Interaction of *Chlamydia trachomatis* with Human Genital Epithelium in Culture

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Primary cultures of human endometrial and ectocervical epithelial cells were examined as a new model system to study genital infection by *Chlamydia trachomatis*. Initial studies demonstrated that these cells were indeed susceptible to chlamydial infection. Inocula, adjusted to produce inclusions in 50 to 80% of equivalent numbers of standard McCoy cells, resulted in infection rates of approximately 15 to 30% for the columnar cells of the endometrium and 5 to 10% for the squamous cells of the ectocervix. Exposure of cultures to DEAE-dextran and centrifugation-assisted inoculation, manipulations reported to enhance infection of HeLa and McCoy cells, did not alter the number of inclusion-positive genital cells. Addition of cycloheximide to the post-inoculation culture medium slightly increased numbers of inclusion-bearing cells while growth of genital cells in hormone-supplemented medium resulted in a variable effect on inclusion development and a significant reduction in the association of radiolabelled organisms with these cells. The basis for the different levels of infection in McCoy versus genital cell cultures was revealed by immunofluorescence analysis of chlamydial association with host cells immediately after inoculation. Chlamydiae failed to adhere to many cells in the genital cell cultures while adherence to McCoy cells was uniform. In addition, the association of radiolabelled *C. trachomatis* was significantly lower with genital cells than with McCoy cells. Finally, culture conditions were defined which markedly inhibited inclusion development without an immediate loss of chlamydial growth potential. This investigation indicates that primary genital cell cultures are susceptible to chlamydial infection and will be valuable for studies on the nature of *C. trachomatis* interactions with natural human target cells.

INTRODUCTION

Chlamydia trachomatis is a bacterium primarily responsible for ocular and genitourinary tract infections of varying severity. In non-trachoma endemic areas the major reservoir of infection is the human genital tract (Schachter & Dawson, 1978). *C. trachomatis* is one of the most common and economically significant sexually transmitted disease pathogens, affecting some two to four million people in the USA annually. One of the major deterrents to the investigation of this agent has been the lack of an appropriate model system, the development of which has been hindered by the fact that humans are the natural hosts. The obligate intracellular nature of *C. trachomatis* means that eukaryotic cell cultures must be used for *in vitro* study of this organism. HeLa cells and McCoy cells have been used in the majority of research investigations designed to elucidate mechanisms which govern chlamydial interactions with host cells. Although these cells lines are sufficiently susceptible to infection to warrant their use for routine isolation of the organism from clinical samples (Gordon & Quan, 1965; Kuo *et al.*, 1972; Croy *et al.*, 1975),

Abbreviations: EB, elementary body; DMEM, Dulbecco's modified Eagle's medium; MEM, minimal essential medium.

neither is representative of normal target epithelium *in vivo*. Some investigators have used various manipulations with these cell lines to enhance infection, such as pretreatment with the polycation DEAE-dextran (Kuo *et al.*, 1973; Rota & Nichols, 1973), centrifugation of chlamydial inocula (Darougar *et al.*, 1974) and addition of cycloheximide to the culture medium (Ripa & Mårdh, 1977). The development of an alternative cell model system, using non-transformed epithelial cells derived from permissive human target tissue, would seem essential in studying selected aspects of *C. trachomatis* pathogenesis.

In recent years, there has been renewed interest in the use of human *in vitro* cell culture systems for studying the pathogenesis of bacterial and viral disease agents. Hutchinson *et al.* (1979) used fallopian tube explants to monitor the effect of *C. trachomatis* infection on epithelial ciliary action, in an attempt to describe potential cellular events contributing to infertility. Harrison & Riggan (1979) reported that human amnion epithelial cell monolayers were as susceptible to *C. trachomatis* infection as McCoy cell cultures treated with 5-iodo 2-deoxyuridine and DEAE-dextran.

The purpose of this investigation was to determine if primary cultures of squamous epithelial cells derived from the ectocervix and columnar epithelial cells derived from endometrial glands could serve as models for *C. trachomatis* infection.

METHODS

Chlamydial strains. A human urogenital isolate of *C. trachomatis*, E/UW-5/Cx, kindly provided by C. C. Kuo and S. P. Wang, University of Washington, Seattle, was used. For one set of experiments a lymphogranuloma venereum strain, L2/434/Bu, was used for comparison with E/UW-5/Cx. This strain was donated by the same investigators.

Cell culture media. MEM. Eagle's minimal essential medium (Gibco) supplemented with 10% (v/v) heat-inactivated foetal bovine serum (AFBS; Sterile Systems Inc.), 4.2 mM-sodium bicarbonate, 1.6 mM-glutamine (Eastman Kodak), 0.02% streptomycin (Pfizer) and 0.01% kanamycin (Beecham), was used to prepare *C. trachomatis* stock cultures in McCoy cell monolayers.

MEM/D-valine. Before infection with chlamydiae, ectocervical cell cultures were maintained in Eagle's MEM with D-valine substituted for L-valine (Gibco) and supplemented with 12% AFBS, 10 mM-glutamine, 0.02% streptomycin, 0.01% kanamycin, 1.25 µg amphotericin B ml⁻¹ (Squibb) and 5 µg hydrocortisone 21-hemisuccinate ml⁻¹ (Sigma).

DMEM. Endometrial cells were grown in high-glucose Dulbecco's modified Eagle's medium (Gibco) containing 12% AFBS, 0.11 mg sodium pyruvate ml⁻¹, 10 mM-glutamine, 0.02% streptomycin, 0.01% kanamycin, 1.25 µg amphotericin B ml⁻¹ and 4 µg insulin ml⁻¹ (Sigma). Unless stated otherwise, DMEM was also used as the culture medium after inoculation of both McCoy and genital cell cultures with *C. trachomatis*.

