

***Chlamydia trachomatis* infection of human fallopian tube organ cultures**

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The pathogenic events that precede *Chlamydia trachomatis* salpingitis in the human fallopian tube have not been fully described. We used a model of human fallopian tubes in organ culture (HFTOC) infected with strain E/UW-5/CX of *C. trachomatis* to study these events. The model supported sustained *C. trachomatis* infection as demonstrated by recovery of viable *C. trachomatis* from medium and tissue over 5–7 d. However, the level of infectivity was low. Maximal infection occurred at 72 h after initial inoculation. In contrast to gonococcal infection of the HFTOC, *C. trachomatis* did not damage overall ciliary function of HFTOC. However, a local direct cytotoxic effect characterized by loss of microvilli and disruption of cell junctions was noted when multiple chlamydial elementary bodies attached to mucosal cells. Beginning at 24 h, and continuing throughout the course of *C. trachomatis* infection of HFTOC, ruptured epithelial cells releasing elementary bodies were noted. Chlamydial inclusions were seen in the mucosa by 72 h in ~6% of both ciliated and nonciliated epithelial cells. Mucosal inclusions contained all forms of the *C. trachomatis* developmental cycle. These data suggest that factors present in the human fallopian tube may limit susceptibility to chlamydial infection but support the use of the HFTOC model in the study of the pathogenesis of *C. trachomatis* salpingitis.

Introduction

Chlamydia trachomatis has emerged as the most common sexually transmitted bacterial pathogen in developed countries (Washington *et al.*, 1987; Aral & Holmes, 1990). In women, genital infection due to *C. trachomatis* may be asymptomatic or result in mucocervicitis, acute urethritis or salpingitis. Salpingitis is potentially the most devastating of these infections. Damage to the fallopian tube caused by *C. trachomatis* may lead to recurrent salpingitis, tubo-ovarian abscess, ectopic pregnancy and infertility. A knowledge of the steps by which *C. trachomatis* causes salpingitis is critical to the development of strategies for controlling and preventing this infection.

Although the human fallopian tube organ culture (HFTOC) model has been used to define the patho-

genesis of gonococcal salpingitis (McGee *et al.*, 1976; Ward *et al.*, 1974), only one study has used this model to study *C. trachomatis* pathogenesis. Hutchinson *et al.* (1979) used fallopian tube organ cultures of both bovine and human origin to study infection by *C. trachomatis*. They noted that HFTOCs *in vitro* could be infected with recovery of *C. trachomatis* during the third to fifth day of infection. However, chlamydial inclusions could not be detected in the HFTOCs and the microscopic events occurring during the infection were not determined. In the current study we used light and electron microscopy as well as quantitative culturing to define events in the infection of HFTOCs with *C. trachomatis*.

Methods

Micro-organisms and culture techniques. The *C. trachomatis* strain used in this study was serotype E/UW-5/CX. The E strain of *C. trachomatis* is well characterized (Kuo *et al.*, 1972) and has previously been shown to be capable of infecting explants from primate and human fallopian tubes of endometrial and ectocervical epithelium in

Abbreviations: EB, elementary body; HFTOC, human fallopian tubes in organ culture; IFU, inclusion-forming unit; LM, light microscopy; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

culture (Patton, 1985; Patton *et al.*, 1983, 1987*a, b*). To maintain stocks of this strain, it was passaged in HeLa 229 cells for nine passages, concentrated to 1×10^8 inclusion-forming units (IFU) ml^{-1} and stored at -80°C in HEPES-sucrose-calcium (HSC) buffer (Bird & Forrester, 1981).

