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Zinc and *Chlamydia trachomatis*[1] (42113)

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Abstract. Zinc was noted to have significant effects upon the infection of McCoy cells by each of two strains of *Chlamydia trachomatis*. With a high or low *Chlamydia* inoculant, the number of infected cells increased up to 200% utilizing supplemental zinc (up to 1×10^{-4} *M*) in the inoculation media compared with standard *Chlamydia* cultivation media (8×10^{-6} *M* zinc). Ferric chloride and calcium chloride did not effect any such changes. Higher concentrations of zinc, after 2 hr of incubation with *Chlamydia*, significantly decreased the number of inclusions. This direct effect of zinc on the *Chlamydia* remained constant after further repassage of the *Chlamydia* without supplemental zinc, suggesting a lethal effect of the zinc. Supplemental zinc (up to 10^{-4} *M*) may prove to be a useful addition to inoculation media to increase the yield of culturing for *Chlamydia trachomatis*. Similarly, topical or oral zinc preparations used by people may alter their susceptivity to *Chlamydia trachomatis* infections.

Zinc is essential for all living organisms (1). It serves numerous functions including those of metalloenzyme activation (especially at lower concentrations) and membrane stabilization and enzyme inhibition (at higher concentrations) (1, 2, 3). Because zinc is active in so many different aspects of cellular physiology and the effects of zinc may be different dependent upon its concentration, it is not surprising that zinc has been reported to perform seemingly contradictory functions.

With specific regard to zinc and infection, some data suggest that zinc could impede the development of infection and other data implies that zinc may promote the development of infection (3). For example, prolonged zinc deficiency in mammals is associated with depressed T-lymphocyte function (but near normal B-lymphocyte function) that may predispose to the development of infection (3). Zinc (administered in certain concentrations at specific times) seems to protect mice from the lethality of administered endotoxin, perhaps by stabilizing lysosomal membranes (4). Other data which suggest that zinc may inhibit the development of infection include the toxic effect of zinc (10^{-5} *M* or greater) on various microorganisms *in vitro* (3, 5) and reports that zinc therapy may be responsible for a more favorable outcome of the common cold, herpes virus eye and skin infections, and rats and other animals infected with certain bacteria (3, 6–10). Some of the same studies just mentioned also noted different effects if the time of zinc administration, amount of zinc administered, or bacteria used were changed (3, 8, 10). For example, using the same experimental conditions, intraperitoneal administration of zinc 1 hr prior to bacterial infection of rats with a strain of *Salmonella typhimurium* was associated with increased mortality compared with control rats; however, if a strain of *Francisella tularensis* or *Streptococcus pneumoniae* was used instead, decreased mortality compared with nonzinc (saline) control animals was noted (8). Further, zinc (in physiologic concentrations) could promote the development of infection by causing the elaboration of microbial virulence factors (3). Examples of this include zinc causing increased production of toxin by certain bacteria (3, 11, 12) and zinc enhancing the adherence of piliated bacteria to receptor cells (3, 13).

Zinc and *Chlamydia* have not been systematically investigated; however, preliminary

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work of others has shown that millimolar concentrations of zinc inhibit or kill *Chlamydia trachomatis* under certain incubation conditions (14, 15). These microorganisms cause a variety of infections in humans and other animals (16). Cultivation of these organisms in the laboratory may be difficult and time consuming unless certain procedures are performed (16, 17). These organisms are obligatory intracellular parasites and are currently isolated most often after growth in McCoy or HeLa cell cultures (16, 17). Because of the difficulty in propagating these microorganisms, numerous special techniques have been advocated. Most frequently recommended are techniques that inhibit the McCoy or HeLa cells such as cycloheximide treatment, or less often irradiation or iododeoxyuridine (17–21). Because *Chlamydia* and mammalian cells have net negative surface charges (13, 22), other techniques to increase infectivity have been the use of polycations such as diethylaminoethyl (DEAE)–dextran and centrifugation (17, 18, 20, 23). Different types of *C. trachomatis* may respond in a different manner to these various treatments (16, 17). Still other work has suggested that factors as subtle as the hormones present in the incubation media may effect the growth of *Chlamydia* (24, 25).

