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Estrogens and *Chlamydia trachomatis* (42396)

BARRETT SUGARMAN*,† AND PHYLISHA AGBOR*

**Veterans Administration Medical Center and Baylor College of Medicine, Houston, Texas 77030, and †Michigan State University, East Lansing, Michigan 48824*

Abstract. Isolates of *Chlamydia trachomatis* were inoculated in nonreplicating McCoy cells and incubated for 48 hr with various concentrations of hormones. Only the estrogens, particularly 17-β-estradiol, had an affect on the subsequent infection of the McCoy cells by the *Chlamydia.* Exposure to 2–4 ng/ml (10^{-8} *M*) estradiol during inoculation and incubation was associated with no change in the initial binding of *Chlamydia* to McCoy cells, but significantly more (about twofold) *Chlamydia* inclusions in the McCoy cells after 48 hr incubation. This effect was dose dependent, could be blocked with anti-estrogens, and also occurred with replicating McCoy cells. Localization studies suggest that this effect on the infectivity of *Chlamydia trachomatis* is dependent upon initial interactions of estrogens with McCoy cells. Both light microscopy and electron microscopy of the McCoy (fibroblast) cells showed no morphological changes after exposure to the estrogens under the incubation conditions employed in these studies. Estrogens may modify host susceptibility to *Chlamydia* infections in a manner independent of morphological changes in mammalian cells.

The hormonal milieu may alter host susceptibility to a variety of infections. Most often, estrogens have been studied in this regard. For example, estrogens may increase the susceptibility of mice to *Listeria monocytogenes* and disseminated gonococcal infections due to alterations in the host immune system (7, 14, 15). Other studies, mostly of fungi, suggest that the ability of microorganisms to bind hormones and changes induced subsequently may alter host susceptibility to infections in a manner directly related to the hormonal milieu (10, 13). Further, recent studies have suggested that the binding of microorganisms to receptor cells may also be modified by hormones, especially estrogens (3, 6, 19).

Infections with *Chlamydia trachomatis* may also be more frequent after exposure of the host to female sex hormones (16, 20, 21). Because *Chlamydia* infections so frequently involve the genitourinary tract and it is well known that sex hormones modify the morphology of target cells in this area, prior work has focused on this aspect. In fact, earlier studies suggest that gross morphological alterations in receptor cells induced by female sex hormones could well account for changes in susceptibility to infection (16, 20, 21).

Prior studies with hormones suggest that their presence may alter *in vitro Chlamydia* infections (4, 11, 18). Addition of cortisol to replicating McCoy cells was associated with an increased number of subsequently infected cells in one report (4). Estradiol enhanced inclusion counts of guinea pig inclusion conjunctivitis chlamydial agent in replicating HeLa cells, but not McCoy cells, in another study (11).

Hormones could alter susceptibility to *Chlamydia* infection by altering the immune response of the host, altering other host factors such as mucus production or host cell structure, altering the virulence of the *Chlamydia,* or by other mechanisms. We attempted to systematically study the effect of hormones on *C. trachomatis* infection of cells. We initially chose nonreplicating (exposed to cycloheximide) fibroblastic receptor to cells to see if other mechanisms not dependent upon receptor cell growth might modify susceptibility to infection (especially since the outer layers of mucous membranes that are frequently infected by *Chlamydia* are not actively replicating).

Material and Methods. *Chlamydia.* Human isolates of *C. trachomatis* (a D-serotype of nongonococcal urethritis isolate and a lymphogranuloma venereum isolate) infectious elementary bodies (17) were stored in complete media with antibiotics (see below) with

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20% supplemental v/v fetal bovine serum (Sterile Systems, Inc., Logan, Utah) in aliquots stored in liquid nitrogen. Inoculations for each experiment were made from the same container after it was quickly warmed at 37°C. Inocula used were of known levels of infectivity, calculated from trials using other containers prepared in an identical manner. Inocula were used which did not cause appreciable lysis of the cells used for cultivating the *Chlamydia*. Both high (about 10% control McCoy cells ultimately infected) and low (about 2%) level inoculants were used for these experiments.

Hormones. Hormones were crystalline pure preparations (Sigma Chemical Co., St. Louis, Mo.) except for follicle-stimulating hormone and luteinizing hormone from porcine and equine pituitaries. Hormones and the antiestrogens were dissolved in 0.05 *M* phosphate-buffered saline without calcium or magnesium (PBS; pH 7.3). Estrogens, progesterone, and testosterone were dissolved in 100% ethanol initially, and then in PBS, with final ethanol concentrations less than 0.1% (v/v). The antiestrogen tamoxifen was graciously provided by Stuart Pharmaceuticals (Wilmington, Del.).

