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# Enhancement of Adherence and Growth of *Chlamydia trachomatis* by Estrogen Treatment of HeLa Cells

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**Treatment of HeLa 229 cultures with 17-β-estradiol or with diethylstilbesterol, a synthetic estrogen analog, prior to infection with *Chlamydia trachomatis* UW31 (serovar K) or LGV440 (serovar L1) led to a 50 to 60% enhancement of chlamydial inclusion formation. After infection, the presence of estrogen was required for the enhancement. The optimal concentration of estrogen required was $10^{-10}$ M. At least 18 h of preinfection treatment plus 12 h of postinfection treatment was necessary. The adherence of purified radioactive elementary bodies of *C. trachomatis* to estrogen-treated HeLa cells was stimulated in an estrogen dose- and exposure-dependent manner. The requirements for both pre- and postinfection exposure to the hormone suggest that alterations in the cell membrane as well as in the metabolic capacity of the host cells is required for intracellular chlamydial development. Cycloheximide did not prevent estrogen enhancement of chlamydial adherence or subsequent intracellular development of inclusions.**

A higher-than-normal frequency of isolation of *Chlamydia trachomatis* from women with genital infections who used oral contraceptives has been reported (7, 13), although a similar correlation was not made by others (5, 9). Increased susceptibility to a variety of genital infections has been associated with the use of oral contraceptives or phases of the menstrual cycle. Direct evidence for the role of ovarian hormones in altering the course of genital infections is, however, available only in a few animal models (10, 11). Treatment of HeLa cells but not McCoy cells with 17-β-estradiol (E2) was found to render them more susceptible to infection with *C. psittaci* GPIC (8). We have extended these observations on the enhancement by E2 and also by diethylstilbesterol (DES), a synthetic nonsteroidal estrogen, of chlamydial adherence and growth in HeLa 229 cells by using two serovars of *C. trachomatis*, UW31 (serovar K) and the lymphogranuloma venereum serovar LGV440 (serovar L1).

## MATERIALS AND METHODS

**Preparation of radioactively labeled chlamydiae.** Elementary bodies (EBs) of *C. trachomatis* UW31 (serovar K) and LGV440 (serovar L1), labeled during intracellular replication in monolayer cultures of HeLa 229 cells with either $^{32}$P or [5′,6′-$^{3}$H]uridine and purified (2, 4), were used for the adherence experiments with hormone-treated cells.

**Chlamydial adherence assay.** Details of the chlamydial adherence assay have been published previously (2–4). Briefly, $1.5 \times 10^5$ cells were plated in 24-well multidishes (2 cm$^2$ per well) and incubated in growth medium at 37°C. After 16 h the medium was replaced with one containing 5% (vol/vol) "stripped serum", prepared by the treatment of fetal bovine serum with dextran-charcoal (1), and 8 h later E2, DES, or the antiestrogen tamoxifen was added to appropriate wells and the incubation was continued. Prior to the assay, the monolayers were washed once with *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES)-buffered Earle balanced salts solution (EH), labeled EBs were added in a final volume of 150 μl per well, and the adherence of the EBs to HeLa cultures in duplicate wells after 60 min at 5°C with continuous rocking was measured (2–4).

Adherence data are presented after correction for variations in cell number that are often encountered after certain treatments. Results are expressed as radioactivity from cell-bound chlamydiae per $10^5$ HeLa cells.

**Inclusion assay.** Tissue culture chamber slides (growth areas: four-chamber, 2.1 cm$^2$; eight-chamber, 0.81 cm$^2$) (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.) were used for the inclusion assay. HeLa cells at $3 \times 10^5$ in 1 ml (four-chamber slide) or $1.2 \times 10^5$ in 0.4 ml (eight-chamber slide) were plated in each chamber and incubated at 37°C for 18 h. The medium was removed, the monolayers were washed with EH, and medium containing stripped serum was added to each chamber. After incubation at 37°C for 8 h, E2, tamoxifen, or DES was added to replicate chambers. Stock solutions of E2, tamoxifen, and DES were prepared in absolute ethanol and diluted in EH prior to addition to the cell cultures so that the final ethanol concentration was <0.0001%. After incubation for various periods, the medium was removed from each chamber, and the monolayers were washed twice with EH and infected with appropriately diluted chlamydiae. Dilutions were made in EH containing 10% (vol/vol) stripped serum. The inoculum volume was 100 or 50 μl per chamber. The slides were incubated at 37°C for 1 h, the inoculum was removed, the monolayers were washed once with EH, and medium containing the estrogens or the antiestrogen and cycloheximide (1 μg/ml) was added. After incubation at 37°C for 44 h (LGV440) or at 35°C for 64 h (UW31), the monolayers were fixed with methanol and stained with Giemsa. A total of 20 microscope fields were examined under a 40× objective as described previously (2, 4) to enumerate inclusion-containing cells in each chamber. All treatments were done in duplicate or, in some cases, in quadruplicate chambers.

