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The Binding of Chlamydia Trachomatis and Zinc to McCoy Cells (Mouse Fibroblasts)

Summary: Zinc was found to have profoundly different effects upon the infection of McCoy cells (mouse fibroblasts) by two strains of Chlamydia trachomatis dependent upon the time and concentration of zinc exposure. Radiolabeled zinc-65 became McCoy cell-associated in a manner independent of incubation temperature, but highly dependent on incubation time and zinc concentration. This effect was maximal after 30 to 60 minutes of incubation. Correspondingly, incubation of a chlamydia inoculant with McCoy cells and supplemental zinc (10^{-5} to 10^{-4}M) for 1 h was associated with significantly (approximately twofold) more binding of the chlamydia to the McCoy cells compared with control media (8 \times 10^{-6}M Zn). More prolonged incubation of the chlamydia and McCoy cells with supplemental zinc was associated with significantly fewer chlamydia inclusions. Concentrations of 5 \times 10^{-4}M zinc or higher were also found to be toxic to the McCoy cells after 48 h of incubation. Brief exposure to supplemental zinc may augment infection of cells by chlamydia: however, more prolonged exposure to the same concentrations of zinc lessens cellular infection by chlamydia.

Introduction

All living organisms require zinc for its presence in numerous metalloenzymes, membrane stabilizing effects, and numerous other functions (1, 2). Zinc, especially at higher concentrations, can also display opposing effects such as enzyme inhibition (1, 2). Previous studies have suggested that even brief exposure to supplemental zinc may alter mammalian susceptibility to various infections (2, 3). These results have also been contradictory, suggesting enhanced susceptibility to some infections, but reduced susceptibility to others dependent upon factors including the concentration of zinc, time of zinc exposure, and microorganisms studied (2, 3). Because zinc is present in so many topical and oral over-the-counter preparations and physician prescribed compounds, detailed examination of the relationship between zinc and infection is of importance (2).

Several investigators have preliminarily studied the relationship between zinc and Chlamydia trachomatis. Zinc, in concentrations of 10^{-3}M or greater, when incubated with C. trachomatis and receptor cells for 48 h or longer is associated with an inhibitory effect on the development of chlamydia inclusions (4–6). Further passage of these specimens without supplemental zinc suggests that this effect is a lethal effect of the zinc on the chlamydia (6). Similarly, zinc has been found to inhibit or kill numerous other microorganisms at these concentrations: for certain viruses by the inhibition of cleavage of posttranslational polypeptide precursors, and by inhibition of cellular functions of other microorganisms (2, 7, 8). Recently, the presence of 10^{-4}M zinc at the time of chlamydia inoculation into McCoy cells was noted to increase the subsequently infected number of McCoy cells (6). This period of sup

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plemental zinc exposure was for 1h, after which additional media without supplemental zinc was added (6). Further, this study noted that more prolonged exposure of the system to supplemental zinc was associated with a lethal effect of zinc on the chlamydia (6).

Most microbiological media contain $10^{-7}$ to $10^{-6}$M zinc and mammalian serum concentration of zinc is approximately $2 \times 10^{-5}$M (16). It is interesting to note that concentrations of supplemental zinc approximating physiological concentrations may have profound effects upon subsequent infection (2, 4–6). Because zinc binds to numerous proteins present in serum and media and on cells (2, 9), meticulous attention must be devoted to work with zinc prior to the formation of conclusions regarding actual zinc concentration and infection. These experiments were performed in order to better delineate the exact relationship between zinc, chlamydia, and infection. Zinc, after systemic or topical application, can readily achieve concentrations similar to those reported in earlier in vivo and in vitro studies (2). The high frequency of chlamydiaal infections (10) coupled with the frequency of zinc supplementation form the basis for better determining whether zinc exposure would predispose or prevent the recipient from acquiring chlamydial infections.