Assay. McCoy cells (mouse fibroblasts) were grown to near confluency on 12-mm diameter round glass coverslips in complete medium with antibiotics [CMA; Eagle's minimal essential medium (GIBCO, Grand Island, N.Y.)] supplemented with 10% v/v fetal bovine serum (Sterile Systems; Inc.), 40 μg/ml gentamicin (Sigma), 2.5 μg/ml amphotericin B (Squibb, Princeton, N.J.) and 2 m*M* glutamine (Sigma) in a humidified atmosphere of 95% air and 5% CO at 37°C.

The coverslips were placed in 1-dram glass vials and an 0.2-ml inoculum of *Chlamydia* with various concentrations of hormones was added to each vial. These vials were centrifuged at 2800*g* for 60 min at 32°C, and then 2 ml of 37°C overlay media [CMA with 0.72 mg/ml (4 m*M*) glucose (Sigma) and 1 μg/ml cycloheximide (Sigma)] with the same hormones at the same concentrations was added to each vial. These vials were incubated in the same 37°C humidified atmosphere previously mentioned for 48 hr. Cultivated *Chlamydia* were detected with an iodine staining procedure. Briefly, overlay media was removed from the vials, 1 ml of methanol was added, quickly removed, and another 1 ml added for 15 min at ambient temperature. This was removed and 1 ml of (5% w/v KI, 5% iodine) iodine stain (Jones' Iodine) reagent was added to each vial and incubated for 20 min at ambient temperature. The iodine was removed and the coverslips were placed on a slide with clear nail polish and examined under 400× light microscopy for intracytoplasmic (glycogen-containing) inclusions. Every cell was examined in some of the experiments, and in the others, 100 cells in each of 10 randomly selected fields were examined from each coverslip. Initial concentrations of the hormones used (depicted in Table I) represent several times the normal maximum nonpregnant human serum concentrations. For each experiment, identically prepared control diluents without supplemental hormones were also studied. Baseline concentrations of hormones in the CMA were less than 0.1% of the final concentration of hormones added in Table I except for the baseline concentrations of insulin (0.7 μU/ml), and L-thyroxine (0.016 μg/ml). To assay the binding of *Chlamydia* to McCoy cells, monoclonal antibodies (Syva Corp., Palo Alto, Calif.) to *Chlamydia* were added to slightly less confluent McCoy cells (to allow individual counting of the bound or-

TABLE I. EFFECT OF HORMONES ON *Chlamydia* INFECTION OF MCCOY CELLS[a]

Hormone (amount)	McCoy cells with inclusions
None (control)	2.3 ± 0.6
17-β-Estradiol (1 ng/ml)	3.1 ± 0.5[b]
Estriol (1 ng/ml)	3.0 ± 0.7[b]
Estrone (1 ng/ml)	2.6 ± 0.8
Follicle-stimulating hormone (60 mIU/ml)	2.2 ± 0.5
Hydrocortisone (0.4 μg/ml)	2.4 ± 0.6
Insulin (20 μIU/ml)	2.3 ± 0.7
Luteinizing hormone (80 mIU/ml)	2.1 ± 0.6
Progesterone (20 ng/ml)	2.2 ± 0.8
Testosterone (20 ng/ml)	2.3 ± 0.4
L-Thyroxine (20 μg/ml)	2.5 ± 0.7

[a] Data expressed as mean percentage McCoy cells with *Chlamydia* inclusions ±1 SD. Each percentage represents about 1000 cells with inclusions.

[b] $P < 0.05$ compared with control.

ganisms) after 1 hr centrifugation of the *Chlamydia* (D-serotype) inoculant with hormones and McCoy cells and rinsing of the McCoy cells. After rinsing off unbound antibodies, bound *Chlamydia* to each of 100 McCoy cells in five randomly selected fields per slide were counted with a fluorescent microscope. Experiments were repeated at least five times.

Statistics. Differences were analyzed with nonparametric Wilcoxon signed rank tests and rank sum tests, with $n \geq 6$.

Results. *McCoy cells.* McCoy cells used averaged $1.6 \pm 0.2 \times 10^5$ cells/cm^2 at the time of *Chlamydia* inoculation. More than 99.9% of cells were viable using Trypan blue staining (12).

Effect of hormones. Table I shows the effect of various hormones on subsequent infection of McCoy cells by a low level inoculant of *C. trachomatis* (D-serotype). At these hormone concentrations (several times the normal maximum nonpregnant human serum concentrations) only the estrogens, especially 17-β-estradiol, had any effect on subsequent infectivity. These effects were highly reproducible and more prominent with high level inoculants (Fig. 1).

