Inhibition of Growth of *Chlamydia trachomatis* by the Calcium Antagonist Verapamil

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Treatment of BGM (African Green Monkey kidney) cells with the calcium antagonist Verapamil resulted in a reduced yield of chlamydial infectious particles. The inhibitory effect was concentration-dependent, the maximal effect being achieved at 200 μ M-Verapamil, which produced a 99.99% reduction of infectious particle yield. Electron microscopy showed that control *Chlamydia trachomatis*-infected BGM cells contained typical large inclusions in which most of the particles were elementary bodies, whereas Verapamil-treated infected cells contained small inclusions consisting predominantly of reticulate bodies. The findings indicate a possible therapeutic use of this calcium antagonist as an anti-chlamydial drug.

INTRODUCTION

Chlamydia trachomatis, an obligate intracellular bacterium, with a genome of 660×10^6 Da (Sarov & Becker, 1968, 1969; Moulder, 1982), is the causative agent of trachoma, inclusion conjunctivitis and lymphogranuloma venereum. It is now well established that certain serovars of *C. trachomatis* (D through K) are the most common cause of non-gonococcal urethritis and post-gonococcal urethritis, cervicitis, endometritis, neonatal pneumonitis and epididymitis. Recent studies have also implicated *C. trachomatis* as one of the major causes of pelvic inflammatory disease, which may lead to infertility (reviewed by Ladany & Sarov, 1985).

Chlamydiae undergo a unique developmental cycle within the host cell. The small (300 nm) infectious elementary body (EB) characterized by an electron-dense core, enters the host cell by endocytosis and differentiates to a large (1000 nm) metabolically active reticulate body (RB). The RBs multiply by binary fission and differentiate to the infectious EBs, which are released after host cell lysis (reviewed by Ward, 1983).

In extensive studies with *C. trachomatis*, Ward & Salari (1982) suggested that Ca^{2+} ions, among other substances, were important in the control mechanism governing chlamydial infectivity of HeLa cells. Adhesion of chlamydiae to host cells was found to be associated with a flux of Ca^{2+} across the cell membrane.

Since treatment of chlamydial infection is an important clinical need, the aim of this study was to find out whether the calcium antagonist Verapamil, which is used as a therapeutic agent mainly in cardiovascular disease, would affect *C. trachomatis* growth in cell culture.

METHODS

Tissue culture. African green monkey kidney (BGM) cells were grown in RPMI 1640 medium, supplemented with 10% (v/v) foetal calf serum, 2 mM-glutamine, 100 U penicillin ml^{-1} , 100 μ g streptomycin ml^{-1} and 12.5 U mycostatin ml^{-1} (all purchased from Biological Industries, Beth Haemek, Israel).

Abbreviations: EB, elementary body, RB, reticulate body, IFU, inclusion-forming unit.

Preparation of infectious, purified, EB particles. *C. trachomatis* biovar lymphogranuloma venereum (L2/434/Bu) was grown in BGM cells in RPMI 1640 supplemented with 10% (v/v) foetal calf serum, 1% (w/v) glucose, 0.15% sodium bicarbonate, 2 mM-L-glutamine, 100 µg streptomycin ml⁻¹, 10 µg Fungizone ml⁻¹, and 1 µg cycloheximide ml⁻¹. Chlamydiae were harvested from BGM monolayers grown in 175 cm² (basal area) polystyrene flasks (Nunc) as described by Caldwell *et al.* (1981). Purified EBs were suspended in SPG buffer (0.01 M-sodium phosphate buffer, pH 7.2, containing 0.25 M-sucrose and 5 mM-L-glutamic acid) and stored at -70 °C until use.

Effect of Verapamil on the yield of infectious C. trachomatis particles in BGM cells. Prior to infection, BGM cells were thoroughly washed with medium free of antibiotics and seeded in 96-well microwell plates (Nunc) at 2.5×10^4 cells per well. After 48 h, the medium was aspirated and the cells were infected with purified *C. trachomatis* at an input multiplicity of infection (m.o.i.) of 1.0. After 90 min at 37 °C for adsorption, the inoculum was removed, cells thoroughly rinsed and fresh medium added (as described above for the preparation of infectious, purified EB). Verapamil (Teva, Jerusalem) was added at various concentrations, either during the 90 min of the adsorption period only, or after the adsorption period for 45 h post-infection. At 45 h after infection, the cells were scraped into their overlaying medium. Duplicate wells were pooled and their contents were frozen at -70 °C. In order to measure the chlamydial yield, the thawed samples were sonicated for 40 s (Bransonic sonifier). Tenfold dilutions were prepared in growth medium and titrated in triplicate on BGM cells as described below.