Growth, harvest and titration of chlamydiae. Chlamydiae for stock cultures were grown in McCoy cell monolayers, according to the methods described by Kuo *et al.* (1977) and Newhall *et al.* (1982) in MEM containing 0.5 µg cycloheximide ml⁻¹ (Gallard Schlesinger). Infected monolayers were removed from roller bottles 72–96 h post-inoculation, transferred to 100 ml bottles containing sterile glass beads (3 mm diameter) and sonicated for 5 min in a sonic water bath (Heat Systems, Ultrasonics Inc.). The sonicate was centrifuged at 478 g for 10 min at 4 °C to remove McCoy cell debris. The supernatants were removed and centrifuged at 10 780 g for 30 min at 4 °C to pellet chlamydiae. The resulting crude elementary body (EB) pellets were resuspended in a buffer containing 0.02 M-NaH₂PO₄ · H₂O, 0.02 M-Na₂HPO₄, 0.02 M-sucrose and 5 mM-glutamine per ml distilled water (2SPG) and frozen at -70 °C until needed.

For the preparation of radiolabelled organisms, chlamydiae suspended in 5 ml 2SPG were adsorbed to the McCoy cell monolayers in 840 cm² roller bottles for 2 h at 35 °C. The inoculum was then removed and 100 ml MEM, containing 0.1 mCi (3.7 MBq) [³H]uridine (Research Products International Corp.), was added to the culture and incubation was continued for 72–96 h. The chlamydiae were harvested and partially purified as described above. The radiolabelled organisms were then further purified by Percoll (Pharmacia) density gradient centrifugation according to the method of Newhall *et al.* (1982). Finally, purified EB were washed with 2SPG to remove the Percoll, sonicated with a microtip probe (Heat Systems, Ultrasonics Inc.) at 40 Hz for 90 s to break up EB aggregates, resuspended to a particle count of 1×10^{10} per ml 2SPG (Wyrick *et al.*, 1978), and frozen at -70 °C. A sample of each frozen chlamydial stock was rapidly thawed at 37 °C and prescreened for infectivity titres in coverslip cultures of McCoy cells before inoculation of genital cell cultures. After centrifuge-assisted inoculation, infected monolayers were incubated at 35 °C in an atmosphere of 5% CO₂ for 48 to 72 h, stained with Giemsa Gurr (BDH) and percentages of inclusion-bearing cells were determined. Chlamydial stocks were subsequently diluted to produce infection in approximately 50%–80% of the McCoy cells in a coverslip monolayer.

The number of infectious EB per stock population is routinely 1% for purified preparations, even at a multiplicity of infection (m.o.i.) of less than one EB per McCoy cell. At high m.o.i. the possibility exists that titres may be artificially low if more than one infectious EB is ingested per host cell, and the developing EB- or reticulate body-containing phagosomes fuse (Ward, 1983; see legend for Fig. 1*d*). This would result in a host cell exhibiting a single chlamydial inclusion.

Isolation and culture of human genital cells. Tissue was obtained from women of childbearing age undergoing hysterectomy for non-malignant disease under a protocol approved by the institutional committee for the protection of human rights. The uterus was bisected from cervix to fundal dome and inspected for gross evidence of disease intrinsic to ectocervix and endometrium. Only tissues showing no macroscopic evidence of underlying pathology were used.

The method of Vesterinen *et al.* (1980*a*) was used for the establishment of squamous ectocervical cell cultures. Briefly, the underlying stroma was removed from ectocervical strips and the remaining epithelial layers were cut into 1 mm² explants. Three to four explants were placed, stroma side down, on 13 mm diameter plastic tissue culture coverslips (Thermonox; Flow Laboratories) held in 24 well tissue culture plates or 7–10 explants were placed directly on the surface of 35 × 10 mm tissue culture dishes (Falcon). MEM/D-valine was added to the culture vessels and explants were incubated at 35 °C in an atmosphere of 5% CO₂. This medium prevents contaminating fibroblast growth due to the inability of fibroblasts to convert D-amino acids into their essential L-enantiomers (Gilbert & Migeon, 1975). Squamous epithelial cells, capable of this conversion, grew out in a radial fashion from adherent explants to form confluent cell cultures within 10 to 15 d. The epithelial nature of these cultures has been described (Vesterinen *et al.*, 1980*a, b*). Ectocervical cultures were fed with fresh MEM/D-valine at 4 d intervals before *C. trachomatis* challenge.

A modification of the procedure of Satyaswaroop *et al.* (1979) and Varma *et al.* (1982) was used for the isolation and culture of human endometrial glands. Strips of endometrium were slivered using a scalpel and treated with 0.25% type IV collagenase (Sigma) in Hanks' balanced salts solution supplemented with 25 mM-HEPES buffer (Sigma), 200 µg penicillin and 200 µg streptomycin per ml, pH 7.4 (HBSS/HEPES) for 60 to 90 min at 35 °C in an atmosphere of 5% CO₂. Endometrial glands, containing columnar-cuboidal epithelial cells, were released by enzyme digestion while most of the remaining stroma was reduced to single cell constituents. The glands were separated from the stromal cells by differential centrifugation at 57 *g* for 15 s. Sedimented glands were resuspended and washed three times in HBSS/HEPES and finally resuspended in DMEM. The suspension was distributed, at a density of 20 glands ml⁻¹, to 24 well culture plates containing plastic coverslips or directly into 35 × 10 mm culture dishes. All vessels used for the culture of endometrial tissue preparations were pretreated with 0.01 mg bovine plasma fibronectin (BRL) per ml HBSS for 60 min at 35 °C. Columnar epithelial cells grew out in a swirl pattern from adherent glands to form nearly confluent monolayers within 4 to 10 d. Cultures were fed with fresh DMEM at 3 to 4 d intervals before chlamydial inoculation.