Because *Chlamydia* are more difficult and time consuming to cultivate than many other common human pathogens, much emphasis has recently been devoted to alternative identification methods (16, 17, 26–28). Monoclonal antibody immunofluorescence and enzyme immunoassay techniques are commercially available for rapid identification of *Chlamydia* infections and may prove to be of great value; however, cultivation of the organism remains the absolute standard of proof of infection (16, 17, 26–28).

Because zinc may alter the course of various infections and the net surface charge of cells and subsequent microorganism–mammalian cell interactions (3, 13), we decided to systematically study the effect of zinc on *Chlamydia trachomatis.*

Materials and Methods. *Chlamydia.* Human, nongonococcal urethritis isolates of infectious *C. trachomatis* elementary bodies (16) were stored in complete medium with antibiotics (CMA, described below) with 20% v/v fetal bovine serum (Sterile Systems, Inc., Logan, Utah) in containers in liquid nitrogen. Inoculations for an individual experiment (controls and zinc treatment) were made from the same container after quickly warming at 37°C. Inocula were used with known levels of infectivity, calculated from trial assays using other containers prepared in an identical manner. Inocula were used that did not cause appreciable lysis of the cells used for cultivating the *Chlamydia,* but did produce different levels of infectivity.

Assay. McCoy cells (mouse fibroblasts) were grown to near confluency on 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, St. Louis, Mo.], 2.5 μg/ml amphotericin B [E. R. Squibb, Princeton, N.J.] and 2 m*M* glutamine [Sigma]) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

The coverslips were placed in 1 dram glass vials and an 0.2 ml inoculum of *Chlamydia* in various zinc concentrations 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 without supplemental zinc (CMA with 0.72 mg/ml [4 m*M*] glucose [Sigma] and 1 μg/ml cycloheximide [Sigma]) 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 (Jone's Iodine) reagent was added to each vial and incubated for 20 min at ambient temperature. The iodine was removed and the coverslips placed on a slide with a 1:1 iodine:glycerol mixture. The coverslips were sealed 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.

Zinc experiments. Zinc chloride was prepared in ultra-pure water of at least 18 MΩ/cm resistivity and was incorporated into the *Chlamydia* inoculum for the concurrent incubation experiments at concentrations of 8 $\times 10^{-6}$ *M* zinc (CMA or overlay media alone, as assayed by atomic absorption spectrometry) to 5 $\times 10^{-4}$ *M* zinc. In other experiments, supplemental zinc was added to the McCoy cells in CMA and incubated for up to 2 hr at 37°C in a humidified 95% v/v air and 5% CO_2 atmosphere. After this, the media was removed and *Chlamydia* inoculated as previously described. In still other experiments, the *Chlamydia* infectious elementary bodies in CMA were incubated with or without supplemental zinc at various temperatures for up to 2 hr after which they were inoculated into McCoy cells as previously described.

Statistics. All incubations were performed in duplicate, with the mean counted as a single datum point. All experiments were repeated at least five times, and differences analyzed with the Wilcoxon rank sum test (no presumption of normal distribution).

Results. *Zinc effect.* Incubation of the *Chlamydia* with supplemental zinc at the time of McCoy cell inoculation increased the number of inclusions. This was true for each of the strains of *C. trachomatis,* and high or low *Chlamydia* inocula. The incubation conditions and zinc concentrations used had no effect by themselves on the McCoy cells as judged by light microscopy analysis of control and zinc-treated cells, including trypan blue viability analysis [(29) data not shown]. Higher concentrations of zinc were not used because of toxicity to the McCoy cells. These results are summarized in Table I. Reading of the coverslips without knowledge of the contents failed to note any qualitative differences or aberrancy comparing the inclusions from control and zinc-treated groups. To avoid the possibility of an increased number of inclusions causing McCoy cell lysis, inocula were used which did not cause appreciable cell lysis under any of the incubation conditions used.

