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Inhibition of *Chlamydia trachomatis* Growth in McCoy, HeLa, and Human Prostate Cells by Zinc

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Zinc salts (10^{-4} and 10^{-5} M) inhibited the number of *Chlamydia trachomatis* inclusions in McCoy, HeLa, and primary human prostate epithelial cell cultures. Addition of zinc salts 1 h before or 24 h after inoculation with *C. trachomatis* was found to inhibit growth. Both *C. trachomatis* serotype D and a lymphogranuloma venereum strain were inhibited by the zinc salts. Although the mechanism of inhibition is not known, the continued presence of the zinc appeared necessary for maximal effect. At the concentrations tested, zinc was not directly toxic to the McCoy cells. These results suggest that the levels of zinc in prostatic secretions may be sufficient to preclude the recovery of chlamydia in the diagnostic laboratory or to inhibit chlamydia from infecting the prostate in vivo.

Chlamydia trachomatis has been established as a frequent cause of sexually transmitted diseases (16, 26). The organism has been found in 30 to 70% of all cases of nongonococcal urethritis and has been associated with acute epididymitis in men (3, 12, 14) and mucopurulent cervicitis and endometritis in women (4, 23). Although *C. trachomatis* has not been definitively shown to cause acute or chronic prostatitis, some investigators have implicated it with their serological results (24).

Zinc salts have been documented to inhibit the growth of several viruses (2, 5, 8, 18–20, 27, 28), bacteria, and protozoa (2, 10). Zinc has been demonstrated to be an antibacterial factor in human prostatic fluid secretions and appears to play a role in preventing bacterial prostatitis (9). It is found in high concentrations in both seminal plasma and prostate tissue (21) and has been implicated in the inhibition of chlamydial growth (6, 11, 22). Because of these reports, we evaluated the role of zinc salts in the inhibition of *C. trachomatis* infection in McCoy, HeLa 229E, and primary human prostate epithelial cell cultures.

MATERIALS AND METHODS

***C. trachomatis* strains.** *C. trachomatis* D/UW139/Ur (kindly provided by E. R. Alexander, University of Washington, Seattle) was obtained from a patient with nongonococcal urethritis. This strain had been serially propagated in McCoy cells. *C. trachomatis* LGV-BCM/1/Bu was isolated from a patient with lymphogranuloma venereum (LGV) at the Ben Taub General Hospital, Houston, Tex., and also passed in McCoy cells. Stock cultures of each strain were stored in 1-ml aliquots at −70°C in sucrose-phosphate buffer with antibiotics (30).

Cell cultures. For isolation of *C. trachomatis*, a modification of the method of Wentworth and Alexander (30) was used. Three cell culture systems were used in different experiments. (i) McCoy cells (kindly provided by E. R. Alexander) were planted in 1-dram glass vials containing 12-mm glass cover slips (Bellco Glass, Inc., Vineland, N.J.) at concentrations of 2×10^6 cells per ml and then incubated at 37°C under 5% CO_2 for 18 h in Eagle minimum essential medium containing 10% fetal calf serum plus gentamicin (10 μg/ml) and amphotericin B (2.5 μg/ml). This medium (CMA) was removed, and 1 ml of control media or various zinc concentrations was added to each vial. The cells were then incubated at 35°C for 60 min before inoculation with *C. trachomatis*. (ii) HeLa 229E cells (kindly provided by T. Hatch, University of Texas, Austin) were used in parallel experiments and grown in a manner similar to the McCoy cells. (iii) Human prostate epithelial cells were derived from prostatic tissue obtained from patients undergoing transurethral resection. A coagulase solution of 1 to 1.5 mg of crude coagulase type 1 (Sigma Chemical Co., St. Louis, Mo.) per ml diluted in 2× CMA was used to remove stromal cells from glandular structures (17; P. J. Giles, Ph.D. thesis, Baylor College of Medicine, Houston, Tex., 1978). Minced tissue was incubated in CMA at 37°C under 5% CO_2 for 24 to 48 h, and the resulting suspension was separated so that the stromal cells were dispersed as single cells and prostatic glands remained as large pieces. The glands were allowed to settle by gravity in a 15-ml conical tube for 10 to 15 min. Supernatant fluid containing the bulk of the fibroblasts was removed, and this procedure was repeated twice more in fresh CMA with coagulase. The resulting pellet was resuspended in 5 to 15 ml of 2× CMA. A 1-ml sample of the suspension was then seeded onto 12-mm glass cover slips contained in 1-dram glass shells (Kimbell Co., Toledo, Ohio) and incubated at 37°C under 5% CO_2. As a marker of prostatic glandular epithelium, tartrate-inhibitable acid phosphatase determinations were performed. Human prostate epithelial cell cultures were treated in a similar fashion to McCoy cells when *C. trachomatis* was inoculated.