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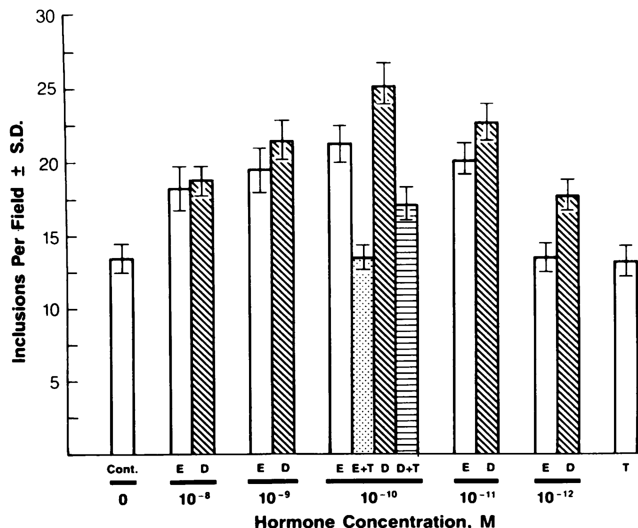


FIG. 1. Hormone concentration dependence of chlamydial inclusion formation in HeLa cells. Monolayer cultures in eight-chamber Miles Scientific slides were treated in duplicate for 18 h with the stated concentrations of E2 (E), DES (D), E2-tamoxifen (E+T), or DES-tamoxifen (D+T) before infection with *C. trachomatis*. Tamoxifen, when present, was at a concentration of $10^{-7}$ M. The estrogens or estrogen-tamoxifen were also present in the postinfection medium, which contained 1 μg of cycloheximide per ml.

**Statistical analyses.** Standard deviations and *t* tests were performed by using the Stats Plus and HSD Stats programs (Human Systems Dynamics, Northridge, Calif.) in an Apple IIe microcomputer (Apple Computers Inc., Sunnyvale, Calif.). Graphs were generated by using the Analytical Graphics program (Scientific Software Products, Indianapolis, Ind.).

**Chemicals.** E2, DES, tamoxifen, trypsin, cycloheximide, and glutamine were from Sigma Chemical Co., St. Louis, Mo. Powdered tissue culture medium and bovine fetal and calf serum were from Irvine Scientific, Santa Ana, Calif. Radiochemicals were purchased from ICN Radiochemicals, Irvine, Calif.

## RESULTS

**Effect of estrogens on chlamydial infectivity.** A significantly higher number of inclusion-containing cells were detected in HeLa cells that had been exposed to E2 or DES prior to infection with *C. trachomatis* UW31 (K) or LGV440 (L1) and then maintained in hormone-supplemented medium. The enhancement was hormone dose dependent, the optimum concentration being $10^{-10}$ M E2 or DES (Fig. 1 and 2), which produced increases of 53 and 65% with UW31 and LGV440, respectively. The antiestrogen tamoxifen at $10^{-7}$ M completely abolished the enhancement caused by $10^{-10}$ M E2 (Fig. 1 and 2) and reduced, although incompletely, the enhancement elicited by DES (Fig. 1). Tamoxifen itself had no effect on the development of inclusion-containing cells incubated in medium containing stripped serum. It should be noted that the postinfection media contained cycloheximide (1 μg/ml).

Data summarized in Fig. 1 and 2 and in Tables 1 and 2, as well as those not shown, representing approximately 20 experiments with different chlamydial stocks, were subjected to analysis by the Student *t* test. HeLa cultures subjected to the pre- and posttreatment with $10^{-10}$ M estrogen, E2, or DES showed a 40 to 60% increase in the number of cells containing chlamydial inclusions ($P < 0.001$). The antiestrogen tamoxifen at $10^{-6}$ or $10^{-7}$ M prevented the E2-induced stimulation completely and partially prevented the stimulation by DES.

