the assumption that receipt of nutrients and other signals at the basolateral surface was also important for transformed cells. Indeed, the only difficulty encountered was catching the HeLa cells at the polarized monolayer stage, because the cells did eventually pile on top of one another, as they also did when grown on plastic. The infectivity titers of purified *C. trachomatis* serovar E EB after two passages in HeLa cells grown on plastic cover slips were compared with those after two passages in HeLa cells grown on filter inserts. In essence, the infectivity titer of the EB harvested continually from HeLa cells grown on the permeable substratum was 55.8% greater than the infectivity titer of the EB harvested from the HeLa cells grown on plastic (data not shown). TEM of mature chlamydial inclusions in the polarized HeLa cells revealed an impressive number of EB (Fig. 4A). Usually, a significant number of reticulate and intermediate bodies are visible even in late inclusions.

As a second parameter, the polarity of the infectious process was tested with another genital biovar of *C. trachomatis*, biovar lymphogranuloma venereum (LGV-2) which characteristically invades the lymphatic tissue in infected individuals. Infectious EB were added to the apical surface chamber of polarized cells. By 48 h postinfection, some inclusions had ruptured, releasing the LGV progeny from the basolateral surface (Fig. 4B). In contrast, release of progeny of *C. trachomatis* serovar E (a lumenal pathogen) from the basolateral surface has never been observed despite examination of many polarized infected cells from 72 to 120 h.

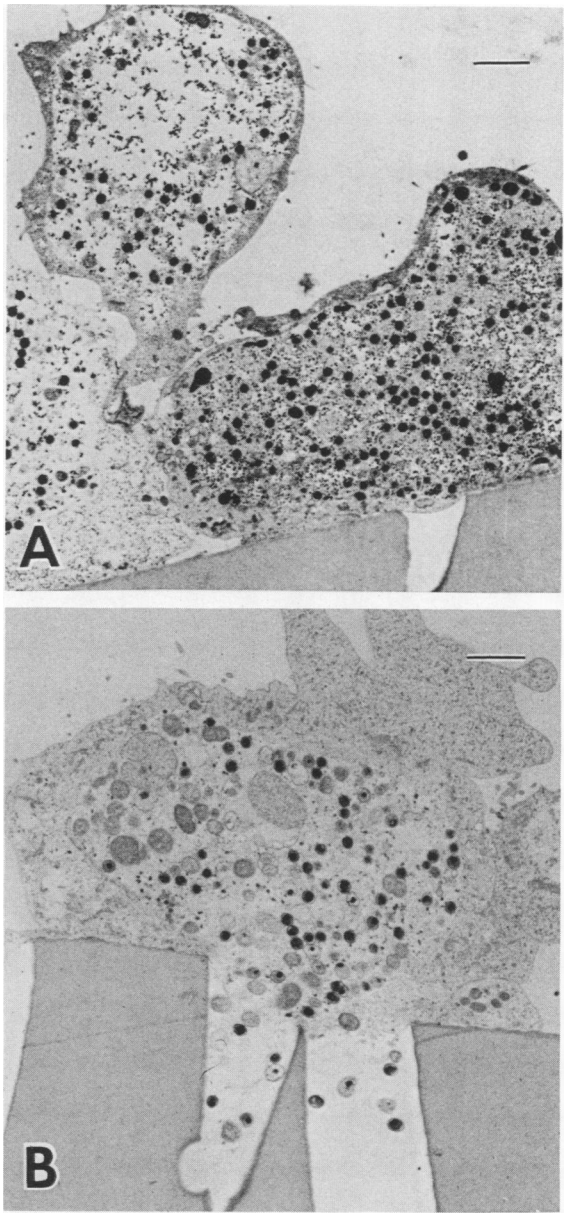

FIG. 4. Transmission electron micrographs depicting late-stage *C. trachomatis* infection in HeLa cells grown on collagen-coated polycarbonate filters. (A) Numerous EB visible in the glycogen-containing (granular matrix) inclusion. (B) Release of LGV progeny at the ruptured basolateral surface. Bar = 0.5 μm.