The epithelial purity of endometrial cell cultures was determined using alkaline phosphatase activity as a marker (Siegfried *et al.*, 1984). The monolayers typically contained more than 90% alkaline phosphatase positive cells. HeLa 229 cultures were used as positive controls while L929 and McCoy fibroblast cultures served as negative controls.

Chlamydial infection of human genital cell cultures. Genital cells were used for chlamydial infection when the cultures were subconfluent (8 to 10 d for the ectocervical cells; 5 to 7 d for the endometrial cells). These cultures contained >90% viable cells, as determined by trypan blue exclusion, at the time of chlamydial inoculation. EB inoculum (0.2 ml) was added to each coverslip (column height 1 mm) and the cultures were centrifuged at 375 *g* for 20 min. Monolayers were washed twice with fresh medium and finally 1 ml DMEM was added to each infected monolayer. The extent of infection was monitored by determining the percentages of inclusion-bearing cells with Giemsa stain, after incubation at 35 °C in 5% CO₂ for 72 h. Uninoculated genital cells, stained with Giemsa, served as negative controls in each experiment. No evidence of indigenous chlamydial infection was found in any of the human cell cultures used.

Association of radiolabelled C. trachomatis E/UW-5 with host cells. To measure the association of radiolabelled *C. trachomatis* EB with confluent cultures of genital and McCoy cells, ³H-labelled chlamydiae (³H-E/UW-5), at a m.o.i. of 50 to 100 particles per host cell, were centrifuged at 375 *g* for 20 min onto 1–2 × 10⁶ cells grown in 35 mm diameter tissue culture dishes. After inoculation, supernatants and cell monolayers were collected, placed on ice and precipitated in 5% (w/v) trichloroacetic acid, and assayed according to the method of Wyrick *et al.* (1978). Duplicate monolayers were incubated in DMEM for 48 to 72 h to determine percentages of inclusion-bearing cells visible with Giemsa stain.

Statistics. Student's *t*-test was used to analyse and compare differences in (i) the extent of *C. trachomatis* infection of McCoy cells and genital cells, (ii) the effects of various host cell manipulations on percentages of inclusion-positive genital cells and association of ³H-E/UW-5 with host cells, and (iii) the effects of cell culture medium on inclusion development in genital and McCoy cell cultures. Results for which *P* was <0.05 were defined as significant.

RESULTS

Chlamydial infection of human genital cells. The results of initial studies conducted to determine if *in vitro* cultures of human genital cells would be susceptible to *C. trachomatis* infection are presented in Table 1. Two representative experiments are shown for each chlamydial biovar. Both genital cell types were susceptible to *C. trachomatis* challenge, but McCoy cells supported more inclusion development than either genital cell type. In all cases the difference in percentages of inclusion-positive McCoy cells versus genital cells was significant ($P \leq 0.005$). The results from these experiments also indicated that columnar epithelial cells from endometrium supported chlamydial inclusion development to a greater extent than squamous epithelial cells derived from ectocervical explants ($P \leq 0.01$). The time points indicated in Table 1 were optimal for counting the chlamydial inclusions in McCoy and genital cells.

Fig. 1(a, b) shows Giemsa-stained inclusions in ectocervical and endometrial cells infected with *C. trachomatis*. Inclusions in the human genital cells were confirmed as *C. trachomatis* inclusions with a fluorescein-labelled monoclonal antibody (Microtrak Culture Confirmation Test, Syva Co., Palo Alto, Calif., USA) (Fig. 1c).

Effects of various manipulations on the extent of chlamydial inclusion development in genital cell cultures. Further studies were done to examine the effects of various manipulations reported to influence the extent of chlamydial infection in tissue culture cells. Genital cell cultures were (i) treated with DEAE-dextran (M_r 500000; Pharmacia) at $30 \mu\text{g ml}^{-1}$ before chlamydial inoculation (Kuo *et al.*, 1972), (ii) exposed to cycloheximide ($0.5 \mu\text{g}$ per ml DMEM) after inoculation, or (iii) grown in the presence of female sex hormones (Kaufman *et al.*, 1980): 10^{-7} M-17- β -oestradiol (Calbiochem) alone or 10^{-8} M-17- β -oestradiol in combination with 10^{-7} M-progesterone (Sigma).

The results of these treatments on the percentage of inclusion-bearing cells in the genital cell cultures are presented in Table 2. Each experiment represented tissue from a different patient. Pretreatment of either host cell with DEAE-dextran did not result in a significant increase in visibly infected cells. The addition of cycloheximide did result in a significant increase in the percentage of inclusion-positive ectocervical cells in experiments 1, 2, 3 and 6 (Table 2a; $P \leq 0.04$) and inclusion-positive endometrial cells in experiments 4 and 6 (Table 2b; $P = 0.02$). Finally, growth of ectocervical cells in hormone-supplemented medium had no obvious effect on percentages of inclusion-positive cells (Table 2a), but there were some cases where decreases in percentages of inclusion-positive cells were noted for endometrial cultures maintained in DMEM supplemented with hormones (Table 2b). With medium containing 17- β -oestradiol alone these decreases were significant for experiments 1 and 6 ($P \leq 0.03$), while cell cultures maintained in medium supplemented with a combination of 17- β -oestradiol and progesterone exhibited significant decreases, as observed in experiments 1, 5 and 6 ($P \leq 0.01$).