Localization of zinc effect. Pretreatment of the *Chlamydia* inocula (elementary bodies) with zinc for 30 min at 37°C, followed by inoculation into the McCoy cells, had no effect upon subsequent number of inclusions with up to 1 $\times 10^{-4}$ *M* Zn. Under these conditions, 5 $\times 10^{-4}$ *M* Zn incubation was associated with fewer inclusions; however, the results were not statistically significant. Incubation of the *Chlamydia* for 2 hr with at least 5 $\times 10^{-4}$ *M* Zn did cause significantly fewer inclusions to form after inoculation into the McCoy cells. Further repassage (of identically treated vials without supplemental zinc in the media used for repassage) yielded only a few inclusions, suggesting a lethal, and not inhibitory effect upon the *Chlamydia* (Fig. 1).

Incubation of zinc with the McCoy cells for 1 hr, followed by removal of the medium and inoculation of *C. trachomatis*-A resulted in a residual effect of the zinc, but a lesser effect than that noted with concurrent incubation with zinc. Control cells ultimately had 6.2 ± 1.2% of the cells with inclusions; McCoy cells incubated with 5 $\times 10^{-5}$ *M* zinc had 5.9 ± 1.3%; cells incubated with 1 $\times 10^{-4}$ *M* zinc had 8.7 ± 1.5% ($P < 0.05$); cells incubated with 5 $\times 10^{-4}$ *M* zinc had 8.2 ± 1.6% ($P < 0.05$ compared with control). Incubation

TABLE I. EFFECT OF ZINC CONCENTRATION ON THE NUMBER OF *C. trachomatis* INCLUSIONS

	Zinc concentration of *Chlamydia* inocula[a]			
C. trachomatis	8 $\times 10^{-6}$ M (Media alone)	5 $\times 10^{-5}$ *M*	1 $\times 10^{-4}$ *M*	5 $\times 10^{-4}$ *M*
Isolate A[b]	1.3 ± 0.2	1.6 ± 0.3	2.3 ± 0.3[d]	2.0 ± 0.3[d]
Isolate A[c]	8.7 ± 1.5	11.9 ± 1.8[d]	15.1 ± 3.3[d]	14.2 ± 3.4[d]
Isolate B	2.1 ± 0.4	2.3 ± 0.6	3.3 ± 0.9[d]	3.5 ± 0.8[d]

[a] Data are expressed as percentage of McCoy cells with inclusions as mean ± SD of the mean.
[b] Low inocula.
[c] High inocula.
[d] $P < 0.05$ compared with control media (8 $\times 10^{-6}$ *M* Zn).

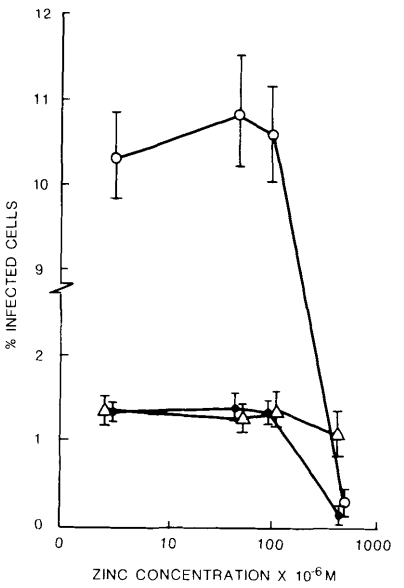

FIG. 1. Effect of incubation of *C. trachomatis*-A with different zinc concentrations prior to inoculation into McCoy cells. ●, 2 hours incubation; ○, further repassage of identically treated vials (without supplemental zinc in the repassage media), △, 30-min incubation; I, 1 SD.

with higher concentrations of zinc or with 1×10^{-4} *M* zinc (or greater) for more than 2 hr was toxic to the McCoy cells (data not shown).