Entry of *C. trachomatis* in polarized HeLa cells. The importance of growing HeLa cells in a polarized fashion was further demonstrated when we examined the morphological features of the entry process of *C. trachomatis* by TEM. By using the standard protocol for chlamydial inoculation of HeLa cell monolayers grown on plastic surfaces, EB were found associated with smooth areas of the HeLa cell surface, in indentations and invaginations, and internalized in membrane-bound vesicles. Coated pits and coated vesicles were rarely observed. This was in striking contrast to the features of the entry process in polarized HeLa cells in which EB were often localized in coated pits. Figure 5A and B are representative electron photomicrographs of the type of polarized monolayers that can be obtained with HeLa cells

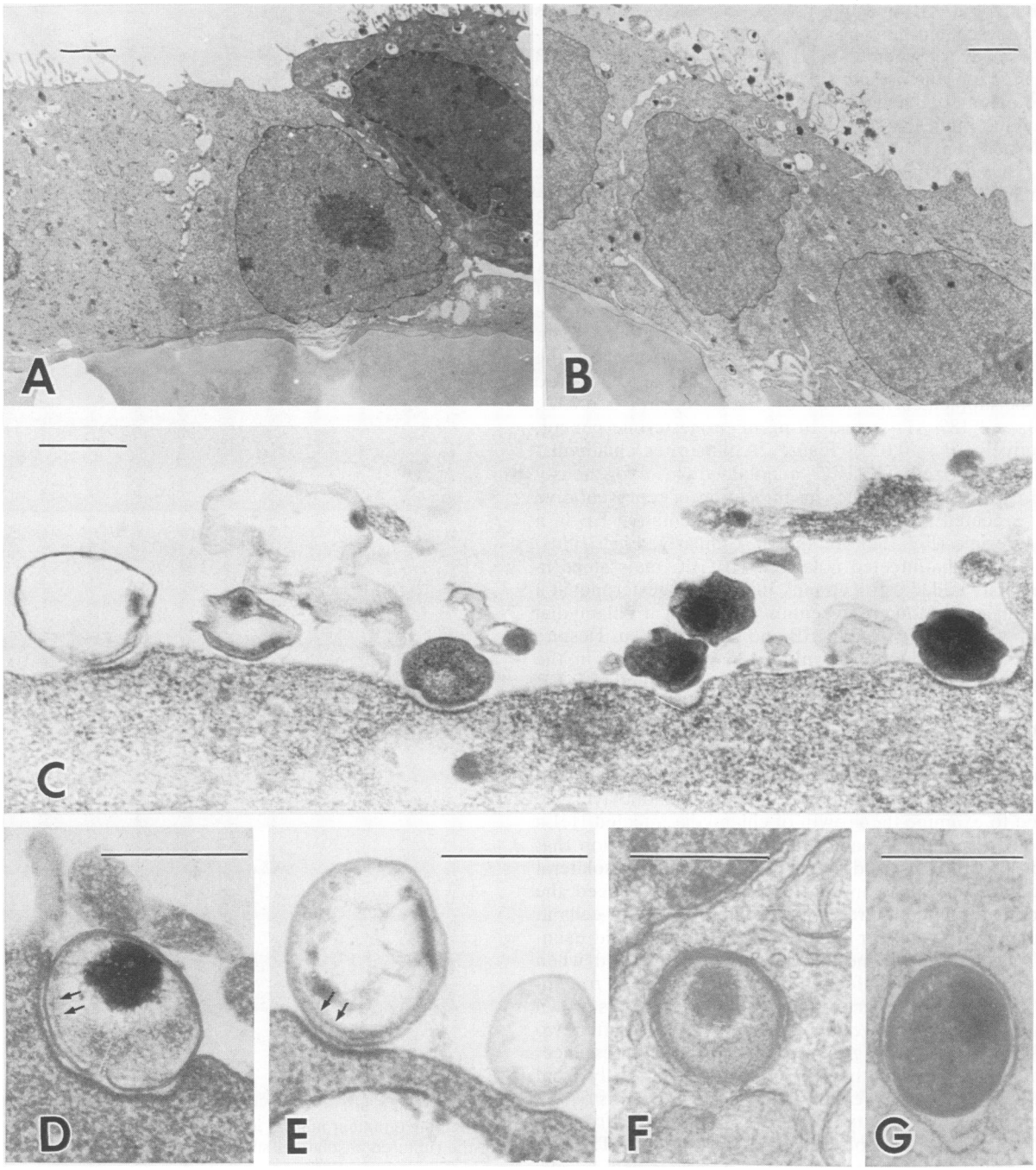

FIG. 5. Transmission electron photomicrographs of *C. trachomatis*-infected HeLa cells grown on collagen-coated polycarbonate filters. (A and B) Representative polarized monolayers. Bar = 0.5 μm. Panel C illustrates the frequency with which EB and envelope structures can be visualized in coated pits at the apical surface of a single polarized HeLa cell. Bar = 0.1 μm. (D and E) At a higher magnification, the hairlike structures of the chlamydial projections (arrows), oriented toward the coated pit, can be visualized. Bar = 0.1 μm. Representative examples of an EB in a coated vesicle (F) and in an endosome (G). Bar = 0.1 μm.

grown on the collagen-coated filters. Figure 5C illustrates the frequency with which chlamydial EB and envelopelike structures can be found in coated pits along the apical surface of a single polarized cell. At a higher magnification, the hairlike structures of the chlamydial projections (arrows; 20), oriented toward the coated pit, can be visualized (Fig. 5D and E). Examples of an EB in a coated vesicle and in a smooth endosome are shown in Fig. 5F and G, respectively.

Chlamydial association with HeLa cells exposed to female hormones. Despite the fact that HeLa cells do not possess estrogen (17) or progesterone receptors, several investigators (3, 31) have reported that preexposure of HeLa cells to estrogen enhances chlamydial binding and infection, measured by increased inclusion counts. Both activities were reduced by the antiestrogen tamoxifen. Since strains of HeLa cells can behave in different ways and exhibit distinct characteristics, we examined, by several methods, the binding of our strain of *C. trachomatis* serovar E to HeLa cells