Chlamydial inoculation. Unless specified otherwise, chlamydial inoculation was performed in the following manner. At the end of the 60-min incubation period, supernatant

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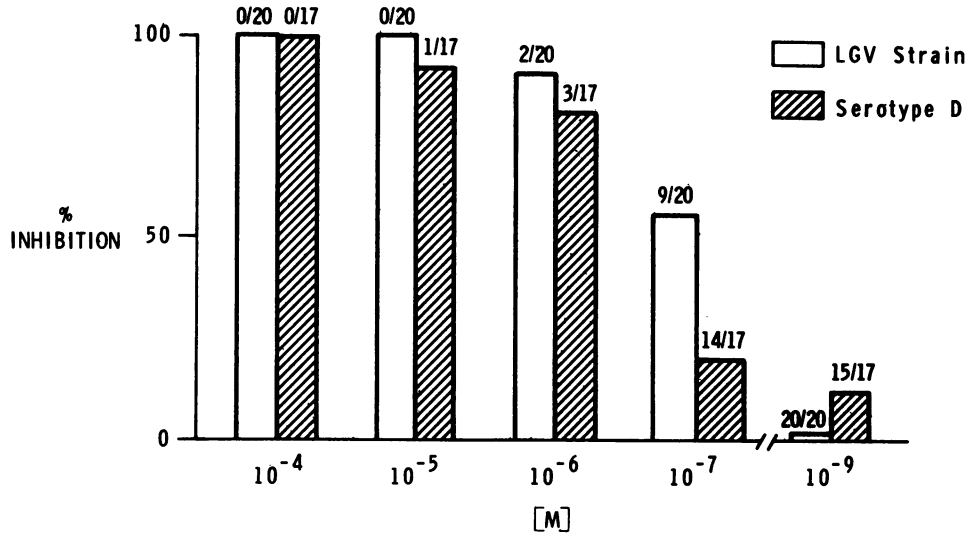

FIG. 1. Effect of various concentrations of $ZnCl_2$ on *C. trachomatis* serotype D and LGV strain found in McCoy cells. Bars represent percent inhibition of inclusions compared with control untreated cover slips. The numbers above each bar are the mean number of inclusions per 30 fields of treated cover slips over the mean number of inclusions per 30 fields of untreated control cover slips.

fluid was removed and the cells were inoculated with a 1:10 dilution of a laboratory pool of *C. trachomatis* serotype D or the LGV strain. These strains of *C. trachomatis* were diluted in CMA to produce at least 15 to 20 inclusions per 30 microscopic fields in McCoy cells. *C. trachomatis* was added in 0.2-ml amounts, and the vials were centrifuged at 1,000 × *g* for 60 min. Maintenance medium containing 1 μg of cycloheximide per ml was added to each vial, and the vials were incubated at 35°C and under 5% CO_2. After 72 h, the cover slips were stained with either standard iodine (McCoy cells or human prostate cells) or Giemsa (HeLa 229E cells) (7). In passage experiments, both zinc-treated and untreated infected McCoy cells were frozen quickly in a dry ice-acetone bath and immediately thawed in a 37°C water bath. The resulting suspension was centrifuged at 600 × *g* for 5 min and diluted with an equal volume of double-strength sucrose-phosphate buffer. A 0.2-ml portion of this suspension was layered onto pretreated McCoy cells and examined for inclusions 72 h later. For most tested variables, at least five cover slips were read. Inclusions in 30 random fields per cover slip were counted with a ×40 oil immersion objective (final magnification, ×480). Media and cell control slides were included in each experiment.

Zinc salts. Several different concentrations of zinc chloride (certified A.C.S.; Fisher Scientific Co., Fair Lawn, N.J.), zinc sulfate (J. T. Baker Chemical Co., Phillipsburg, N.J.), and zinc oxide (Sigma) were used after being diluted in Hanks balanced salt solution. Concentrations greater than 10^{-3} M zinc salts were toxic to the tissue culture cells. Solutions of zinc salts used for making dilutions were analyzed for zinc content by atomic absorption analysis (Spectrix Co., Houston, Tex.) and found to have the designated concentration. CMA (with fetal bovine serum) was found to have an approximately 10^{-6} M concentration of zinc. The zinc was bound to the protein fraction of the fetal bovine serum, which had a concentration of 10^{-2} M zinc when assayed alone. Two samples of prostate tissue obtained for cell culture were found to have 1.3×10^{-7} M zinc by atomic absorption analysis.