The monolayer of HeLa cells was infected by pretreating these cells with DEAE-dextran ($30 \mu\text{g ml}^{-1}$ in HSC buffer) for 20 min at room temperature. The monolayer was then rinsed with Hanks' balanced salt solution and inoculated with 1–2 ml of the serotype E inoculum (10^8 IFU). The inoculum was prepared by rapidly thawing the chlamydiae and briefly sonicating them to eliminate clumping of elementary bodies (EBs). The flasks containing the infected monolayers were then incubated at 37°C for 1 h with the flasks being rotated every 5–10 min. The inoculum was then removed, and the monolayer was rinsed with Hanks' balanced salt solution and was covered with 20–40 ml of medium containing cycloheximide and glucose. The flasks were then incubated at 37°C in 5% (v/v) CO_2 for 48 h. At 48 h, the inoculum gave ~90% infectivity of the HeLa cell monolayer. The medium was removed and 5 ml of HSC and about 60–70 glass beads (3 mm diameter) were added, to dislodge the cells. The cells were collected and chlamydial inclusions were released by sonication for 30 s at full power. A low-speed centrifugation (1200 r.p.m. for 10 min) removed the cell debris. The supernatant from the low-speed centrifugation was centrifuged at 16000 r.p.m. to pellet the EBs. The pellet was resuspended in 20 ml HSC, sonicated to remove EB clumps and 1 ml portions were frozen.

The inoculum was titrated by the following method. The chlamydial suspension was thawed, sonicated and diluted 10-fold from 10^{-1} to 10^{-7} in HSC. All dilutions were performed at 4°C . HeLa cell monolayers grown on 12 mm coverslips were each infected in duplicate with 0.1 ml of the dilutions of *C. trachomatis*. The infected vials were centrifuged at 2500 *g* for 60 min at 37°C . After centrifugation, the inoculum was aspirated, and 1 ml of medium containing cycloheximide and glucose was added to each vial followed by incubation at 37°C in 5% CO_2 for 48 h. After incubation, the coverslips were fixed in 95% (v/v) ethanol and stained with fluorescein-labelled monoclonal antibodies (Syva Microtrak). Stained inclusions were counted and the log of the average number of IFU per coverslip was plotted against the dilution used for inoculation. To calculate the IFU per ml of the initial suspension, the antilog of the y -intercept was multiplied by the dilution factor. After calculating this number, the IFU contained in any less dilute concentration could be obtained.

Infection of HFTOC. HFTOC were established as previously described (McGee *et al.*, 1976). Briefly, fallopian tubes were obtained from nonpregnant, premenopausal women undergoing hysterectomy for prolonged uterine bleeding or fibroid tumours as well as from women undergoing salpingectomy for sterilization. Tubes were removed from the patient and placed in Eagle's minimal essential medium (MEM) containing Earle's salts and L-glutamine and buffered with 0.05 M-HEPES to a pH of 7.5. The HEPES-MEM also contained colistin ($3 \mu\text{g ml}^{-1}$) and vancomycin ($5 \mu\text{g ml}^{-1}$). After removal of the adventitial tissues, the fallopian tubes were cut longitudinally and 3–4 mm² pieces were cut with a scalpel blade and transferred to tissue culture dishes containing 3 ml HEPES-MEM plus antibiotics for 24 h. In all experiments the organ cultures were washed free of antibiotics after the 24 h incubation and maintained in media free of antibiotics during the experiment. The cultures were maintained at 37°C in 5% CO_2 .

The HeLa-passaged serotype E strain was used to infect the HFTOC. EBs were quickly thawed and placed on ice. The EB suspension was sonicated for 30 s and diluted in HSC to approximately 1×10^8 IFU ml^{-1} and 50 μl of this inoculum was added to each HFTOC. For chlamydial infection, HFTOC were prepared using 24-well tissue culture plates. A single piece of fallopian tube tissue (3–4 mm²) was placed in each well and covered with 1 ml of antibiotic-free medium

and incubated for periods up to 10 d. At 4 h, 8 h, 24 h, 48 h, 72 h, 5 d and 10 d, tissues were removed and processed for light microscopy (LM), scanning electron microscopy (SEM), and transmission electron microscopy (TEM). Ciliary activity of organ cultures was also assessed at those time intervals by the techniques previously described (McGee *et al.*, 1976). To quantify chlamydial infection, HFTOC samples were washed and homogenized; then the homogenate was used to infect HeLa cell monolayers. Samples of the tissue culture medium from the infected HFTOC were also used to infect HeLa cell monolayers in order to further quantify the infection of the HFTOC. At each time interval uninfected tissues were processed for microscopy, examined for ciliary activity and cultured as a control for tissue integrity as well as for previous undiagnosed chlamydia infection.