Association of radiolabelled C. trachomatis with genital cells. As a second approach to determine what effect(s) DEAE-dextran addition and hormone supplementation might have on chlamydial interactions with host cells, experiments were done to determine the extent of association of radiolabelled *C. trachomatis* with treated host cells immediately after inoculation. A representative experiment is shown in Table 3. Addition of DEAE-dextran resulted in a slight but non-significant enhanced association of radiolabelled organisms with all three cell types. Growth in medium supplemented with hormones resulted in a significant decrease in the percentage of host cell associated $^3\text{H-E/UW-5}$ with both genital cell types ($P \leq 0.02$). In contrast, the association of $^3\text{H-E/UW-5}$ with McCoy cells was not reduced by culture in hormone-supplemented medium. Giemsa stain analysis of inclusion-positive cells in duplicate cultures highlighted the differences observed for $^3\text{H-E/UW-5}$ association with treated cells with the exception of the DEAE-dextran group. Finally, these results were confirmed in a second experiment utilizing genital cells from a different patient.

In the experiment described in Table 3, the ratio of *C. trachomatis* particles per host cell was 100:1 for endometrial and McCoy cells and 50:1 for ectocervical cells. We have no information concerning the concentration of *C. trachomatis* receptors on the different cell types or the level of *C. trachomatis* required for host cell receptor saturation. Therefore, it is difficult to compare the

Table 1. Extent of *C. trachomatis* infection of cultures of ectocervical, endometrial and McCoy cells

13 mm diameter plastic coverslip cultures of ectocervical, endometrial and McCoy cells, containing $1-2 \times 10^5$ host cells, were inoculated with *C. trachomatis* by centrifugation at 375 g for 20 min. Chlamydial inocula were adjusted to produce inclusions in approximately 50 to 70% of equivalent numbers of McCoy cells. The medium for endometrial and McCoy cell cultures was DMEM before and after chlamydial inoculation. The medium for ectocervical cell cultures was MEM/D-valine before and DMEM after chlamydial inoculation.

<i>C. trachomatis</i>	Expt	Cell type	(% inclusion-positive genital cells)/ (% inclusion-positive McCoy cells)*	
			Ectocervical	Endometrial
Immunotype E (UW-5)†	1		5.8 ± 0.94/53.8 ± 1.0	22.0 ± 2.4/60.7 ± 1.2
	2		6.97 ± 0.93/55.7 ± 1.5	18.7 ± 0.6/49.0 ± 0.6
LGV (L2/434)‡	1		9.3 ± 1.5/57.5 ± 2.5	30.6 ± 0.8/79.4 ± 4.1
	2		7.3 ± 1.5/57.5 ± 2.5	36.7 ± 3.1/73.3 ± 1.5

* Infection was determined by counting chlamydial inclusions, visible with Giemsa stain, in an average of 300 cells per coverslip culture. Results are means ± SEM for triplicate cultures.

† Inclusions were counted 72 h post-inoculation.

‡ Inclusions were counted 48 h post-inoculation.

extent of bacterial association with the different cell types. However, the association of ³H-E/UW-5 appeared greater with McCoy cells than with either genital cell type. Similarly, the level of ³H-E/UW-5 association was approximately two-fold greater, on a per cell basis, with endometrial cells than with ectocervical cells (data not shown). These findings were in agreement with the three cell types with respect to the order of sensitivity to E/UW-5 infection determined by inclusion counting (Table 1).

Immunofluorescence analysis of early chlamydial interactions with genital and McCoy cell cultures. In a further attempt to investigate the nature of differences in the percentages of inclusion-bearing McCoy cells versus genital cells and endometrial cells versus ectocervical cells (Table 1), the pattern of E/UW-5 association with host cells was visualized by immunofluorescence with a fluorescein-labelled monoclonal antibody. Immediately after centrifugation-assisted inoculation with E/UW-5, infected cultures were washed twice with HBSS/HEPES, fixed with methanol and reacted with antibody. Fluorescent chlamydial particles were associated with approximately 30% of the cells in endometrial cultures and 15% of the cells in ectocervical cultures. In contrast, approximately 70% of the McCoy cells possessed adherent and/or internalized E/UW-5 particles. In all cell types, the associated chlamydiae appeared evenly distributed over the infected cells. These results indicated that, unlike McCoy cell cultures, a high percentage of the cells in both endometrial and ectocervical cultures failed to adsorb E/UW-5.

Centrifugation-assisted versus adsorption inoculation of genital cells. For adsorption inoculation, the same titre of infectious E/UW-5 was resuspended in a much reduced fluid volume (50 µl) and this inoculum was adsorbed to host cells for 60 min at 35 °C with agitation at 5–10 min intervals. The results (Table 4) indicated that genital cells were as susceptible to chlamydial infection after adsorption inoculation as they were after centrifugation-assisted inoculation. Analogous experiments with McCoy cells gave similar results (data not shown). Increases in centrifugal force from 375 g to 1000 g and in the time of centrifugation from 20 min to 60 min did not result in increased percentages of inclusion-bearing McCoy cells. In both cases approximately 50% of the McCoy cells contained chlamydial inclusions visible with Giemsa stain 72 h post-inoculation.