Zinc chloride (10^{-4} M) was added to uninfected control McCoy cells for 24 h to test for direct toxicity to cell cultures. Total cell counts and a trypan blue exclusion test demonstrated no significant differences in viability and total number of cells between treated and untreated control McCoy cells. Zinc chloride (10^{-4} M) was incubated with 0.1 mCi of ^{51}Cr-labeled ($Na_2{}^{51}Cr_4$, CJS-1; Amersham Corp., Arlington Heights, Ill.) uninfected McCoy cells for 18 h, and no increase in chromium release compared with the base line was detected in the zinc-treated cells.

The data for the effects of zinc on chlamydial activity are presented as percent inhibition. Percent inhibition is defined as 100 minus the mean number of inclusions per 30 fields in the zinc-treated culture divided by the mean number of inclusions per 30 fields in the control cultures.

RESULTS

Several concentrations (10^{-4} to 10^{-7} and 10^{-9} M) of zinc chloride were incubated with McCoy cells before inoculation of *C. trachomatis* serotype D or an LGV strain; 90 to 100% inhibition of growth was found with 10^{-4} or 10^{-5} M zinc chloride against either strain (Fig. 1). Little or no inhibition of chlamydial growth was detected with less than 10^{-7} M zinc chloride. After passage of *C. trachomatis*-infected cells that had been pretreated with zinc chloride, no inclusions were detected. *C. trachomatis*-infected McCoy cells that were not treated with zinc salts could be passaged to uninfected cells.

In the experiments described above, the McCoy cells were pretreated in a variety of procedures to define the role of zinc (Fig. 2). In "PreRx" experiments, McCoy cells were pretreated with zinc chloride for 1 h before inoculation with chlamydia. The cells were then incubated for 72 h and stained, and cover slips were examined for typical inclusions (Fig. 2). In these experiments, a 90% inhibition of *C. trachomatis* growth was observed. In "PreRx and Wash" experiments, zinc chloride (10^{-4} M) was added to McCoy cells for 1 h. The cells were washed three times with CMA, and then the *C. trachomatis* serotype D inoculum was added. If this zinc was washed off, then no significant

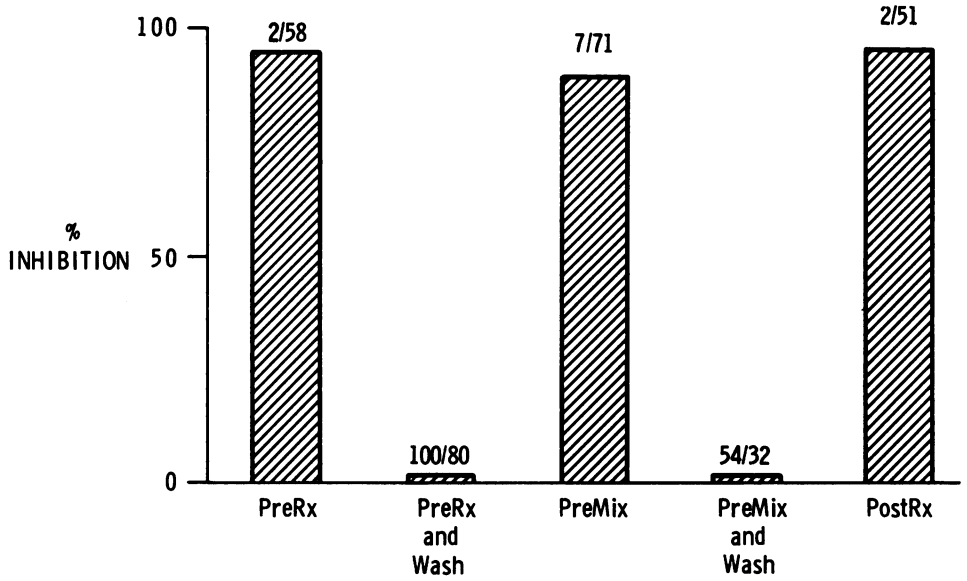

FIG. 2. Effect of pretreatment and posttreatment use of zinc chloride on *C. trachomatis* serotype D inclusions in McCoy cells. Designations: PreRx, McCoy cells treated with zinc chloride before chlamydia inoculation; PreRx and Wash, zinc chloride-treated McCoy cells washed before chlamydia inoculation; PreMix, zinc chloride mixed with chlamydia inoculum for 1 h before being added to McCoy cells; PreMix and Wash, zinc chloride mixed with chlamydia inoculum (followed by spinning and washing) before being added to McCoy cells; PostRx, experiments in which zinc chloride added to McCoy cells 24 h after inoculation with chlamydia. Zinc chloride (10^{-4} M) was used in all experiments. *C. trachomatis* serotype D was used in the chlamydia inoculum. Results of experiments are reported as percent inhibition of inclusions compared with control untreated cover slips. The numbers above each bar are the mean number of inclusions per 30 fields of treated cover slips over the mean number of inclusions per 30 fields of untreated control cover slips.