Other metals. Concurrent incubation of *C. trachomatis* with ferric chloride or calcium chloride (1×10^{-4} *M*) did not reproduce the effects noted with zinc chloride; however, incubation with zinc acetate did. These results are depicted in Fig. 2.

Discussion. These data show that zinc can alter the susceptibility of McCoy cells to infection by *C. trachomatis* in at least two different manners. First, at concentrations of the same magnitude as that of normal human serum (2×10^{-5} *M*) (3, 30) up to 10^{-4} *M*, zinc present in the media at the time of *Chlamydia* inoculation into McCoy cells increases the percentage of subsequently infected McCoy cells compared with inoculation at lower zinc concentrations. This could mean that supplemental zinc, under specific circumstances, may act as a virulence agent by increasing susceptibility of mammalian cells to infection. Under conditions of stress, mammalian serum zinc (and iron) levels promptly decrease in a temporary manner (with associated liver sequestration of zinc (3, 30)). This could serve as a protective mechanism, removing these nutrients from potentially invading microorganisms (3). Of course, this is speculative, and the binding of zinc to proteins in serum and incubation media may alter these concentration comparisons (3, 30). Second, direct exposure of *C. trachomatis* elementary bodies to higher concentrations of zinc or for longer periods of time may kill the *Chlamydia*. Thus, topical or systemic zinc preparations may impede infections caused by various bacteria (3), fungi (3), viruses (3, 5–7, 9), and even *Chlamydia* (14, 15).

These experiments again show the importance of incubation conditions and that apparently contradictory results can occur with different concentrations of zinc and time of zinc exposure (3, 10, 14, 15). In the *Chlamydia* experiments reported here, concentrations of zinc that *Chlamydia,* McCoy cells, or the *Chlamydia* inoculum and McCoy cells were initially incubated with did not remain constant as the media was removed, changed, or new media added after the initial zinc incubations. This fact, coupled with residual binding of zinc to cells after its removal (2) and binding of zinc to proteins in the media (3, 30) make determination of actual zinc concentration and site of zinc activity difficult. However, the results previously mentioned and preliminary data with ^{65}zinc and

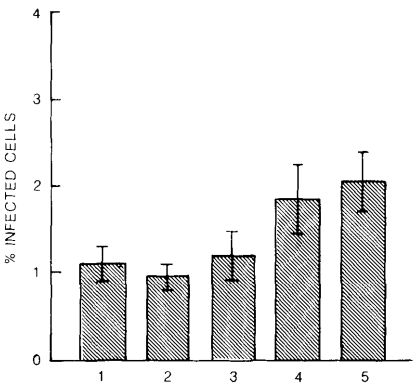

FIG. 2. Effect of inoculation of *C. trachomatis*-A into McCoy cells with supplemental heavy metals. 1, no supplemental metal; 2, calcium chloride; 3, ferric chloride; 4, zinc acetate; 5, zinc chloride (all metals at 1×10^{-4} *M*); I, 1 SD.

biochemical sulfhydryl blockers suggest that the increased *Chlamydia* infectivity seen with supplemental zinc is related to zinc binding to the McCoy cells. Perhaps zinc bound to McCoy cells changes their net surface charge (making it less negative) and thus easier for *Chlamydia* (which also have a net negative surface charge) to interact with and infect the McCoy cells (13, 22). Extra zinc (10^{-4} *M*) in the media at the time of *Chlamydia* inoculation into McCoy cells may make it easier to cultivate *Chlamydia,* especially when the inoculant is of low infectivity and only a few McCoy cells might become infected. Further, supplemental dietary zinc or topical zinc may alter mammalian susceptibility to *Chlamydia* infection; however, any *in vivo* relevance of these findings remains to be determined.

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