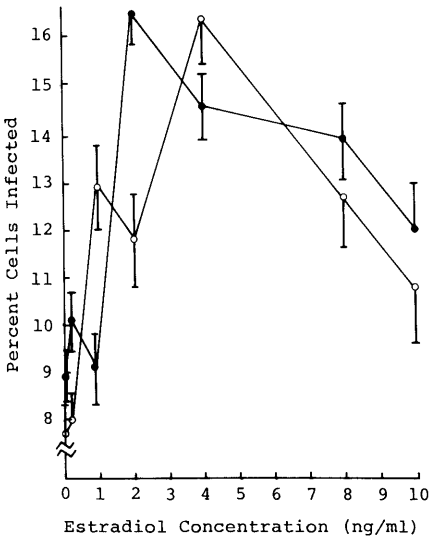

FIG. 1. Effect of various 17-β-estradiol concentrations on the number of *Chlamydia* inclusions in McCoy cells. ○, D-serotype; ●, LGV isolate. Vertical bars represent ±1 SD.

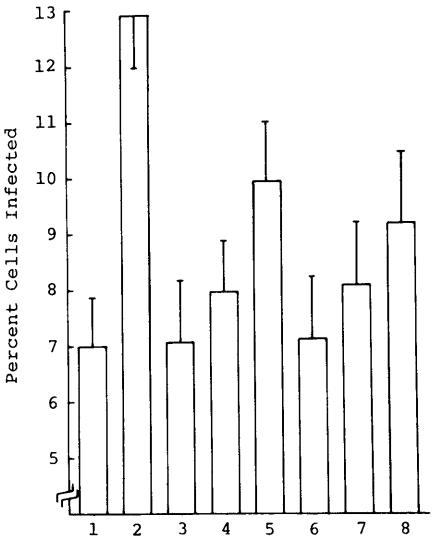

FIG. 2. Effect of anti-estrogens alone and with estrogens on the number of *Chlamydia* inclusions in McCoy cells. D-serotype *Chlamydia* incubated for 48 hr with 1 = control; 2* = estradiol, 2 ng/ml; 3 = tamoxifen, 0.2 ng/ml; 4 = tamoxifen, 2 ng/ml; 5* = tamoxifen, 20 ng/ml; 6 = estradiol, 2 ng/ml, and tamoxifen, 0.2 ng/ml; 7 = estradiol, 2 ng/ml, and tamoxifen, 2 ng/ml; 8* = estradiol, 2 ng/ml, and tamoxifen, 20 ng/ml. Vertical bars represent ± 1 SD. *$P < 0.05$, compared with No. 1 control.

Effect of increasing estrogen concentrations. The dose-response curve in Fig. 1 shows that with nanogram per milliliter concentrations of estradiol, increasing concentrations were associated with greater infectivity of the McCoy cells using high level inoculants of both *Chlamydia* isolates. This effect was maximal with 2–4 ng/ml estradiol (7.4×10^{-9} to 1.5×10^{-8} *M*) and significant ($P < 0.02$). Anti-estrogens (tamoxifen and nafoxidine) muted the increase associated with estradiol and displayed a weak estrogenic effect at the highest concentrations. Representative data are depicted in Fig. 2.

Effect of hormones on McCoy cells. Using 1000× oil immersion light microscopy, the McCoy cells incubated for 48 hr with the hormones in Table I and up to 10 ng/ml estradiol in overlay media appeared no different than control cells. There was also no difference in cell protein or viability (data not shown). Scanning electron microscopy of confluent McCoy cells on coverslips was performed after