Effect of culture medium on E/UW-5 inclusion development. During our early studies on the interaction of *C. trachomatis* with human genital cell cultures, infections using MEM/D-valine resulted in a marked alteration, and, usually, reduction of inclusion development (Table 5). When inclusions did appear, they exhibited a distinct morphology, generally containing empty

Fig. 1. *C. trachomatis* E/UW-5 inclusions (arrowed) in experimentally infected genital cell cultures 72 h post-inoculation. (a) Ectocervical cells and (b) endometrial cells stained with Giemsa; bars, 50 μ m. (c) Endometrial cells stained with fluorescent antibody; bar, 100 μ m. (d) E/UW-5 inclusion morphology in MEM/D-valine; bar, 50 μ m.

Table 2. *Effects of DEAE-dextran, cycloheximide and hormones on the extent of C. trachomatis E/UW-5 inclusion development in ectocervical and endometrial cell cultures*

Host cell cultures on plastic coverslips, treated accordingly, were inoculated with *C. trachomatis* by centrifugation. Ectocervical cells were grown in MEM/D-valine before and DMEM after chlamydial inoculation. Endometrial cells were cultured in DMEM before and after chlamydial inoculation.

Expt	Cell treatment*	Percentage of inclusion-positive cell†			
		None	DEAE-dextran	Cycloheximide	Oestrogen + progesterone
(a) Ectocervical cells					
1		4.7 ± 1.0	5.2 ± 0.4	8.1 ± 0.3‡	4.5 ± 0.1
2		3.6 ± 0.2	7.9 ± 0.3‡	10.9 ± 0.8‡	6.3 ± 0.3
3		8.0 ± 0.4	8.1 ± 0.8	10.4 ± 0.3‡	—
4		5.8 ± 0.9	7.0 ± 0.1	5.4 ± 0.7	—
5		13.9 ± 0.6	—	17.0 ± 1.2	14.2 ± 2.2
6		11.6 ± 0.9	—	15.5 ± 0.8‡	9.8 ± 0.3
(b) Endometrial cells					
1		11.5 ± 0.3	15.0 ± 0.2	—	6.8 ± 1.2‡
2		13.5 ± 1.4	13.8 ± 0.8	—	13.9 ± 1.0
3		20.0 ± 1.7	20.9 ± 3.1	24.6 ± 0.5	—
4		18.7 ± 0.6	18.5 ± 1.0	23.3 ± 1.7‡	—
5		21.6 ± 0.2	22.2 ± 4.2	28.0 ± 4.0	22.4 ± 0.2
6		17.4 ± 1.5	16.8 ± 1.3	22.4 ± 0.8‡	11.4 ± 1.4‡

* DEAE-dextran at 30 µg per ml HBSS; cycloheximide at 0.5 µg per ml DMEM after inoculation; cells grown in medium with 17-β-oestradiol (10⁻⁷ M) or medium with a combination of progesterone (10⁻⁷ M) and 17-β-oestradiol (10⁻⁸ M).

† Determined by Giemsa staining 72 h post-inoculation. In (a) the corresponding levels of inclusion-positive McCoy cells, grown in DMEM, were 49%, 54% and 71% for experiments 1 and 2, 3 and 4 and 5 and 6, respectively. In (b) the corresponding levels of inclusion-positive McCoy cells were 36%, 49%, 54%, 59%, 52% and 47% for experiments 1 to 6, respectively. Results are means ± SEM for duplicate to triplicate coverslip cultures.

‡ Indicates where *P* values obtained from comparisons of the various cell treatments versus no treatment were <0.05.

Table 3. *Effects of DEAE-dextran and hormones on the association of ³H-labelled C. trachomatis E/UW-5 with genital and McCoy cell cultures*

Endometrial and McCoy cell cultures, containing 1 × 10⁶ cells per 35 mm diameter culture dish, were maintained in DMEM before chlamydial inoculation. Ectocervical cell cultures, containing 2 × 10⁶ cells per 35 mm diameter culture dish, were maintained in MEM/D-valine before chlamydial inoculation. Host cells were inoculated with ³H-labelled *C. trachomatis* by centrifugation. The chlamydial particle to host cell ratio was 100:1 for endometrial and McCoy cells and 50:1 for ectocervical cells.

Cell type	Cell treatment*	Percentage cell-associated TCA-precipitable counts†		
		None	DEAE-dextran	Oestrogen + progesterone
Endometrial		26.0 ± 0.5 (15.8)‡	31.7 ± 0.3 (15.7)	16.4 ± 0.8§ (8.3)
Ectocervical		21.1 ± 1.8 (11.2)	27.0 ± 1.6 (10.2)	13.4 ± 1.6§ (6.3)
McCoy		45.6 ± 1.3 (59.5)	52.4 ± 3.0 (60.3)	53.3 ± 6.3 (56.3)

* As described in Table 2.

† Percentages of cell-associated TCA-precipitable (ppt) counts were calculated as follows: % = [(cell-associated ppt counts)/(cell-free ppt counts + cell-associated ppt counts)] × 100.

‡ Numbers in parentheses are the percentage of inclusion-positive cells visible with Giemsa stain 72 h post-inoculation.

§ *P* values for ³H-E/UW-5 association with genital cells in hormone-supplemented medium versus non-supplemented medium were ≤0.02.

Table 4. *Infection of genital cell cultures after centrifugation-assisted or adsorption inoculation with C. trachomatis E/UW-5*

Before chlamydial inoculation endometrial cells were grown in DMEM and ectocervical cells were grown in MEM/D-valine. The post-inoculation culture medium for both cell types was DMEM. Chlamydial particles suspended in 0.2 ml DMEM were centrifuged onto host cells at 375 *g* for 20 min. Equivalent numbers of chlamydial particles, suspended in 50 μ l DMEM, were adsorbed to host cells at 35 °C for 60 min.