inhibition in total chlamydial inclusions was noted. In "PreMix" experiments, zinc chloride (10^{-4} M) and the chlamydia inoculum were added together in equal volumes for 1 h, after which the suspension was spun at 1,000 × *g* for 10 min. After the supernatant was removed, the remaining 0.2-ml pellet was inoculated onto McCoy cells and stained for inclusions 72 h later. Under these conditions, approximately 90% inhibition in chlamydial inclusions was observed. However, if the mixtures of zinc and chlamydia were spun and then washed before inoculation, as in the "PreMix and Wash" experiments, then chlamydial inclusions were unaffected compared with the controls. In "PostRx" studies, McCoy cells were inoculated with the chlamydia inoculum and incubated 24 h before either zinc chloride or fresh medium was added. After 48 h, the zinc-treated and control cells were stained and the inclusions were counted. In the "PostRx" experiments, chlamydial inclusions were reduced by >90% compared with controls.

In separate experiments, zinc chloride, zinc sulfate, zinc

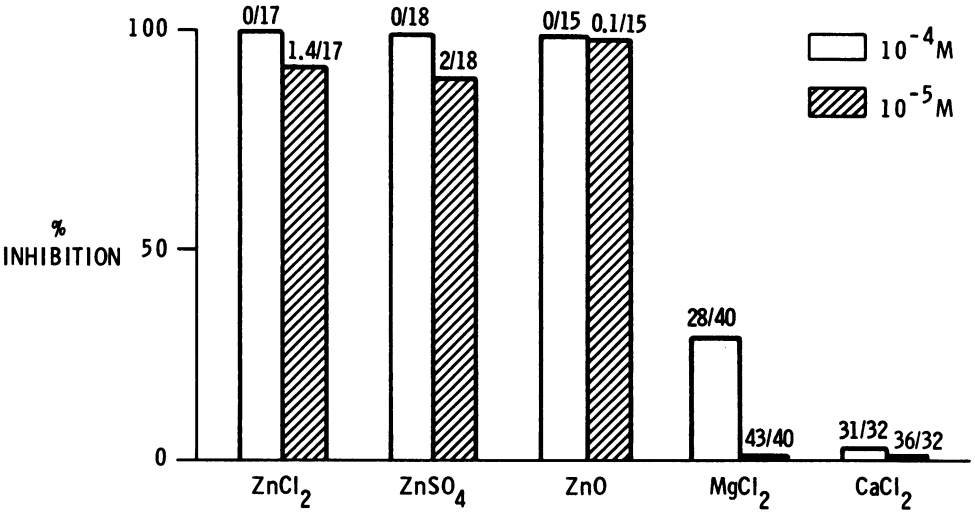

FIG. 3. Effect of two concentrations (10^{-4} and 10^{-5} M) of zinc salts and $MgCl_2$ and $CaCl_2$ on *C. trachomatis* serotype D inclusions in McCoy cells. Bars represent percent inhibition of inclusions compared with untreated control cover slips. The numbers above each bar are the mean number of inclusions per 30 fields of the treated cover slips over the mean number of inclusions per 30 fields of untreated control cover slips.