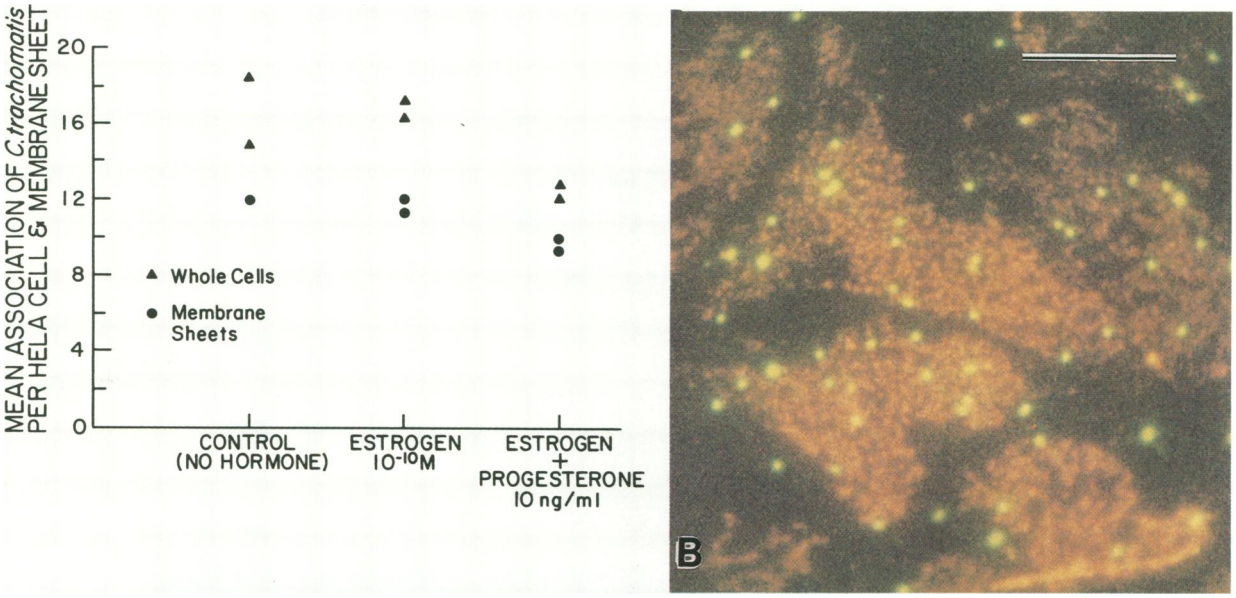

FIG. 6. *C. trachomatis* bound to HeLa membranes. (A) The mean number of fluorescent (FITC), labeled chlamydiae associated per HeLa cell in a total of 50 Evans blue counterstained whole cells or R-phycoerythrin-conjugated anticlathrin-labeled HeLa membrane sheets on poly-L-lysine-coated cover slips. Each point is representative of one experiment, performed with duplicate cover slips. (B) A representative fluorescence photomicrograph of Formalin-fixed HeLa cell membrane sheets doubly stained with FITC-labeled anti-*C. trachomatis* monoclonal antibody and R-phycoerythrin-streptavidin-amplified, biotin-conjugated second-affinity antibody directed against monoclonal antibody to the clathrin heavy chain. Bar = 4 μm.

grown on plastic surfaces and exposed to estrogen (10^{-10} M) and estrogen plus progesterone (10 ng/ml). In the first method, the number of chlamydiae intimately associated with microvilli and the surface of HeLa cells was quantitated by TEM. A total of 500 to 800 HeLa cells were counted per sample, each performed in quadruplicate. The values of the percent binding of chlamydiae by HeLa cells in one (of two) representative experiments were 50% for control HeLa cells (no hormone), 56.6% for HeLa cells grown in estrogen-supplemented medium, and 25.6% for HeLa cells grown in medium supplemented with 10^{-10} M estrogen plus 10 ng of progesterone per ml (data not shown).

In the second method, the number of FITC-labeled EB bound to HeLa cells or to isolated membrane sheets derived from HeLa cells (see below) was determined by fluorescence microscopy (Fig. 6). In both instances, preexposure of HeLa cells to estrogen did not alter the number of chlamydiae bound. However, there was again a reproducible and statistically significant reduction ($P = 0.002$) in the binding of EB to isolated HeLa cell membrane sheets and HeLa cells cultured in medium supplemented with estrogen plus progesterone (Fig. 6A).