Expt	Cell type	Percentage of inclusion-positive cells after:*	
		Centrifugation	Adsorption
1	Endometrial	25.0 $\pm$ 1.8	23.3 $\pm$ 0.6
2	Endometrial	20.0 $\pm$ 1.7	20.7 $\pm$ 0.5
1	Ectocervical	8.0 $\pm$ 0.4	6.8 $\pm$ 0.5
2	Ectocervical	5.8 $\pm$ 0.9	5.1 $\pm$ 0.4

* Determined by Giemsa staining 72 h post-inoculation. Results are means $\pm$ SEM for triplicate coverslip cultures.

Table 5. *Effect of cell culture medium on C. trachomatis E/UW-5 inclusion development in genital and McCoy cell cultures*

Host cells were inoculated with *C. trachomatis* by centrifugation. Endometrial and McCoy cell cultures were maintained in DMEM before inoculation. Ectocervical cell cultures were maintained in MEM/D-valine before inoculation.

Expt	Cell type	Post-inoculation medium . . .	Percentage of inclusion-positive cells*	
			DMEM†	MEM/D-valine
1	Endometrial		25.0 $\pm$ 1.8	4.6 $\pm$ 0.6
2	Endometrial		18.7 $\pm$ 0.6	2.2 $\pm$ 0.4
1	Ectocervical		7.9 $\pm$ 0.3	0.41 $\pm$ 0.1
2	Ectocervical		9.3 $\pm$ 0.8	0.25 $\pm$ 0.1
1	McCoy		52.3 $\pm$ 1.3	1.0 $\pm$ 0.3
2	McCoy		53.2 $\pm$ 0.5	6.0 $\pm$ 2.4

* Determined by Giemsa staining 72 h post-inoculation. Results are means $\pm$ SEM.

† With all cell types the level of infection was significantly greater in DMEM than in MEM/D-valine ($P \leq 0.001$).

centres with a few chlamydiae lining the periphery of the inclusion membrane (Fig. 1*d*). This reduction of E/UW-5 inclusion development was significant for both genital and McCoy cell cultures ($P \leq 0.001$). Since there were no differences in the initial attachment and internalization of E/UW-5 particles by host cells in MEM/D-valine or DMEM, we decided to investigate the possibility that inhibition of inclusion development in MEM/D-valine was the result of nutrient deprivation (Allan & Pearce, 1983).

Various methods were used in an effort to reverse the inhibition of inclusion development observed in MEM/D-valine, including addition of 4 mM-L-valine to basal MEM/D-valine, addition of cycloheximide at 0.5 μ g per ml MEM/D-valine after chlamydial inoculation and culture of host cells in DMEM after chlamydial inoculation. With endometrial cells (Table 6*a*), no post-inoculation culture condition was capable of returning the level of inclusion development to that observed in DMEM or DMEM plus cycloheximide. The addition of cycloheximide and L-valine to basal MEM/D-valine did result in a slight increase in percentages of inclusion-bearing cells when compared to MEM/D-valine alone. However, these percentages were significantly lower than the percentage of inclusion-positive endometrial cells cultured in DMEM ($P \leq 0.003$). In contrast, addition of either cycloheximide or L-valine to basal MEM/D-

Table 6. *Effects of various culture conditions on C. trachomatis E/UW-5 inclusion development*

Coverslip cultures of endometrial and McCoy cells were maintained in DMEM before centrifugation-assisted inoculation with *C. trachomatis*. Ectocervical cells were maintained in MEM/D-valine before inoculation with *C. trachomatis*. At 0, 24, 48 and 72 h post-inoculation the culture medium was changed from MEM/D-valine to one of the indicated conditions.

Medium	Post-inoculation medium shift time (h)	Percentage of inclusion-positive cells*			
		0	24	48	72
<i>(a) Endometrial cells</i>					
DMEM		21.0 ± 0.3	10.3 ± 1.5	4.9 ± 0.4	0.6 ± 0.2
DMEM + cycloheximide†		27.4 ± 0.6	14.7 ± 0.4	8.8 ± 0.2	1.1 ± 0.4
MEM/D-valine		1.3 ± 0.2	0.8 ± 0.3	1.1 ± 0.4	0.1 ± 0.1
MEM/D-valine + cycloheximide†		4.8 ± 1.2	5.7 ± 0.9	2.6 ± 0.4	0.9 ± 0.2
MEM/D-valine + L-valine‡		7.1 ± 0.5	2.3 ± 0.2	2.0 ± 0.3	1.1 ± 0.4
<i>(b) Ectocervical cells</i>					
DMEM		7.0 ± 0.9	5.4 ± 0.3	2.3 ± 0.2	0.8 ± 0.2
DMEM + cycloheximide†		12.4 ± 0.2	8.8 ± 1.9	3.2 ± 0.1	2.0 ± 0.3
MEM/D-valine		0.2 ± 0.1	0.3 ± 0.3	0.1 ± 0.1	0.0
MEM/D-valine + cycloheximide†		12.9 ± 0.5	7.7 ± 1.6	3.2 ± 1.3	1.3 ± 0.5
MEM/D-valine + L-valine‡		4.7 ± 0.2	2.1 ± 0.1	0.9 ± 0.2	1.3 ± 0.1
<i>(c) McCoy cells</i>					
DMEM		52.3 ± 1.3	35.8 ± 2.9	10.9 ± 0.8	2.1 ± 0.4
DMEM + cycloheximide†		77.8 ± 2.7	47.9 ± 2.3	14.9 ± 0.5	1.6 ± 0.6
MEM/D-valine		1.0 ± 0.3	0.8 ± 0.1	1.9 ± 0.4	0.4 ± 0.02
MEM/D-valine + cycloheximide†		78.2 ± 1.4	46.8 ± 0.3	11.6 ± 0.5	1.6 ± 0.7
MEM/D-valine + L-valine‡		51.1 ± 1.0	26.9 ± 0.8	11.8 ± 0.6	0.8 ± 0.1

* Inclusions were counted in an average of 300 cells per Giemsa-stained coverslip culture 72 h after shifts in media. Triplicate samples were run for each time and each medium condition. Results are means ± SEM.