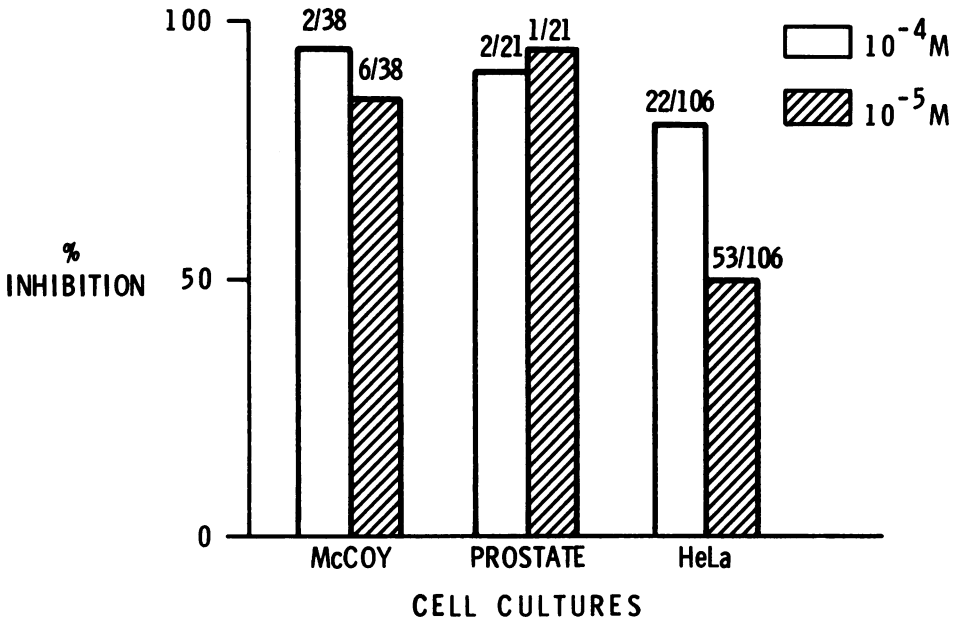

FIG. 4. Effect of two concentrations (10^{-4} and 10^{-5} M) of $ZnCl_2$ on *C. trachomatis* serotype D inclusions in McCoy, human prostate, and HeLa cells. Bars represent percent inhibition of inclusions compared with control untreated cover slips. The numbers above each bar are the mean number of inclusions per 30 fields of the treated cover slips over the mean number of inclusions per 30 fields of untreated control cover slips.

oxide, magnesium chloride, or calcium chloride was added for 1 h at 10^{-4} or 10^{-5} M to uninfected McCoy cells (Fig. 3). The supernatants were partially removed as in the previous experiments, and a standard inoculum of *C. trachomatis* serotype D was added. Greater than 90% inhibition of chlamydial inclusions was noted with each of the zinc salts but not with the magnesium or calcium chloride.

Zinc inhibition of chlamydial inclusion formation was also demonstrated in HeLa 229E and human prostate epithelial cells (Fig. 4). When 10^{-4} or 10^{-5} M zinc chloride was incubated in HeLa 229E or human prostate cells before inoculation with *C. trachomatis* serotype D, 50 and 90% inhibition of inclusions, respectively, were observed.

DISCUSSION

These experiments demonstrate that *C. trachomatis* growth can be inhibited in vitro by zinc salts. Greater than 90% inhibition was produced with the chloride, sulfate, and oxide salts of zinc, and in both human continuous (HeLa 229E) or primary (prostate epithelial) cell cultures as well as McCoy cells. Both *C. trachomatis* serotype D and an LGV strain were inhibited by zinc chloride. In experiments in which the chlamydia and zinc were mixed before inoculation and then washed (PreMix and Wash), no inhibition could be documented. The presence of zinc in the media appeared to be necessary for inhibition to take place. Inhibition of chlamydial inclusions was established even when zinc chloride was added 24 h after chlamydia had been inoculated into McCoy cells.

The zinc concentrations that caused inhibition of chlamydial inclusions were comparable to those found in seminal plasma (21). Hanna et al. (11) have reported the inhibition of an LGV strain of *C. trachomatis* when seminal plasma was added to McCoy cells before inoculation; however, they did not report the zinc concentrations. Mardh et al. (22) found a similar effect of seminal and prostatic fluid on chlamydial growth but did not find a similar inhibitory effect of zinc chloride in concentrations that are reported in this study. Zinc concentrations which were inhibitory to chlamydia in their study were toxic to the cell cultures used in our study.

The mechanism of chlamydial-growth inhibition by zinc is not clearly delineated by our results. Although previous investigators had attempted to clarify the sequence of chlamydia adherence and internalization (1), recent studies by Murray and Ward (25, 29) have defined the central role of calmodulin in *C. trachomatis* endocytosis. Future studies will focus on documenting the effect of zinc on calmodulin in chlamydia-infected cells.

We describe here for the first time the infection of primary human prostatic epithelial cells with *C. trachomatis*. Except for one study with primary human amnion cells (13) and another with human thyroid cells (15), in vitro studies of *C. trachomatis* have involved the use of transformed cell lines of both human and animal origin (1, 7). The amount of zinc found in the processed human prostate epithelial cells discussed here was too low to inhibit chlamydial growth. It is not known whether the prostate can be infected in vivo, although serological studies show such infections to be uncommon (24). Nevertheless, the presence of increased concentrations of free zinc in prostatic fluid could decrease the recovery of chlamydia from this site.

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