Microscopy (i) SEM. HFTOC to be examined by SEM were fixed in 2% (w/v) glutaraldehyde (pH 7.3) in 0.1 M-sodium cacodylate. Post-fixation was accomplished in 1.5% (w/v) osmium tetroxide in the same buffer. HFTOC were dehydrated in ethanol, critical-point dried, coated with gold/palladium and examined in a scanning electron microscope (model S-500; Hitachi Scientific Instruments).

(ii) LM and TEM. HFTOC to be examined by TEM were fixed in 2.5% glutaraldehyde in 0.13 M-phosphate buffer (pH 7.2). After fixation for 12–24 h, the tissues were transferred to 0.13 M-phosphate buffer (pH 7.2) containing 7.5% (w/v) sucrose. HFTOC were post-fixed for 1–2 h in 1.5% osmium tetroxide, dehydrated in graded solutions of ethanol and embedded in Spurr resin (Electron Microscopy Scientific). Sections of tissue 1–2 μm thick were stained with 1% (w/v) toluidine blue in 1% (w/v) borax for 15 to 30 s; the stained sections were examined by LM. Sections 40–60 nm thick were stained with uranyl acetate and lead citrate before examination in a transmission electron microscope (model 300; Philips Electronics).

Results

Infection of HFTOCs by C. trachomatis

Both culture medium and HFTOC homogenates were cultured to quantify *C. trachomatis* infection of HFTOC. Because *C. trachomatis* is an obligate intracellular parasite, medium and tissue assayed after the first 24 h of infection should reflect active intracellular multiplication.

Fig. 1(a) shows the number of *C. trachomatis* IFU recovered from the medium of infected HFTOC. The original inoculum was 5×10^6 IFU ml^{-1} . At 24 h post-infection there were 1.3×10^2 IFU ml^{-1} in the HFTOC culture medium. More IFU (5.2×10^3) were recovered at 48 h, with the maximum number (1.86×10^4) recovered at 72 h. Chlamydiae were recovered at 5 d post-infection (3.9×10^3) and up to 7 d post-infection. Culture medium from uninfected HFTOC did not yield *C. trachomatis*, indicating the absence of concomitant natural infection.

Recovery of *C. trachomatis* from homogenates of HFTOC is shown in Fig. 1(b). Following infection with 5×10^6 IFU ml^{-1} , the counts of IFU (mg tissue^{-1}) were 1.2×10^1 at 24 h, 4.3×10^1 at 48 h, 2.6×10^2 at 72 h and 2.6×10^1 at 5 d post-infection. Infection in HFTOC persisted for at least 5 d.

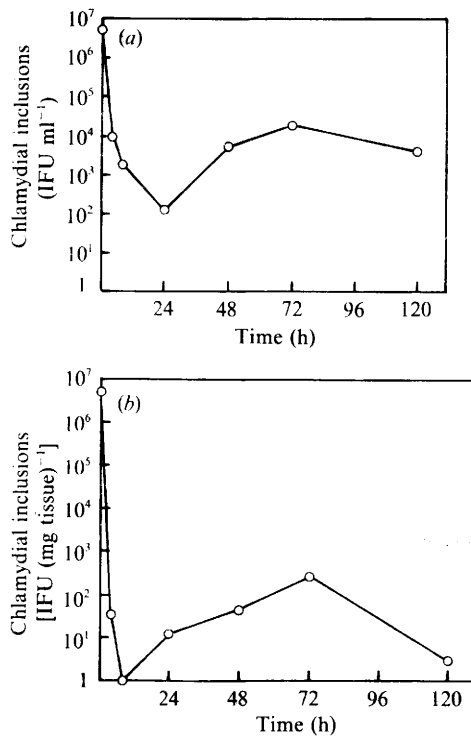


Fig. 1. Recovery of *C. trachomatis* from (a) the culture medium and (b) homogenates of infected HFTOC. The initial infection contained 5×10^6 IFU ml⁻¹.

Effect of *C. trachomatis* infection on ciliary activity of HFTOC

Ciliary activity as monitored by percentage peripheral ciliary activity and ciliary vigour was assessed (McGee *et al.*, 1976) during the course of *C. trachomatis* infection of HFTOC. In three separate experiments no loss of ciliary activity was detected by either criterion when *C. trachomatis*-infected HFTOC were compared with uninfected cultures (data not shown). These data are in sharp contrast to gonococcal infections of HFTOC, which show marked damage to ciliary activity using identical assays (McGee *et al.*, 1976).