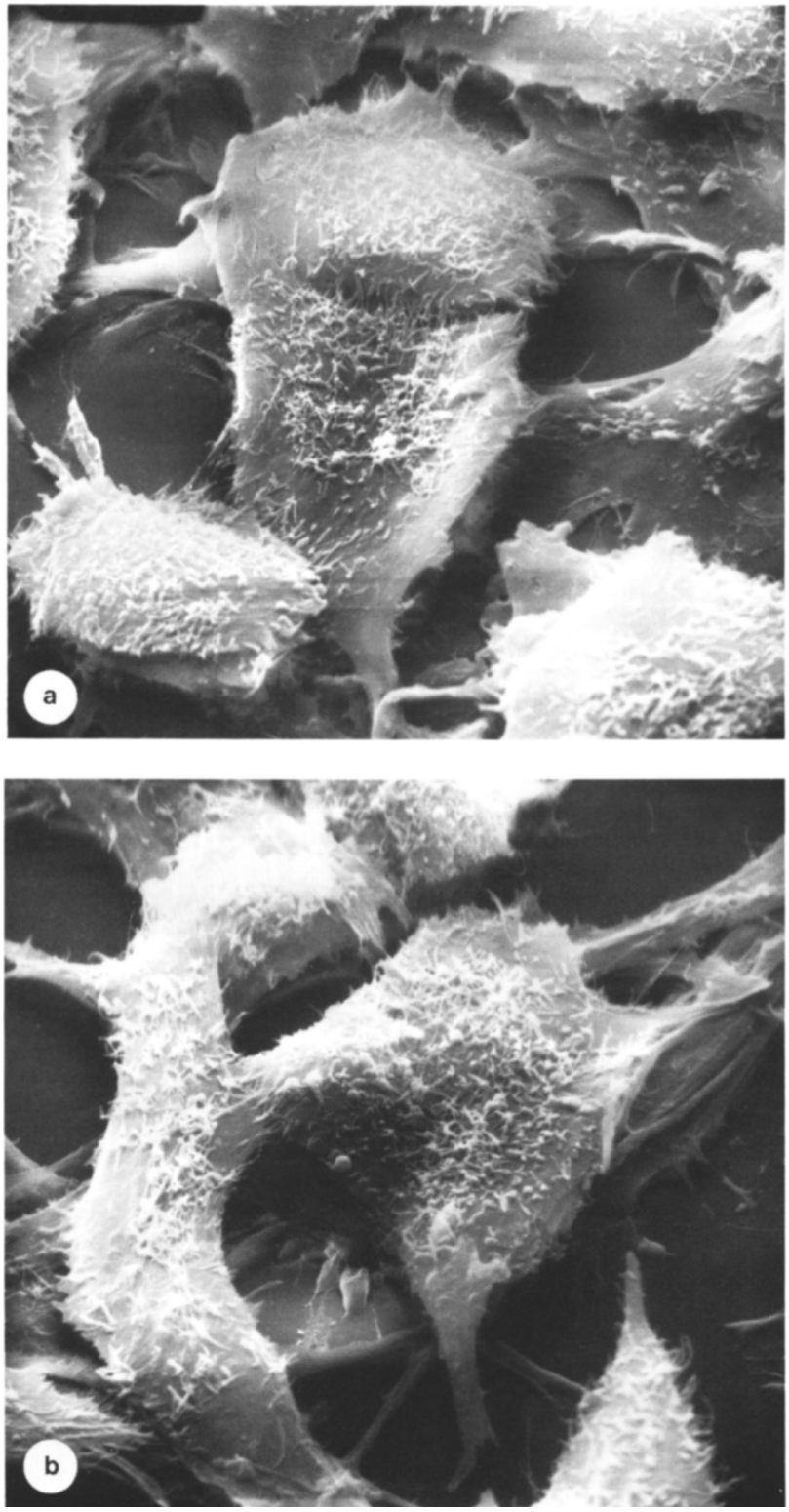

FIG. 3. Photomicrographs of McCoy cells exposed to estradiol for 48 hr, 2700×. (a) Control media; (b) 10 ng/ml estradiol.

48 hr incubation at 37°C with up to 10 ng/ml estradiol. No differences between control and estradiol-exposed cells in shape, size, number, number of microvilli, or large pseudopods was noted (Fig. 3).

Localization studies. Preincubation of confluent McCoy cells for 24 hr with supplemental nanogram per milliliter estradiol and overlay media (containing 1 μg/ml of the eukaryotic protein synthesis inhibitor, cycloheximide) was followed by removal of the media, two rinses, replacement with fresh overlay media (without supplemental estrogens), and high level *Chlamydia* inoculation. An increase in McCoy cells with inclusions was noted, similar to the results with 48 hr of concurrent supplemental estrogen incubation; however, these effects remained more prominent with higher estradiol concentrations (Fig. 4). Preincubation with estradiol and anti-estrogens muted this effect (Fig. 4).

Binding of Chlamydia to McCoy cells. After 1 hr incubation, no difference in the number of bound *Chlamydia* per McCoy cell was noted for control media (11.6 ± 3.8 *Chlamydia* per McCoy) or 2 ng/ml supplemental estradiol (11.7 ± 3.5 *Chlamydia* per McCoy; $n = 6$). Only a small percentage of bound *Chlamydia* after 1 hr ever developed into intracellular inclusions (above data and Fig. 1).