A recent study by Berthois et al. (1) reported that phenol red, used as a pH indicator in tissue culture media, bears a structural resemblance to some nonsteroidal estrogens. At the concentrations found in tissue culture media, phenol red causes significant stimulation of cell proliferation and specific protein synthesis in estrogen-responsive cells. Comparison of the protein profiles of our hormone-exposed HeLa cells, grown in medium with and without phenol red, revealed no differences in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis band patterns (Fig. 7A). It thus appears, at least from the limited studies performed here, that the physiology of the strain of HeLa cell used in our laboratory is not altered on exogenous exposure to estrogen (10^{-10} or 10^{-8} M) in regard to binding by *C. trachomatis* serovar E.

To determine whether the reduced association of chlamydiae with HeLa cells exposed to progesterone was due to a reduction in the number of clathrin baskets assembled at the HeLa cell plasma membrane, two quantitative assays were performed: radioisotopic labeling and laser scanning microscopy. Both methods depended on the identification of clathrin baskets, associated with isolated HeLa cell membrane sheets, by primary anticlathrin antibody. The signal was amplified by biotin-conjugated second-affinity antibody bound by either ^{125}I-labeled streptavidin or FITC-labeled streptavidin. For the primary antibody probe, polyclonal antibody directed against purified coated vesicles (Fig. 7B) and monoclonal antibody directed against the 180-kilodalton clathrin heavy chain (Fig. 7C) were tested. The monoclonal antibody was used in the quantitative assays because it provided a cleaner signal. The relative quantity of clathrin on the unstripped HeLa cell membranes, i.e., maximized for coated pit formation, was determined by radioactivity (Table 2) and by laser scanning microscopy (Table 3; Fig. 7D, E, and F). As a control, triplicate cover slips of membrane sheets were exposed to high pH buffer under conditions known to reduce the number of coated pits associated with the membrane; these membranes are referred to as stripped (21).