† Cycloheximide at 0.5 µg (ml culture medium)⁻¹.

‡ L-Valine at 4 mM.

valine returned the percentage of inclusion-positive ectocervical cells and McCoy cells to those levels observed when DMEM plus cycloheximide or DMEM alone were used as the post-inoculation media, respectively (Table 6*b* and *c*). In concurrent experiments, where MEM/D-valine was maintained in the cultures for 24, 48 and 72 h post-inoculation and then shifted to a growth-permissive condition, a nearly constant decrease in the percentage of inclusion-positive cells was observed, indicating that the growth potential of internalized E/UW-5 was gradually lost in MEM/D-valine. These results were confirmed on two separate occasions with each cell type.

DISCUSSION

Chlamydial genital infection in the adult female human involves the columnar epithelium lining mucosal surfaces of the uterus (Swanson *et al.*, 1975; Mårdh *et al.*, 1981) and fallopian tubes (Moller *et al.*, 1979) but not the squamous epithelium of the ectocervix (Evans, 1982; Mitao *et al.*, 1984). *C. trachomatis* is capable of infecting the atrophic squamous epithelium of the anoestrogenic prepubertal vagina to cause vaginitis (Bump, 1985). In the current study interaction with two different genital cell types was investigated. The columnar epithelial cell cultures were susceptible to chlamydial infection, yielding a reproducible infection rate of approximately 20% despite heterogeneity in the genetic background of tissue donors. However, this infection rate was far less than might be expected for a major target cell, perhaps suggesting some intrinsic resistance of endometrial epithelium to chlamydial infection; a factor which could contribute to the nature of *C. trachomatis* infection *in vivo*. In humans, *C. trachomatis* is noted for giving rise to chronic infections which are often inapparent or asymptomatic. A limitation in the number of susceptible cells on a given mucosal surface suggests a possible mechanism, other than host immune response, which may play a role in controlling the severity of infections *in vivo*.

Limited growth of chlamydiae in the squamous cell cultures derived from ectocervical explants also occurred. It is not clear whether infection in these cells represents a previously unrecognized reservoir for chlamydial infection, an *in vitro* alteration in susceptibility to *C. trachomatis* challenge, or a possible contamination with susceptible squamocolumnar epithelial cells. The squamous cells that emerge from ectocervical explants are viable, dividing, nucleated cells most probably originating from the basal layers of the explants. Our culture methods may therefore have by-passed the normal organization of host-protective cell layers to expose susceptible cells which may not normally encounter infectious chlamydiae during the course of a natural infection.

Several explanations are possible for the low infection rates of genital cells compared with McCoy cells. One possibility, a partial adaptation of *C. trachomatis* to McCoy cells resulting from propagation in this cell line, was not directly examined in this study. Another is that internalized chlamydiae failed to develop into visible inclusions. This certainly occurred in cultures of both genital and McCoy cells but only in a small percentage of those cells. The major determinant in the degree of host cell susceptibility appeared to be a difference in the initial association of E/UW-5 with the three cell types. Immunofluorescence staining of cell-associated chlamydiae, immediately after inoculation, demonstrated that many cells in endometrial and ectocervical cultures were resistant to challenge at this early stage of infection. Supporting evidence was obtained from comparisons of the level of ³H-E/UW-5 association, adjusted for equivalent numbers of the different host cells; an order of association identical to the order of sensitivity determined with Giemsa and immunofluorescence staining techniques was observed. Other investigators have also observed an irregular distribution of adherent micro-organisms with primary cultures of human epithelium. Stephens *et al.* (1983) reported that piliated *Neisseria meningitidis* bound preferentially to non-ciliated cells in nasopharyngeal organ cultures. Studies on the attachment of *Neisseria gonorrhoeae* to explants of human genital tract epithelium revealed that gonococci adhered in higher numbers to the transitional cells of cervical tissue than to squamous or fallopian tube epithelium (Draper *et al.*, 1980). Sobel *et al.* (1982), using a vaginal explant culture system, observed bacterial and fungal adherence at higher levels to epithelial cells in the process of desquamation. It is tempting to speculate that resistance to chlamydial attachment results from the absence of specific receptors on many of the genital cells, perhaps as a function of cell cycle stages and/or different states of cellular differentiation.