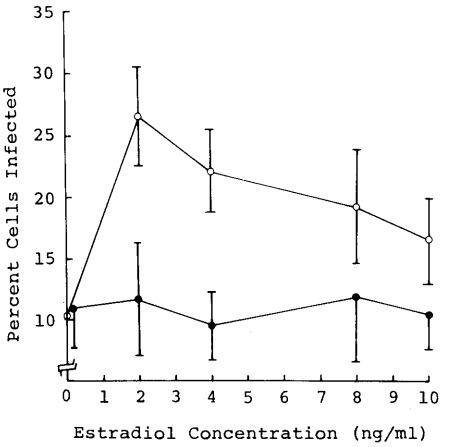

FIG. 4. Effect of preincubation of McCoy cells with varying concentrations of estradiol (○) or estradiol and 2 ng/ml tamoxifen (anti-estrogen) (●) on the number of *Chlamydia* inclusions. McCoy cells were incubated for 24 hr at 37°C with estradiol ± tamoxifen, rinsed twice, inoculated with *Chlamydia*, and incubated for 48 hr. Vertical bars represent 1 SD. $P < 0.05$ for each estradiol concentration compared with control.

Replicating cells. Experiments were repeated with slightly less than confluent McCoy cells and overlay media without cycloheximide. Everything else was performed as in the earlier mentioned experiments. After 48 hr incubation, there was no difference in the number of McCoy cells after control or supplemental estrogens exposure; however, cells exposed to 17-β-estradiol, prednisolone, and hydrocortisone (but not other hormones) had more inclusions than control cells (Table II).

Other Chlamydia. Results for all these experiments were analogous with the LGV *Chlamydia* isolate and low (about 2% control McCoy cells with inclusions) or high (about 10% control cells with inclusions) level inoculants (data not shown).

Discussion. These data show that increasing concentrations of estradiol are associated with greater infectivity of McCoy cells by *C. trachomatis,* but not increased binding of *Chlamydia* to the McCoy cells. Binding of *Chlamydia* to receptor cells is a rapid, integral step in the internalization and infection of mammalian cells by this organism; however, avid binding of some *Chlamydia* isolates to certain mammalian cells with only a small percentage displaying subsequent *Chlamydia* internalization has been reported before (1, 2, 5, 8, 9). Specificity of estrogens in causing this effect was noted by the failure of other hormones to do so, the dose-responsiveness of this effect, and the ability of antiestrogens to mute this effect. This effect was noted whether the McCoy cells were replicating or not. Findings of more guinea pig inclusion conjunctivitis *Chlamydia* inclusions in replicating HeLa cells, but not McCoy cells, after exposure to estradiol (that could also be blocked with tamoxifen) under comparable conditions were recently reported (11).

Animal models of *C. trachomatis* infections have shown that sex hormones may alter infectivity of the microorganisms; however, these studies noted marked morphological changes of the infected cells caused by the hormones. The *in vitro* studies reported here show that another mechanism may also be responsible

TABLE II. EFFECT OF HORMONES ON *Chlamydia* INFECTION OF REPLICATING McCOY CELLS[a]

Hormone (amount)	McCoy cells with inclusions
None (control)	10.3 ± 2.2
17-β-estradiol (2 ng/ml)	18.6 ± 2.9[b]
Follicle-stimulating hormone (60 mIU/ml)	10.7 ± 2.6
Insulin (20 μIU/ml)	9.9 ± 2.4
Hydrocortisone[c] (μg/ml)	
0.4	22.4 ± 3.5[b]
1	24.7 ± 3.8[b]
10	21.2 ± 3.6[b]
Prednisolone[c] (μg/ml)	
1	13.6 ± 2.7[b]
10	29.1 ± 4.3[b]
40	24.3 ± 3.8[b]

[a] Data expressed as mean percentage McCoy cells with *Chlamydia* inclusions ±1 SD.
[b] $P < 0.05$ compared with control.
[c] These concentrations had no effect on the number of inclusions in nonreplicating cells.

in a manner independent of morphological changes of the mammalian cells. Because *Chlamydia* infect a variety of cells and organs (17) including many which are not known to be responsive to sex hormones, the finding that another mechanism may alter susceptibility to infections may well have clinical significance. Further, these studies show that estrogens can alter susceptibility to *Chlamydia* infection in a manner independent of hormone-induced changes in the immune system (7, 14, 15) since this *in vitro* system would not be altered by any such actions.

The localization of this estrogen-mediated effect on infectivity to the McCoy cells and lack of effect on *Chlamydia* binding and McCoy cell morphology suggest that it is likely mediated by changes in *Chlamydia* internalization. Hormones, in particular estrogens, can alter host susceptibility to *Chlamydia* infection independent of the immune system and morphological changes of mammalian cells.

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