Events in the interactions of *C. trachomatis* and human fallopian tube mucosa as noted by SEM

Microscopic events during *C. trachomatis* infection of HFTOC were studied with techniques previously used to define gonococcal infection of this model. Fig. 2 shows uninfected HFTOC after 7 d incubation. The mucosal surface was undisturbed, showing numerous ciliated and nonciliated cells. The microvilli of the nonciliated cells were prominent and uniform. The ciliated cells were intact. These characteristics of the mucosa were maintained in uninfected HFTOC through 7–10 d of incubation.

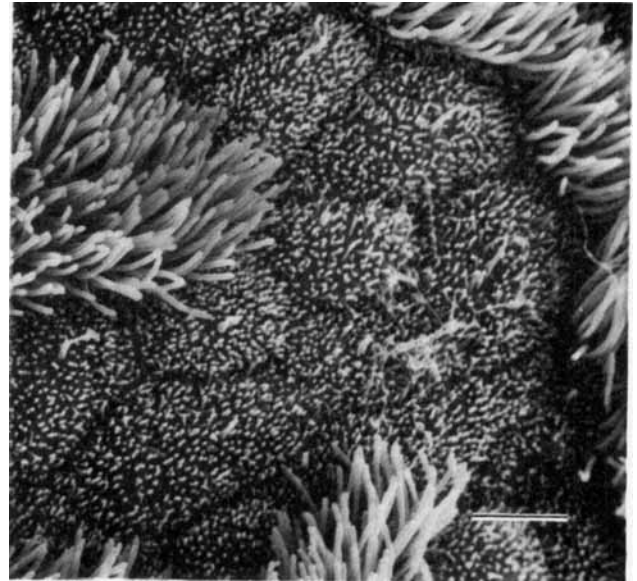


Fig. 2. Scanning electron micrograph of uninfected HFTOC after 7 d of culture. Bar, 2 μ m.

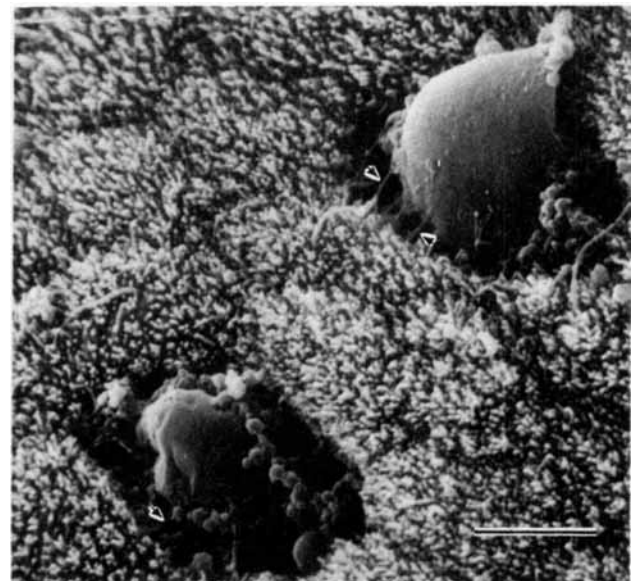


Fig. 3. Scanning electron micrograph of clusters of EBs attaching to nonciliated epithelial cells at 4 h post-infection. Where aggregates of elementary bodies attach there is local cytototoxicity marked by the loss of microvilli on these cells and a disruption of cellular junctions. The arrows indicate residual strands connecting the infected cell to the rest of the mucosa. Bar, 4 μ m.