**Effect of varying the pretreatment period of HeLa cells with hormones.** As had been observed with E2 enhancement of inclusion formation by *C. psittaci* GPIC (8), an absolute requirement also existed for extended treatment of HeLa cells with either E2 or DES before infection with *C. trachomatis*. No enhancement was observed when cells were subjected to 6 h of exposure to $10^{-10}$ M E2 before infection with LGV440 (Table 1). When cells were subjected to 18 and 24 h of exposure to E2 before infection, a 40% increase in the number of cells containing LGV440 inclusions was noted. With UW31, increases of 36 and 52% could be detected in cells pretreated for 12 and 24 h, respectively. Postinfection media contained E2 until fixation and staining of the cells. HeLa cells cultured in normal calf serum exhibited almost twice as many inclusion-containing cells as did those incubated in stripped serum-containing medium (data not shown).

**Effect of varying the posttreatment period of HeLa cells with hormones.** Similar to the observations with GPIC infection of HeLa cells (8), E2-induced enhancement of inclusion development in *C. trachomatis*-infected HeLa cells was observed only when the HeLa cultures, previously treated for 24 h with $10^{-10}$ M E2, were maintained in hormone-containing medium after infection with *C. trachomatis*. Data summarized in Table 2 show that no enhancement occurred unless the pretreated infected cultures were exposed to the hormone for at least 12 h for UW31 and 24 h for LGV440.

**Effect of estradiol on inclusion formation in McCoy and CHO cells.** Enhancement of GPIC inclusion formation was not seen in the mouse fibroblastic cell line McCoy treated with estrogen (8). On the other hand, several separate experiments with *C. trachomatis* LGV440 showed that a

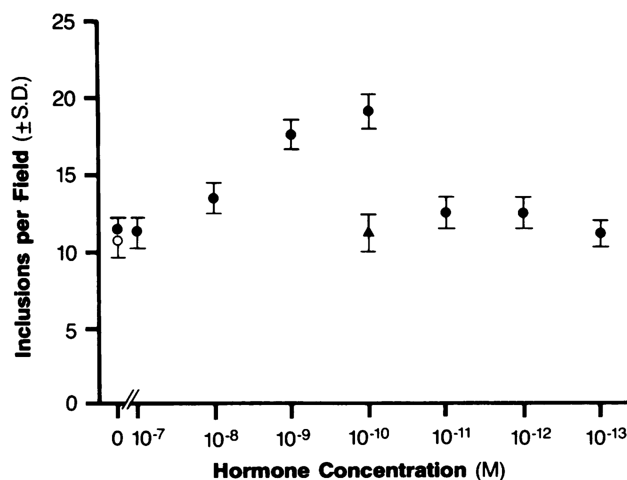


FIG. 2. E2 concentration-dependent enhancement of inclusion formation in HeLa cells infected with *C. trachomatis* LGV440. HeLa cultures were exposed to the indicated concentrations of E2 for 18 h before infection and subsequently maintained in the hormone-supplemented stripped serum and cycloheximide (1 μg/ml)-containing medium. In addition, duplicate chambers were treated with $10^{-7}$ M tamoxifen (○) or tamoxifen–$10^{-10}$ M E2 (▲).

TABLE 1. Effect of varying the pretreatment period of HeLa 229 cells with $10^{-10}$ M E2 on subsequent *C. trachomatis* inclusion development

| *C. trachomatis* strain and E2 exposure (h before infection)[a] | No. of inclusions/ field (mean ± SD)[b] | *P* relative to control | SEM | % of control |
|---|---|---|---|---|
| UW31 | | | | |
| Control (stripped serum) | 12.1 ± 1.74 | | | 100 |
| 6 | 12.5 ± 1.79 | 0.530 | 0.617 | 103 |
| 12 | 16.25 ± 2.07 | <0.001 | 0.525 | 136 |
| 24 | 18.35 ± 1.79 | <0.001 | 0.428 | 152 |
| Normal serum | 26.4 ± 2.93 | <0.001 | 0.856 | 186 |
| LGV440(L1) | | | | |
| Control (stripped serum) | 13.2 ± 2 | | | 100 |
| 6 | 13.4 ± 2.2 | 0.329 | 0.592 | 102 |
| 12 | 15 ± 1.9 | 0.013 | 0.782 | 114 |
| 18 | 18.5 ± 1.5 | <0.001 | 0.645 | 140 |
| 24 | 18.7 ± 1.5 | <0.001 | 0.601 | 142 |

[a] Postinfection medium contained E2 and cycloheximide (1 μg/ml).
[b] Inclusion-containing cells were counted in 20 fields in duplicate chambers.

small (15%), but significant ($P < 0.001$) increase in the number of chlamydial inclusions developed in McCoy and CHO cells after exposure to $10^{-10}$ M E2 (data not shown).