Immunoperoxidase assay for titration of C. trachomatis. *C. trachomatis* was titrated on HEp-2 cells as described by Shemer & Sarov (1985). The cells were seeded at 2×10^4 to 3×10^4 cells per well in 96-well microwell plates (Nunc). After 48 h, triplicate 50 µl samples of serial 10-fold dilutions of chlamydial inoculum, prepared in growth medium, were added. The plates were fixed 2 d later with 100% ethanol and an immunoperoxidase assay (Sarov & Haikin, 1983) was performed. The final titration results were expressed as inclusion-forming units (IFU) ml⁻¹.

Effect of Verapamil on the number of chlamydial inclusions. Prior to infection, BGM cells were washed, as described above, and were seeded on coverslips in 4-well multidish titration plates (Nunc) at 2×10^5 cells per well. After 48 h, the growth medium was replaced with fresh medium and infected with purified *C. trachomatis* (m.o.i. = 1). After the 90 min adsorption period, and washing and rinsing, medium with and without various concentrations of Verapamil was added and the BGM cells incubated for 45 h post-infection. The cells were then fixed with methanol, and stained with Giemsa. Three hundred cells were examined for the presence of chlamydial inclusion bodies by light microscopy.

Effect of Verapamil on DNA synthesis in control cells. BGM cells were grown as described above. After 48 h, the growth medium was replaced with medium without cycloheximide, containing appropriate concentrations of Verapamil and [methyl-³H]thymidine (1 µCi ml⁻¹, 37 kBq ml⁻¹; specific activity, 2000 µCi mol⁻¹, 74 MBq mol⁻¹; Nuclear Research Centre, Negev, Israel) was added. After 24 h and 48 h, TCA precipitates were collected on Millipore filters. Radioactivity was measured in a Packard liquid scintillation spectrophotometer using a toluene-based scintillant (Bio-Lab Ltd, Jerusalem).

Electron microscopy. *C. trachomatis*-infected cells (m.o.i. = 1), with or without Verapamil added after the adsorption period were prepared for transmission electron microscopy in 4-well plates (Nunc). At 24 h and 45 h post-infection, the cells were fixed directly in the original culture wells with 1% (w/v) glutaraldehyde and 4% (v/v) formaldehyde in 0.15 M-sodium phosphate buffer, pH 7.2, overnight at 4 °C. After dehydration with increasing concentrations of ethanol, the cells were scraped into capsules, and the pellets embedded in Araldite-502 (Luft) and sectioned. Sections were examined with a Philips 201C electron microscope at 60 kV following staining with uranyl acetate and lead citrate.

RESULTS

Effect of Verapamil on chlamydial yield. The effect of various concentrations of Verapamil (50 µM to 200 µM) on *C. trachomatis* yield when it was added post-adsorption to *C. trachomatis*-infected cells is illustrated in Fig. 1. Increasing concentrations of Verapamil resulted in increasing inhibition of chlamydial infectivity, up to a 1000-fold reduction at 200 µM-Verapamil. However, when 200 µM-Verapamil was present throughout the adsorption period only, there was only a threefold inhibition of chlamydial yield at 45 h post-infection.

Effect of Verapamil on the number of chlamydial inclusions. BGM cells infected with *C. trachomatis*, with and without the addition of Verapamil after adsorption, were examined by light microscopy, 45 h after infection, for the presence of inclusion bodies following Giemsa staining. Control and 50 µM-Verapamil-treated cells showed a typical large inclusion in almost every cell. With higher concentrations of Verapamil, there was a concentration-dependent reduction in the number of cells containing inclusion bodies: $51 \pm 4\%$ and $28 \pm 3\%$ (mean $\pm$ SD) of the cells contained inclusions at Verapamil concentrations of 100 and 150 µM, respectively. At

Fig. 1

Fig. 2

Fig. 1. Inhibition of the yield of infectious chlamydial particles in BGM cells infected with *C. trachomatis* at m.o.i. = 1 in the presence of various concentrations of Verapamil. Chlamydial titres were determined 45 h post-infection; the mean titres from five experiments are given $\pm$ SD.