Strikingly different results were observed during *C. trachomatis* infections of HFTOC. Fig. 3 illustrates the cytotoxic effects seen at 4 h following attachment of multiple *C. trachomatis* EBs. Nonciliated cells, with multiple EBs attached, began to lose surface integrity as noted by the lack of microvilli and the disruption of

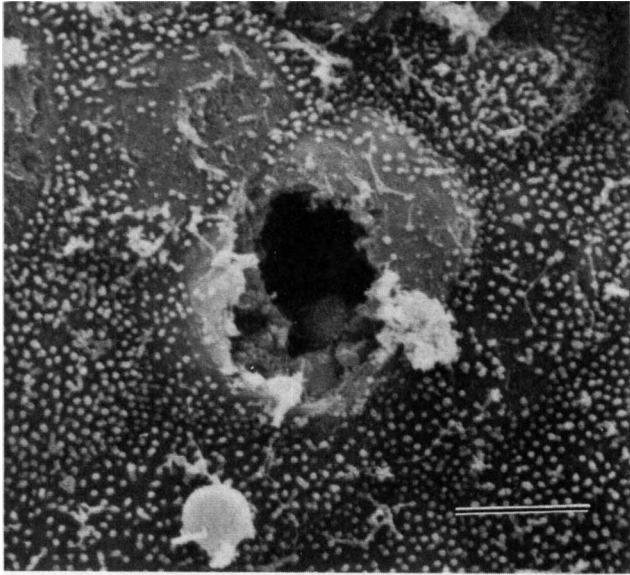


Fig. 4. Scanning electron micrograph of infected HFTOC 72 h after infection, showing a cell with a lesion left by the rupturing of *C. trachomatis* inclusions. Bar, 5 μ m.

cellular junctions. Also noted were residual strands (arrowed in Fig. 3) connecting the infected cell to the rest of the mucosa.

Associated with the initial attachment and infection of HFTOC was the formation of bundles of fused microvilli (not shown) on nonciliated epithelial cells. These bundles of microvilli were not seen in uninfected HFTOC that were processed under identical conditions at the corresponding time period. The role of these aggregated microvilli in *C. trachomatis* infection of the human fallopian tube is unclear.

Fig. 4 illustrates an HFTOC 72 h after infection. Cavities in the mucosa, presumably left by rupturing of the chlamydial inclusions, were noted. These cavities were first seen at 72 h and were present throughout the remaining course of infection. EBs could be seen in some of the ruptured cells.

Intracellular events in the interaction of C. trachomatis and human fallopian tube mucosa as noted by LM and TEM

Intracellular *C. trachomatis* were prominent in LM and TEM preparations of HFTOCs infected for 72 h. Inclusions were seen within the mucosa at this time point in ~6% of both ciliated and nonciliated cells.

Figure 5(a) shows a ruptured epithelial cell at 72 h post-infection, containing EBs, reticulate bodies and intermediate forms. The finding of numerous inclusions

at 72 h corresponded to the recovery of the highest number of *C. trachomatis* IFU from infected supernatants and tissue and the greater number of disrupted epithelial cells noted by SEM. Higher magnification (Fig. 5b) reveals the loss of both phagosomal and surface-exposed cytoplasmic membrane integrity with the release of infectious particles to the epithelial surface. However, the cytoplasmic membrane adjacent to other epithelial cells appears to be intact as shown by a continuous membrane and desmosomes. Fig. 6 shows an infected ciliated epithelial cell. Again various stages of the *C. trachomatis* developmental cycle were noted in the cytoplasm. Interestingly, the chlamydiae were no longer membrane bound.

Discussion

The sequence of events that occurs during infection of the human fallopian tube with *C. trachomatis* has been assumed to be similar to the events seen in *C. trachomatis* infection of animals or tissue culture and *in vivo* chlamydial conjunctival infection. Tuffrey and co-workers (Tuffrey & Taylor-Robinson, 1981; Tuffrey *et al.*, 1982, 1984, 1985, 1986) developed a mouse model of chlamydial genital tract infection using a variety of human isolates of *C. trachomatis*. They found that infection was enhanced by progesterone, which is thought to maintain epithelial cells on the uterine surface. The murine model also offers advantages for immunological studies. Their studies implied that T-cell impairment may play a role in the severity of chlamydial infections and concluded that genetic differences in mice altered their resistance to infection as well as their susceptibility to inflammatory changes. Further, different human isolates do not have equal abilities to produce salpingitis in mice, implying a genetic component to the infectivity of these isolates. Intravaginal inoculation of mice with the agent of mouse pneumonitis (MoPn; a *C. trachomatis* biovar) results in cervical infection, and chlamydial inclusions are found in superficial cervical epithelial cells (Barron *et al.*, 1981). Recently it has been shown that inoculation directly into the ovarian bursa of these mice consistently results in salpingitis (Swenson *et al.*, 1983). Inclusions were readily demonstrated in the oviductal and uterine epithelium. Permanent tubal damage and infertility occurred in the majority of infected mice.