In both assays, there was no significant difference in the amount of clathrin associated with the membrane sheets obtained from HeLa cells exposed to the two female hormone regimens. The sensitivity and preciseness of the laser scanning microscopy measurement revealed a reduced amount of fluorescence ($P = 0.03$) associated with the control membrane sheets when compared with the hormone-exposed membrane sheets. This trend was somewhat apparent in the radiolabeling studies ($P = 0.09$) but tended to be

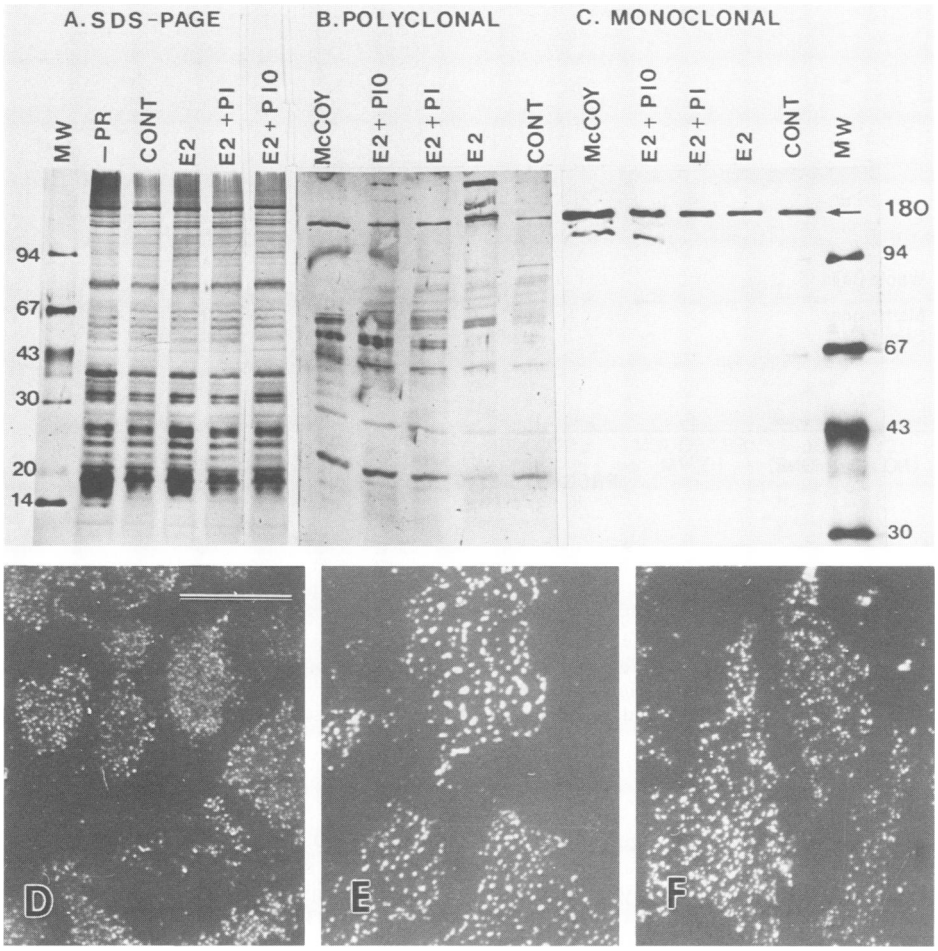

FIG. 7. Analyses of isolated HeLa membranes for hormone effects on clathrin basket assembly. (A) Sodium dodecyl sulfate-polyacrylamide gel stained with silver. (B and C) Western blot (immunoblot) using anticlathrin polyclonal antibody (B) or anticlathrin heavy-chain (180-kilodalton) monoclonal antibody, biotinylated secondary antibody, and alkaline phosphatase-conjugated streptavidin (C). Lanes: MW, molecular weight standards; CONT, membranes isolated from HeLa cells grown in no hormone and in one instance without phenol red (−PR); E2, 10^{-10} M estrogen; E2 + P1, 10^{-10} M estrogen plus 1 ng of progesterone; E2 + P10, 10^{-10} M estrogen plus 10 ng of progesterone. (D, E, and F) Fluorescence photomicrographs of membranes isolated from HeLa cells grown in no hormone (D), 10^{-10} M estrogen (E), and 10^{-10} M estrogen plus 10 ng of progesterone (F) and stained with anticlathrin monoclonal antibody and FITC-labeled streptavidin. Bar = 4 μm.

masked by the variability inherent in the methodology. The control stripping procedure reduced the amount of clathrin associated with the membranes by about 25 to 50%. Thus, one may speculate that the decrease in adherence of chlamydiae to HeLa cells exposed to high physiologic levels of progesterone is probably best explained by interaction of progesterone with the glucocorticoid receptor, which results in profound effects on the HeLa cell membrane.