Fig. 2. Transmission electron micrographs of thin sections of BGM cells infected with *C. trachomatis*, with (a) 150 μ M-Verapamil added after adsorption, and (b) no addition of the drug. Cells were fixed 45 h post infection. (a) Cell treated with 150 μ M-Verapamil, showing a small inclusion with predominantly RBs. (b) Control: part of a large inclusion body showing predominantly EBs. Bars, 1.2 μ m.

those Verapamil concentrations the inclusions appeared smaller than in untreated control infected cells.

Effect of Verapamil on control BGM cells. The effect of various concentrations of Verapamil on DNA synthesis in BGM cells was examined 24 h and 48 h after addition of [³H]thymidine. The only effect observed was a reduction of 12% in DNA synthesis at 48 h with the highest concentration of Verapamil (200 μ M). No noticeable morphological or cytological change in BGM cells could be detected, at this Verapamil concentration, by light or by electron microscopy.

Electron microscopy. Thin sections of *C. trachomatis*-infected cells, with or without Verapamil (100 μ M or 150 μ M) added after adsorption, were examined with the electron microscope. Three hundred cells were scored for each treatment. Cells containing inclusion bodies were examined and the ratio of EBs to RBs in each inclusion body was determined. No significant difference could be detected in the ratio of EBs to RBs at 24 h post-infection between untreated and

Verapamil-treated *C. trachomatis*-infected cells at either Verapamil concentration (about 97% of the particles were RBs). At 45 h post-infection, (Fig. 2), the control infected cells showed large inclusion bodies in which 87.6% of the particles were EBs. In the Verapamil-treated infected cells, there was a decrease in the ratio of EBs to RBs with increase in the concentration of the drug: 43.5% and 26.8% of the particles were EBs at Verapamil concentrations of 100 μ M and 150 μ M, respectively ($P < 0.01$, Student's *t*-test). Moreover, at the higher concentration of Verapamil most of the inclusions appeared much smaller than those in untreated *C. trachomatis*-infected cells.

DISCUSSION

It is now generally accepted that Ca^{2+} and cyclic nucleotides are the major components of an internal signalling system regulating cell activities. Cell injury (Humes, 1986), bacterial and viral attack (Durham, 1978), hormones and many other activities (Berridge, 1975) are mediated by changes in cellular Ca^{2+} fluxes and distribution. Ward & Salari (1982) have suggested that rapid Ca^{2+} flux across the cell membrane, which may lead to the activation of membrane phospholipase A_2 , enhances prostaglandin synthesis and stimulates cGMP synthesis, which together govern the infectivity of chlamydiae (Murray & Ward, 1984). Since Ca^{2+} fluxes are the main regulators of this process, treatment of the host cell with the Ca^{2+} ionophore A23182 enhances infectivity.

In the present study we were able to demonstrate that the calcium antagonist Verapamil markedly inhibited chlamydial growth at concentrations at which host cell DNA replication was hardly affected. Verapamil, when added at a concentration of 150 μ M after adsorption, caused an approximately 1000-fold reduction in chlamydial yield. Light and electron microscopic analysis suggested that there was a reduction in the number of inclusions, and that the differentiation of RBs into EBs was inhibited. A greatly reduced effect of the drug was obtained when it was added only during the adsorption step. It should be noted that the concentration of the drug used in these *in vitro* studies was more than two orders of magnitude greater than the plasma concentration attained in patients receiving therapy. Verapamil may, however, be of value as a local anti-chlamydial agent.

Recent studies in this laboratory with measles and vaccinia viruses (R. Shaiokin-Kestenbaum, and others, unpublished data) have demonstrated that increased Ca^{2+} uptake by the infected cells, as well as treatment with Verapamil, strongly reduces viral yield. Other investigators have also demonstrated an inhibitory effect of Verapamil on replication of human cytomegalovirus (Albrecht *et al.*, 1984), Epstein-Barr virus (Nemerov & Cooper, 1984) and influenza virus (Nugent & Shanley, 1984). Taken together, these results clearly demonstrate that Verapamil exhibits anti-viral and anti-chlamydial activity *in vitro*. Chlamydial infections are inherently chronic, often running a prolonged subclinical course. The therapeutic effects of antimicrobial drugs are variable and in many cases these drugs fail to prevent recurrence of the disease, or may leave the patient symptomless, but still infected (Oriel, 1986). Animal model experiments are required to explore the anti-viral and anti-chlamydial potential of Verapamil *in vivo*, particularly for external or local treatments, alone or in combination with other drugs.

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