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Retinoic acid inhibits the infectivity and growth of *Chlamydia pneumoniae* in epithelial and endothelial cells through different receptors

Mirja Puolakkainen^{1,2}, Amy Lee¹, Tadayoshi Nosaka¹, Hideto Fukushi^{1,3}, Cho-Chou Kuo¹, and Lee Ann Campbell¹

¹Department of Pathobiology, Box 357238, University of Washington, Seattle, WA 98195 ²Infection Pathogenesis Laboratory, Department of Viral Diseases and Immunology, National Public Health Institute and Haartman Institute, Department of Virology, University of Helsinki, Finland ³Department of Veterinary Microbiology, Faculty of Agriculture, Gifu University, Gifu, Japan

Abstract

Chlamydia pneumoniae is a human respiratory pathogen that has also been associated with cardiovascular disease. *C. pneumoniae* infection accelerates atherosclerotic plaque development in hyperlipidemic animals and promotes oxidation of low density lipoprotein *in vitro*. All-trans-retinoic acid (ATRA), an anti-oxidant, has been shown to inhibit *C. pneumoniae* infectivity for endothelial cells by preventing binding of the organism to the M6P/IGF-2 receptor on the cell surface. This current study investigates whether ATRA similarly affects *C. pneumoniae* infectivity of epithelial cells, which are the primary site of infection in the respiratory tract, and the effects on intracellular growth in both endothelial and epithelial cells. Because ATRA binds to both the nuclear RA receptor (RAR) and the M6P/IGF-2 receptor, TTNPB, an ATRA analog, which binds to the RAR but not the M6P/IGF-2 receptor was used to differentiate the receptor mediating the effects of ATRA. The results of this study showed two separate effects of ATRA. The first effect is through interaction with the M6P/IGF-2 receptor on the cell surface preventing attachment of the organism (inhibition by ATRA but not TTNPB) in endothelial cells and the second is through the nuclear receptor (inhibition by both ATRA and TTNPB) which inhibits growth in both epithelial and endothelial cells.

Keywords

Chlamydia pneumoniae; Retinoic acid; Receptor; Hapten inhibition

1. Introduction

Chlamydia pneumoniae is a causative agent of acute respiratory disease, and is also associated with coronary artery disease, atherosclerosis and asthma. Chlamydiae are obligate intracellular organisms that have a unique developmental cycle. After attachment and entry into host cells, the chlamydiae reside in vacuoles that do not fuse with cellular lysosomes, but rather interact

Corresponding author: Dr. Lee Ann Campbell, Department of Pathobiology, Box 357238, University of Washington, Seattle, WA 98195-001, Tel. (206) 543-0317, Fax (206) 5433873, email: lacamp@u.washington.edu.

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with vesicles of the exocytic pathway [1]. *C. pneumoniae* can grow in a variety of human cells, including epithelial, endothelial, macrophages, and smooth muscle cells [2]. Following infection of the respiratory epithelium, the organism disseminates to the vasculature, where the organism has been detected in macrophage and smooth-muscle cell derived foam cells and endothelial cells. Infection of animal models of atherosclerosis with *C. pneumoniae* results in acceleration of atherosclerotic lesion progression [3]. In addition to activation of vascular cells to produce proinflammatory cytokines and other proatherogenic factors, another proposed mechanism for *C. pneumoniae*'s role in contributing to the pathogenesis of atherogenesis is through induction of foam cell formation and oxidation of LDL [4,5,6].

In previous studies focusing on the chlamydial glycan, a high mannose oligosaccharide that promote attachment and internalization of chlamydiae [7], we have demonstrated that ligands of the mannose-6 phosphate/IGF2 receptor affect infectivity of *C. pneumoniae* for endothelial cells [8]. Specifically, phosphomannosylated residues such as mannose-6P that specifically bind to this receptor and all-trans-retinoic acid (ATRA), which has a separate binding site on the M6P/IGF2 receptor [9], competitively inhibit *C. pneumoniae* infectivity and removal of the glycan decrease infectivity *in vitro* and in mouse models of lung infection [10,11].

Binding of ATRA to the cellular M6P/IGF2 receptor affects binding of the phosphomannosylated residues and alters intracellular trafficking of the M6P/IGF2 receptor and its ligands [12]. In addition, ATRA has anti-oxidant properties and can elicit a variety of cellular responses affecting cell growth, differentiation, and metabolism [13,14] and interfere with glycosylation of viral proteins [15]. These effects of ATRA are mediated by binding of retinoids to the nuclear retinoid acid receptor (RAR) and retinoid X receptor (RXR). Because of the potential benefits of the antioxidant properties of ATRA on atherogenesis coupled to the ability to reduce infection of *C. pneumoniae* for endothelial cells, we further investigated whether the effects of ATRA on infectivity of *C. pneumoniae* in epithelial cells, the primary site of initial infection