**Effect of estrogen pretreatment of HeLa cells on chlamydial adherence.** The necessity for an extended exposure to the estrogens before infection of HeLa cells suggested that the hormones may enhance chlamydial infection by increasing the adherence of the chlamydiae to the treated cells. The data summarized in Fig. 3 show that the adherence of labeled purified EBs of *C. trachomatis* UW31 to estrogen-treated cells is significantly stimulated by increasing the exposure to $10^{-10}$ M E2 or DES. As was the case with inclusion formation, enhancement of adherence also required more than 6 h of exposure to E2 or DES.

Maximum enhancement of chlamydial adherence was achieved by treatment of HeLa cells with $10^{-10}$ M E2 or DES (Fig. 4). Inclusion of the antiestrogen tamoxifen during this treatment counteracted the enhancement of adherence of UW31 to estradiol-treated HeLa cells. Tamoxifen in 10,000-fold excess only slightly reduced DES-induced enhancement, but produced a significant inhibition of chlamydial adherence to host cells maintained in medium containing normal bovine calf serum, presumably by preventing endogenous estradiol activity (data not shown).

## DISCUSSION

The data presented in this communication show that although estradiol receptors could not be detected in HeLa cells (8), treatment of these cells with E2 or its nonmetabolizable analog DES resulted in a significant and reproducible increase in the rate of adherence and eventual development of chlamydial inclusions after infection with *C. trachomatis* UW31 (serotype K) or with LGV440 (serotype L1). The absolute requirements for pretreatment of the host and for the continued presence of the estrogen after infection to produce enhancement of chlamydial inclusion formation suggest that the effect of the estrogen is on the host. Adherence of radioactively labeled chlamydiae to the host cells and the number of inclusion-containing cells appearing after infection were found to be enhanced by the estrogens in dose- and exposure-dependent manner. The estradiol antagonist tamoxifen prevented the enhancement by E2 of both chlamydial adherence and inclusion development. Treatment of HeLa but not McCoy cells with E2 has been shown to result in an enhancement of the number of inclusion-containing cells after infection with *C. psittaci* GPIC (8).

The unequivocal stimulation of chlamydial adherence after an extended exposure of the HeLa cells to $10^{-10}$ M E2

TABLE 2. Effect of varying the postinfection exposure to $10^{-10}$ M E2 on *C. trachomatis* inclusion formation in HeLa 229 cells

| *C. trachomatis* strain and E2 exposure (h postinfection)[a] | No. of inclusions/ field (mean ± SD) | *P* relative to control | SEM | % of control |
|---|---|---|---|---|
| UW31 | | | | |
| Control (stripped serum) | 13.9 ± 1.9 | | | 100 |
| 4 | 13.6 ± 1.8 | 0.611 | 0.585 | 98 |
| 8 | 14.3 ± 2.1 | 0.498 | 0.643 | 103 |
| 12 | 19.4 ± 2.3 | <0.001 | 0.769 | 140 |
| 24 | 21.9 ± 2.4 | <0.001 | 0.801 | 158 |
| 64 | 23.1 ± 2.6 | <0.001 | 0.668 | 166 |
| LGV440 | | | | |
| Control (stripped serum) | 12.8 ± 1.7 | | | 100 |
| 4 | 12.3 ± 1.4 | 0.387 | 0.593 | 96 |
| 18 | 13.9 ± 1.8 | 0.169 | 0.740 | 109 |
| 24 | 19.5 ± 1.7 | <0.001 | 0.565 | 152 |
| 42 | 19.6 ± 1.8 | <0.001 | 0.678 | 153 |

[a] Monolayers were exposed to $10^{-10}$ M E2 for 24 h before infection.