In an effort to examine further the resistance/sensitivity of the genital cells to *C. trachomatis* infection we used various manipulations known to alter the susceptibility of HeLa and McCoy cells to the trachoma biovar strains. Treatment with the polycation DEAE-dextran has been reported to increase infections in host cells (Kuo *et al.*, 1972; Rota & Nichols, 1973; Soderlund & Kihlstrom, 1982). In HeLa cells this may involve neutralization of the negatively charged host cell surface, allowing the negatively charged chlamydiae a sufficiently close approach for adsorption to target receptors (Kuo *et al.*, 1973). Chlamydiae apparently lack a smooth lipopolysaccharide and pilus surface appendages used by other mucosal pathogens to bridge electronegative barriers between bacterium and host. Inoculation of cell cultures by centrifugation also enhances infection (Weiss & Dressler, 1960; Darougar *et al.*, 1974). In our hands neither of these two enhancement techniques altered the extent of chlamydial infection in the genital or McCoy cell cultures. The reason(s) for this lack of enhancement is unknown, but these experiments suggest a basic difference, perhaps at the level of the host cell surface, between the cultures we have used and the HeLa and McCoy cultures used in other studies. However, our experience for several years has shown that centrifugation up to 1000 *g* does not increase the number of cells infected by our *C. trachomatis* or *C. psittaci* strains if the EB are suspended in a very small volume of inoculum. The diffusion rate of this non-motile parasite, governed by Brownian motion, in the presence of 0.1 M-salts is evidently adequate to permit infection by adsorption.

During the menstrual cycle, the epithelial and stromal components of human endometrium undergo both structural and biochemical changes in response to variations in steroid hormone concentration. The influence of hormones on genital chlamydial infection in the adult female is

not clear. It has been observed that women taking oral contraceptives appear to be at reduced risk from ascending spread of cervical infection (Senanayake & Kramer, 1980), although it is not known whether this situation is due to a decrease in target cell susceptibility or to increased mucus production in the cervix which in turn blocks the ascent of infectious chlamydial particles. The effect of hormone supplementation of the medium on the sensitivity of cells to chlamydial infection has recently been investigated using some other cell culture systems. HeLa cells, incubated in medium supplemented with 17- β -oestradiol exhibited increased sensitivity to the agent of guinea pig inclusion conjunctivitis (Moses *et al.*, 1984). In contrast, female sex hormones in high concentrations have been reported to 'negatively influence' chlamydial infection in McCoy cells (Mårdh, 1981). Our results on the effect of hormones on infection in genital cells must be interpreted with caution since the physical separation of epithelial cells from the stromal cell component of endometrium may result in a lack of hormone responsiveness in the cultured epithelial cells (Kirk & Irwin, 1980; Satyaswaroop & Mortel, 1981). Measurements of $^3\text{H-E/UW-5}$ association with the genital cells revealed a decrease in association for cells cultured in hormone-supplemented medium, indicating that the effect on host susceptibility may result through alterations in attachment and/or internalization of chlamydiae by these cells. In contrast, association of $^3\text{H-E/UW-5}$ with McCoy cells was unaffected by growth in hormone-containing medium.

There was a marked inhibition of inclusion development when MEM/D-valine was used for the culture of infected ectocervical cells. This effect was reproduced with endometrial and McCoy cell cultures, indicating that cell type was not a determining factor. Since L-valine is a required amino acid for chlamydial growth in McCoy cells (Allan & Pearce, 1983), it is possible that its absence from MEM/D-valine accounted for both the reduction in inclusion number and the abnormal inclusion morphology observed with this medium. Addition of cycloheximide or L-valine to MEM/D-valine after E/UW-5 inoculation returned the level of inclusion-bearing ectocervical and McCoy cells to approximately the same level observed when DMEM was used as the post-inoculation culture medium. This observation was analogous to that described by Hatch (1975) in which *C. psittaci* growth in L cells had been inhibited by isoleucine starvation. Apparently, if a required amino acid is absent or below a threshold level, then chlamydial growth does not ensue. The inhibition may be reversed if the appropriate amino acid is exogenously added or indigenously supplied as a result of host protein turnover in the presence of the eukaryotic protein synthesis inhibitor cycloheximide. Freedom from the inhibitory effect of MEM/D-valine could not be completely achieved with endometrial cells, although a slight increase in the percentage of inclusion-positive cells did occur in the presence of cycloheximide and L-valine. This failure may underlie a difference in the transport of L-valine by endometrial cells or the rate at which this amino acid is consumed and made available for chlamydial metabolism.

It has been suggested by others that the limited availability of required nutrients may in part contribute to the persistent, subclinical nature of chlamydial infections in humans and other animals (Morgan, 1956; Hatch, 1975). It is not difficult to envisage a latent infection in which respiring but non-multiplying chlamydiae survive within phagosomes of host cells, protected from specific immune defences until nutrient conditions arise which favour growth and replication. If such situations occur *in vivo*, then it should be possible to mimic this situation in tissue culture cells. Therefore, the time span over which inhibited inclusion development could be reversed in genital and McCoy cell cultures was examined. In all cell types there was a nearly constant decrease in the number of inclusion-positive cells for each increase in the time at which medium was shifted to a growth-permissive condition. These findings suggest that the growth potential of internalized E/UW-5 is gradually lost in the absence of required L-valine. However, it is possible that we have not used the proper condition(s) required to stimulate resumption of chlamydial multiplication in cells containing viable E/UW-5.

It is evident from our studies that several differences exist between the cultures we have used and the HeLa and McCoy cell cultures used by others. It is likely that future studies will reveal still more differences. There are potential pitfalls in using the primary genital cell cultures, including (i) the quantity of tissue that can be obtained from a given patient, (ii) the limited life

span of primary epithelial cells in culture, (iii) the necessity of making comparisons with tissue from genetically heterogeneous donors and (iv) the continued availability of endometrial and ectocervical tissue. However, we believe that the merits of this system outweigh the disadvantages. Most importantly, the use of these cultures provides us with a unique opportunity to study the interaction of *C. trachomatis* with its natural human target tissue. Experimentation with natural target cells should enhance our chances of identifying features of both host and parasite which contribute to the nature of chlamydial infections *in vivo*.

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