Patton *et al.* (1983) studied acute *C. trachomatis* salpingitis in pig-tailed macaque monkeys. In these studies, a single inoculation caused self-limiting tubal inflammation and little residual damage was demon-

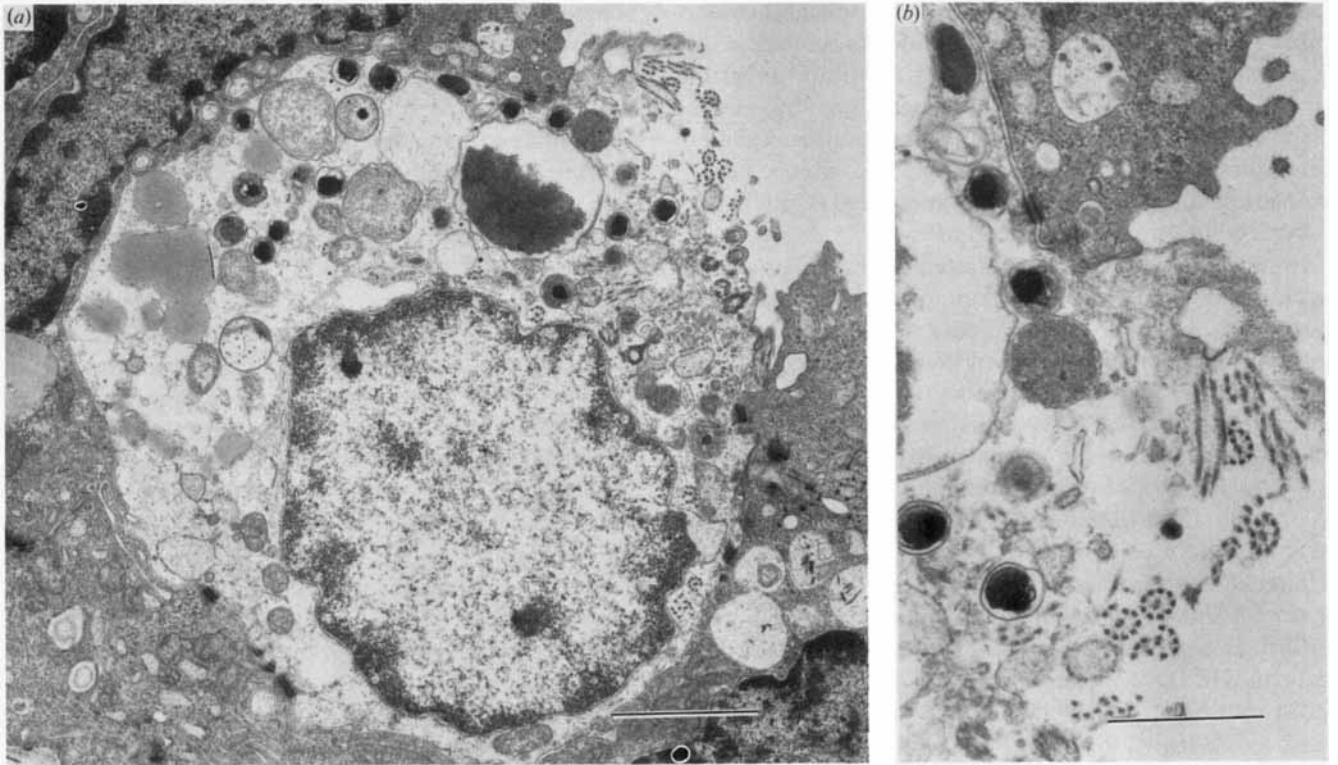


Fig. 5. (a) Transmission electron micrograph of a rupturing epithelial cell at 72 h post-infection. Note that all forms (EBs, reticulate bodies and intermediate forms) of the developmental cycle are visible in the cytoplasm. Bar, 2 μm . (b) Higher magnification of the rupturing cell, illustrating the release of the EBs and intermediate forms, and the disruption of the cell membrane. Bar, 1 μm .