Materials and Methods

Chlamydia: A human, non-gonococcal urethritis isolate of C. trachomatis (D-serotype) and a lymphogranuloma venereum (LGV) isolate obtained from clinical specimens were stored in complete medium with antibiotics (see below) with 20% supplemental v/v fetal bovine serum (Sterile Systems Inc., Logan, UT, USA) in liquid nitrogen. Inoculations used for each experiment were made from one container quickly warmed at 37°C. Inocula were used with known levels of infectivity, calculated from trial assays using other containers prepared identically. Inocula were used which did not cause appreciable McCoy cell lysis. Zinc: Zinc-65 (New England Nuclear, Boston, MA, USA) with specific activity of 4.8 mCi/mg was diluted in unlabeled zinc chloride ($5 \times 10^{-4}$M) and incubated with McCoy cells in fresh CMA (see below) for various periods of time on a shaking apparatus. After this, zinc-65 that was not bound to the confluent layer of McCoy cells was removed by serial washings with CMA containing $5 \times 10^{-4}$M unlabeled zinc chloride (to prevent removal of bound zinc due to a concentration gradient). The remaining, bound zinc-65 was counted after solubilization of the plate contents with 0.1N NaOH followed by pH neutralization and the addition of xylene-surfactant based scintillation fluid. Zinc-65 was counted in a liquid scintillation counter using tritium windows. Calculated counting efficiency was 31%.

McCoy cells and chlamydial incubation: McCoy cells (mouse fibroblasts) were grown to near confluency on 12 mm diameter, round glass coverslips (or 8 cm² plastic dishes for the zinc-65 experiments) in complete medium with antibiotics (CMA, Eagle’s minimal essential medium) (GIBCO, Grand Island, NY, USA) supplemented with 10% v/v fetal bovine serum (Sterile Systems, Inc.), 40 μg/ml gentamicin (Sigma, St. Louis, MO, USA), 2.5 μg/ml amphotericin B (E. R. Squibb, Princeton, N. J.) and 2 mM glutamine (Sigma) in a humidified atmosphere of 95% air and 5% CO₂ at 37°C.

The coverslips were placed in 1 dram glass vials and an 0.2 ml inoculum of chlamydia in defined zinc concentrations was added to each vial. These vials were centrifuged at $2800 \times g$ for 60 min at 32°C, and then 2 ml of 37°C overlay media (CMA with 0.72 mg/ml [4mM] glucose [Sigma] and 1 μg/ml cycloheximide [Sigma]) with defined concentrations of zinc was added to each vial. These vials were incubated at the same 37°C humidified atmosphere previously mentioned for 48 h. Cultivated chlamydia were detected with an iodine staining procedure. Briefly, overlay medium 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 (15% 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 1:1 iodine:glycerol mixture. The coverslips were sealed with clear nail polish and examined under 400X light microscopy for intracytoplasmic (glycogen containing) inclusions. Every cell was examined in some of the experiments, and in the others, hundreds of cells in each of ten randomly selected fields were examined from each coverslip for each individual assay.

Zinc experiments: Zinc chloride was prepared in ultra-pure water of at least 18 megohms/cm resistivity and was incorporated into the chlamydia inoculum, overlay media, or both for the experiments at concentrations of $8 \times 10^{-6}$M zinc (CMA or overlay media alone, as assayed by atomic absorption spectrometry) to $1 \times 10^{-3}$M zinc.

Statistics: All experiments were repeated at least five times on different occasions. Non-parametric Wilcoxon rank sum and signed rank tests were used to determine significance.

Results

McCoy cells: Confluent McCoy cells were quantified with haemocytometry after trypsinization and present at $1.6 \pm 0.2 \times 10^6$ cells per cm². The addition of at least $5 \times 10^{-4}$M zinc in CMA for 48 h was associated with a toxic effect upon these cells visible under 200X light microscopy and a decrease in cell viability with Trypan blue staining from greater than 99% viable (control) to less than 40% (11). Zinc-65 binding to McCoy cells: The binding was dependent upon the incubation time, concentration of zinc, and number of washes. Three washes after incubation were optimal to remove unbound zinc. Maximal binding occurred after 30 to 60 min of incubation with 4–8 $\times 10^{-4}$M zinc. Incubation at ambient temperature yielded results the same as incubation at 37°C. These results are depicted in Figures 1 and 2.

Effect of supplemental zinc on chlamydial inclusions: Zinc was added at defined concentration at the time of inoculation. After 1 h centrifugation, additional overlay medium was added with defined zinc concentrations. Media contained $8 \times 10^{-6}$M zinc when no supplemental zinc was added. Supplemental zinc was added at the time of inoculation, to the overlay media for 48 h incubation, or both. $5 \times 10^{-4}$M zinc present during 48 h incubation was associated with a toxic effect upon the McCoy cells (acidic media and cell lysis). The toxic effect was identical even if no chlamydia were present in the inoculant. Because of this, final zinc concentration in the overlay media did not

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