DISCUSSION

From these morphological studies, it appears that *C. trachomatis* can indeed exploit the receptor-mediated endocytosis pathway for entry into HEGEC. One effect of growing the HEGEC in a polarized orientation was an apparent concentration of coated pits at the apical surface, such that they were more easily visualized. Furthermore, taking advantage of this technological manipulation to mimic the in vivo organizational polarity of cells, albeit transformed and adapted to the laboratory, has provided a more convincing scenario, at least for us, that one method of entry of *C. trachomatis* into HeLa cells is also via coated pits.

Semliki Forest virus has been shown to be internalized by coated pits and coated vesicles in BHK-21 cells (12). Bulk loading of anticlathrin antibodies into CV-1 cells reduced the number of coated pits on the plasma membrane and decreased adsorptive endocytosis of the virus by 40 to 50% (7). Invasion by *Salmonella cholerae-suis* of the apical surface of polarized MDCK cells involved first the disappearance of microvilli in the area surrounding the attached bacteria, followed by the appearance of bacteria in indentations or craters in the MDCK cell surface (8). According to the investigators, coated pits were often, but not always, observed in the host cell membrane in close proximity to the bacteria. Perhaps obligate intracellular parasites require the more efficient entry provided by coated pits and more efficient delivery to the endosomal sorting pathway than do facultative intracellular bacterial pathogens.

There are a number of additional explanations, on a technical level, to account for the different interpretations in different laboratories regarding the method(s) of entry of chlamydiae into target host cells. One explanation is the contrast of embedding resin used for preparation of the

TABLE 2. Relative amount of clathrin on isolated HeLa cell membranes determined by radiolabel[a]

Experiment[b]	Amt (mean[c] [SD]) of clathrin on membranes					
	Control (no hormone)		Estrogen		Estrogen + progesterone	
	Unstripped	Stripped	Unstripped	Stripped	Unstripped	Stripped
1	3,280 (295)	1,870	4,074 (184)	1,615	3,523 (377)	1,861
2	3,181 (176)	2,565	4,202 (1,037)	1,778	4,133 (426)	2,734
3	4,169 (286)	ND[d]	4,596 (431)	ND	4,339 (225)	ND
4	4,865 (691)	ND	4,471 (364)	ND	4,241 (304)	ND
% Stripped[e]	31		59		40	

[a] Membranes were stained with anticlathrin monoclonal antibody, followed by biotinylated second antibody and ^{125}I-labeled streptavidin.
[b] Experiments 3 and 4 were performed on the same day.
[c] Average of quadruplicate samples.
[d] ND, Not determined.
[e] Percent clathrin removed = 100 − (amount of clathrin on stripped membranes/amount of clathrin on unstripped membranes).

samples for TEM. Hodinka and Wyrick (14) did not see *C. psittaci* in coated pits in L cells embedded in Spurr resin, but with the higher-contrast Epon resin, coated pits were numerous. Second, there is some concern about the use of centrifugation-assisted inoculation and its relevance to in vivo events. Pearce (23) has documented a difference, both in the mode of entry as well as in the subsequent intracellular fate, depending on whether the chlamydiae are centrifuged onto host cell monolayers. Third, most of us have used artificially high MOI, especially those of us employing TEM, to be able to visualize the interaction of several chlamydiae per host cell in any given plane of section. A valid criticism is that what is seen is simply fortuitous; with so many EB, it would not be surprising to find some of them in coated pits. To counter this, Hodinka et al. (14) purposely repeated their ultrastructural experiments using a MOI of 10 and still saw EB in coated pits and coated vesicles. They also performed serial sectioning through 10 different *C. psittaci* EB in noncoated pits or indentations in the L-cell plasma membrane; darkly staining coat material could be found in every case at some level. As a consequence of this finding, we feel that the most accurate ultrastructural method for determining the frequency with which chlamydiae utilize the receptor-mediated endocytosis pathway for entry would be to perform serial sectioning through a rather large number of EB, and this would be an enormous undertaking. A more practical quantitative assay would be useful for this purpose or, alternatively, for identification of the eucaryotic receptor(s) involved. Fourth, if tissue culture cell lines are grown as monolayers on plastic surfaces, it is important for the monolayers to be subconfluent. Important eucaryotic functions can be lost when confluency is achieved. This was demonstrated in the study by Soderlund and Kihlstrom (29), who favored the receptor-mediated endocytosis mode. The inhibitors methylamine and monodansylcadaverine were effective only when the monolayers were subconfluent.