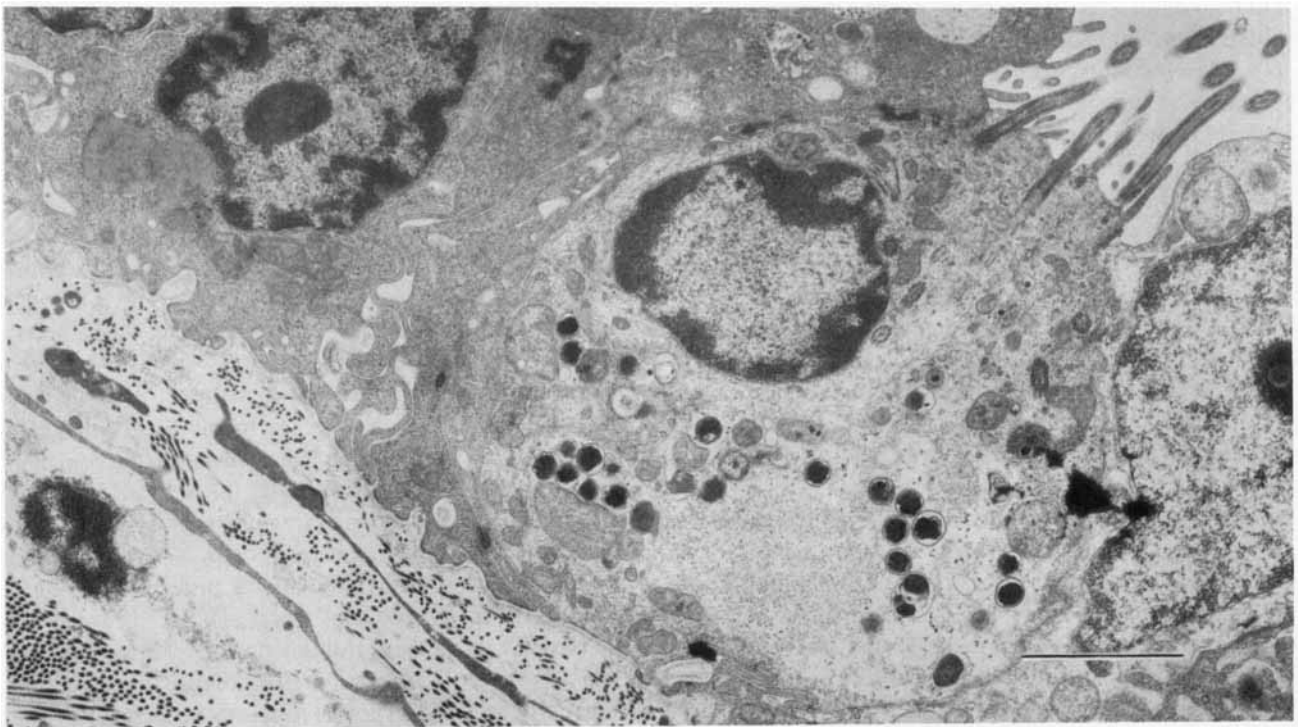


Fig. 6. Transmission electron micrograph of an infected ciliated epithelial cell at 72 h post-infection. Various forms of the developmental cycle are present. Bar, 2 μm .

strated on long-term follow-up. In infected subcutaneous fimbrial transplants in cynomolgus and rhesus monkeys, *C. trachomatis* inclusions were detected within fimbrial epithelial cells up to 7 d post-inoculation by both fluorescent-antibody staining and immunoperoxidase techniques using *C. trachomatis*-specific monoclonal antibodies. *C. trachomatis* was recovered from the autografts up to 5 d after infection.

Swanson *et al.* (1975) demonstrated typical chlamydial inclusions in the columnar epithelium of cervical biopsy specimens obtained from patients who were culture positive for *C. trachomatis* and *Neisseria gonorrhoeae*. Moller *et al.* (1979) studied fallopian tubes removed from two patients with *C. trachomatis* salpingitis. TEM studies failed to reveal inclusions consistent with those produced by *C. trachomatis*. However, immunofluorescence studies showed intracytoplasmic chlamydial inclusions in some of the epithelial cells.