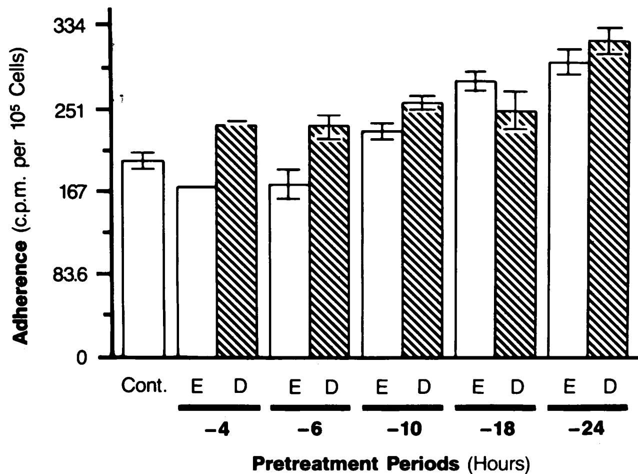


FIG. 3. Effect of varying the period of exposure of HeLa 229 to E2 (E) or DES (D) on the adherence of *C. trachomatis* UW31. Following incubation of monolayers with $10^{-10}$ M E2 (E) or DES (D), the adherence of $^{32}$P-labeled EBs at 5°C after 60 min was measured. Vertical lines on the bars indicate the standard deviation. Less than 1% variation was found in the samples that were given 4 h of pretreatment with the estrogens.

suggests that E2 and DES produce alterations in the host membrane. The necessary alterations require treatment for a prolonged period; no enhancement could be detected after only 6 to 10 h of pretreatment.

The absolute requirement for posttreatment with the estrogen and the significant enhancement of inclusion formation after 12 h of posttreatment recorded here indicate that, during this period of treatment, intracellular (not surface-associated) events in chlamydia-infected HeLa cells are also modulated by the estrogen. Electron-microscopic studies have revealed that the reorganization of intraphagosomal EBs of *C. trachomatis* to the metabolically active form, the reticulate body, occurs several hours after internalization of the chlamydiae (5). It is possible that some unknown alteration of host metabolism induced by the hormone either enhances the kinetics of initiation of chlamydial replicative processes or that in the presence of the estrogen, more internalized EBs become capable of intracellular development, indicating a protective effect by the estrogen.

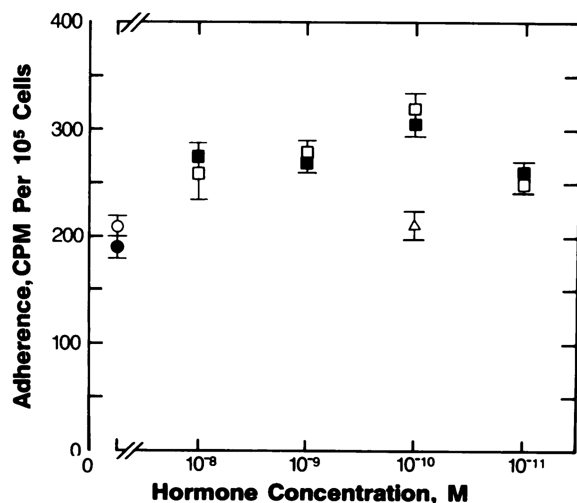


FIG. 4. Adherence of $^{32}$P-labeled EBs of *C. trachomatis* UW31 to HeLa cells pretreated with various doses of E2 or DES for 24 h. Symbols: ○, untreated, in stripped serum; ●, untreated plus $10^{-7}$ M tamoxifen; □, E2 treated; ■, DES treated; △, E2 plus $10^{-7}$ M tamoxifen. Values shown are mean ± standard deviation of duplicate assays.

Although estradiol receptors have not been detected in HeLa cells (8), a requirement for 18 to 24 h of exposure to elicit increased adherence has also been noted for *Escherichia coli* and *Staphylococcus aureus* (12). Replication of *C. psittaci* GPIC within the cytoplasm of HeLa cells similarly required a long pretreatment with estradiol (8). With the data presented here, this strongly suggests that even though gross morphological changes are not apparent, alterations must occur on the HeLa cell surface in an estrogen dose- and exposure-dependent manner. DES produced increased adherence and inclusion by *C. trachomatis* with the same exposure requirement as E2, indicating that the estrogen and not a metabolic product of the hormone is responsible for the effect. Experiments are now in progress to determine the changes in HeLa cell membrane produced by estrogens which affect chlamydial adherence and replication in these cells.

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