Finally, is there any significance to the exploitation by chlamydiae of the receptor-mediated endocytosis pathway beyond a seemingly guaranteed opportunity for entry of an obligate intracellular pathogen? Chlamydial infections are hyperendemic in both avian and mammalian populations, yet overt disease is only the tip of the iceberg. Persistent, clinically inapparent infection is a hallmark of the *Chlamydia* genus. Shedding and subsequent transmission occur in normally infected host. At the cellular level, it is difficult to cure the host cell cytoplasm of this obligate intracellular parasite. The chlamydiae have seemingly successfully exploited the eucaryotic cellular signals and sorting mechanisms, i.e.,

TABLE 3. Relative amount of clathrin on isolated HeLa cell membranes determined by laser scanning microscopy[a]

Treatment and coverslip	Amt[b] (mean ± SD) of clathrin on membranes					
	Control (no hormone)		Estrogen		Estrogen + progesterone	
	Image 1	Image 2	Image 1	Image 2	Image 1	Image 2
Unstripped						
1	60.02 ± 21.58	59.87 ± 22.14	76.08 ± 24.73	78.56 ± 30.35	74.75 ± 31.80	75.29 ± 30.99
2	63.80 ± 25.27	64.11 ± 25.05	63.12 ± 27.65	63.82 ± 27.76	67.39 ± 22.35	67.36 ± 22.39
3	60.66 ± 25.68	58.67 ± 23.82	67.42 ± 22.45	68.40 ± 22.22	ND[c]	ND
Stripped						
1	40.02 ± 9.04	40.48 ± 9.66	53.28 ± 15.95	53.54 ± 15.67	51.84 ± 14.27	51.87 ± 14.26
2	48.56 ± 13.53	49.03 ± 14.25	54.30 ± 19.87	55.14 ± 19.26	51.83 ± 14.54	53.55 ± 15.63
3	54.37 ± 16.60	54.21 ± 16.20	55.87 ± 15.91	56.14 ± 15.84	ND	ND
% Stripped[d]	21.9		21.4		26.6	

[a] Membranes were stained with anticlathrin monoclonal antibody, followed by biotinylated second antibody and FITC-labeled streptavidin. Data presented are from one representative experiment out of two experiments.
[b] Average pixel intensity of center two-thirds of image.
[c] ND, Not determined.
[d] See footnote *e* to Table 2.

receptor-mediated endocytosis-like entry and inclusion transmission at the time of mitosis (24). The former event may somehow ensure that the chlamydiae remain on an endosomal sorting pathway, always contained within a membrane-bound environment, with release of infectious progeny and antigen (25) during persistent versus lytic infection via exocytosis. One might speculate that exocytosed antigen is not processed in a way that can be recognized by class I restricted cytotoxic T lymphocytes (16, 34).

In summary, those of us attempting to dissect the molecular aspects of chlamydial pathogenesis at the cellular level in vitro must always strive to make our model systems as relevant as possible. Although the focus of this study deals with the efficacy of establishing polarized growth of primary HEGEC for examination of chlamydial attachment and entry, the concepts we learned could be applied to some extent to the HeLa cell, a malignant, laboratory-adapted cell line of dubious pedigree which is used by chlamydia researchers throughout the world. The accessibility of these epitheliallike cells has been an important consideration in our use of these cells for obtaining general information regarding the relative advantages of simulated growth in a manner which mimics their architectural organization in vivo. These advantages included (i) an obvious greater differentiation in morphological arrangement and function, which highlighted the affinity of chlamydiae for microvilli and entry in coated indentations; (ii) greater infectivity of chlamydial progeny; and (iii) a means for studying the polarization of the infectious pathway in vitro for systemic chlamydial biovars, i.e., LGV release via the basolateral surface.

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