In an effort to further define the interactions between *C. trachomatis* and human fallopian tube mucosa, we performed a detailed microbiological and microscopic study of HFTOC infected with an E serovar of *C. trachomatis*. The data demonstrated that a sustained, albeit low-level, *C. trachomatis* infection occurs in the model. In contrast to gonococcal infection of HFTOC, where lipopolysaccharides and peptidoglycan monomers released from rapidly dividing gonococci extensively damage both ciliated and nonciliated epithelial cells (Stephens, 1989), infection of HFTOC with *C. trachomatis* had no overall effect on mucosal architecture or ciliary activity. These data support the results of Hutchinson *et al.* (1979), who found that ciliary activity of fallopian tube mucosa was not diminished by *C. trachomatis* infection. However, studies of human fallopian tubes removed during active chlamydial salpingitis demonstrate marked inflammation, loss of cilia, oedema and extensive disruption of the mucosal surface. Taken together, these data suggest the importance of the inflammatory response (not present in the *in vitro* HFTOC model) in the pathogenesis of *C. trachomatis* salpingitis and may indicate that *C. trachomatis*, unlike *N. gonorrhoeae*, may not secrete toxins which produce generalized mucosal damage.

We did observe local, direct cytotoxic effects of *C. trachomatis* on HFTOC mucosal cells. Early in the infection (4–8 h), attachment of multiple *C. trachomatis* EBs to certain epithelial cells produced a loss of microvilli, disruption of cell borders and sloughing of these cells from the mucosal surface while adjacent cells were uninfected. Loss of epithelial cells with attached EBs appeared to be independent of the intracellular development cycle but the exact role of this event in *C. trachomatis* pathogenesis is unclear. These results are in sharp contrast to the attachment of gonococci to

nonciliated epithelial cells of HFTOC, where interaction with microvilli is prominent and mucosal architecture with the adjacent cells is preserved (McGee *et al.*, 1976; Ward *et al.*, 1974). It should be emphasized that loss of microvilli and sloughing of epithelial cells occurred with multiple EBs whereas local cytotoxicity was not observed with attachment of individual EBs.

Moorman *et al.* (1986) used primary cultures of human endometrial and ectocervical epithelial cells to study genital infection of *C. trachomatis*. They noted a difference in the infection in McCoy cell versus genital cell cultures. *C. trachomatis* failed to adhere to many of the genital cell cultures whereas adherence to McCoy cells was uniform. Inocula which gave 50–80% infectivity (inclusion formation) of standard McCoy cells resulted in infection of 15–30% of columnar cells of the human endometrium and 5–10% of the squamous cells of the ectocervix. Exposure to DEAE-dextran and centrifugation did not enhance the number of inclusion-positive genital cells.

Our data would also suggest selectivity of *C. trachomatis* attachment and replication in epithelial cells of HFTOC. The low level of infectivity (~6%) of the epithelial cells may indicate that host factors may limit susceptibility of HFTOC to infection. Maslow *et al.* (1988) found that *C. trachomatis* would attach to approximately 50% of cultured human endometrial gland epithelial cells. The addition of oestrogen increased attachment rates to approximately 80%, while progesterone in combination with oestrogen reduced chlamydial attachment in a dose-dependent fashion. Together these studies suggest a cellular specificity with respect to the attachment and replication of *C. trachomatis* and that these events may be under hormonal influence.

In this study, we were unable to completely identify the events of entry of *C. trachomatis* into HFTOC mucosal cells or the events leading to formation of inclusions. However, LM of infected HFTOC revealed inclusions which were consistent with those seen in human cervical biopsy material (Swanson *et al.*, 1975). Further, this study is the first to demonstrate by electron microscopy all developmental forms of *C. trachomatis* within an infected human fallopian tube mucosal cell. It is interesting that in some of our TEM studies apparent rupture of the inclusion occurred prior to release of *C. trachomatis* from the epithelial cells.

We believe that HFTOC is a unique and relevant model to study the pathogenesis of *C. trachomatis* salpingitis. Hormonal influence on *C. trachomatis* infectivity, effects of antibiotics, role of local immunocompetent cells, and evaluation of mutants with specific genetic lesions are among some of the studies possible using this model.

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