2. Results

2.1. Pretreatment of host cells with retinoic acid and TTNPB inhibit inclusion formation of *C. pneumoniae* in HL cells

In previous studies using ATRA in hapten inhibition experiments in endothelial cells, ATRA, but not TTNPB, an analog of ATRA, was found to significantly inhibit infectivity of *C. pneumoniae* ($P < 0.05$). TTNPB is an agonist of ATRA, which binds to the RAR nuclear receptor, but not the M6P/IGF-2 receptor at the host cell surface, and thus, can be used to differentiate effects of ATRA that result from binding to the M6P/IGF2 receptor or RAR receptor [12]. Therefore, these results indicated that ATRA inhibited binding of *C. pneumoniae* to the M6P/IGF2 receptor [8]. These results were consistent with experiments demonstrating that other ligands, which bind at different sites on the M6P/IGF2 receptor, also affected infectivity of *C. pneumoniae* [8,16]. To determine whether ATRA similarly inhibited *C. pneumoniae* infection of epithelial cells, hapten inhibition experiments were done using HL cells. Interestingly, as shown in Fig. 1, panel A, both ATRA and TTNPB decreased infectivity of *C. pneumoniae* for HL cells ($P < 0.05$ at all concentrations tested), suggesting that the decrease in inclusion formation did not occur through inhibiting attachment of the organism to the M6P/IGF2 receptor.

To further test the hypothesis that the effect of ATRA on inclusion formation in endothelial cells, but not HL cells, was due to inhibition of the attachment of the organism to the host cell surface receptor, metabolically labeled organisms were incubated with HL cells or endothelial cells at 4°C (attachment occurs, but organisms are not internalized) and 37°C (organisms are internalized) in the presences or absence of various concentrations of ATRA. As shown in Fig.

1. panel B, a statistically significant decrease in attachment and internalization was observed in HMEC cells in which ATRA was added to the assay in comparison to control cells. However, this decrease was not saturating, suggesting more than one mode of entry. In contrast, ATRA had no effect on attachment or internalization of *C. pneumoniae* for HL cells at the same concentration indicating that the decrease in inclusion formation was not through inhibition of attachment or internalization.

2.2. Incorporation of ATRA or TTNPB into the growth medium inhibits inclusion formation of *C. pneumoniae* in endothelial and HL cells

If the retinoids were incorporated into the growth medium throughout the growth cycle, both ATRA and TTNPB inhibited inclusion formation of *C. pneumoniae* in HL cells and HMEC cells (Fig. 1, panel C). This suggests that in addition to the effect of ATRA on attachment and internalization of the organism into HMEC-1 cells through the M6P/IGF2 receptor, *C. pneumoniae* growth is inhibited by ATRA through the interaction of ATRA with the RAR receptor. The inhibition of *C. pneumoniae* inclusion formation by 12.5 μ M ATRA was significantly higher in endothelial cells than HL cells. Inclusion of ATRA in the growth medium also inhibited inclusion formation of *C. trachomatis* serovar L₂ in HL cells (data not shown).

The growth inhibition by ATRA and TTNPB of *C. pneumoniae* as measured by the ability to produce infectious particles was dependent on the time that infected cells were incubated in medium containing ATRA. As shown in Fig. 1, panel D, at lower concentrations of ATRA and TTNPB, growth of *C. pneumoniae* and production of infectious progeny could partially be recovered if HL cells were treated for only 24 h post infection. The production of infectious particles decreased with longer treatments with retinoids. At higher concentrations of ATRA (Fig. 1, Panel D) or TTNPB in both HL (data not shown) and HMEC (data not shown), the inhibitory effects became irreversible. Thus, the reversibility of growth inhibition by ATRA and TTNPB is dependent on the exposure time to these retinoids. The concentrations of ATRA and TTNPB used in these studies were not toxic to the host cell line or *C. pneumoniae*.

2.3. ATRA interferes with chlamydial development and alters the inclusion membrane structure

The effects of ATRA on intracellular growth were further investigated in HL cells by fluorescent microscopy and electron microscopy. As shown in Fig 2, in comparison to untreated infected cell cultures (Fig. 2, panel A), the inclusion size is significantly reduced in HL cells in which 12.5 μ M ATRA was included in the growth medium (Fig. 2, panel B). Specifically, in HL cells, the mean inclusion diameters were $9.2 \pm 0.7 \mu$ m (n=17) for untreated infected cells versus $3.6 \pm 0.7 \mu$ m (n=31) for ATRA treated cells ($p < 0.01$). As expected, the burst size was also significantly reduced (n=5, $87.9 \pm 12.5\%$, $p < 0.01$).

As shown in the electron micrographs in Fig. 2, in comparison to untreated infected HL cells showing an inclusion containing elementary bodies (EBs) and reticulate bodies (RBs) (Panel C), HL cells infected with *C. pneumoniae* and incubated in growth medium containing 12.5 μ M ATRA for 72 hrs shows a malformed inclusion containing aberrant forms (Panel D). The inclusion membrane appears fragmented and altered in structure and the host Golgi apparatus appears altered. It should be noted that the *C. pneumoniae* inclusion in untreated HL cells examined at 72 h post infection still contains many RBs, which have not yet reorganized into EBs (Panel C). The latter is due to culturing of cells in the absence of cycloheximide, which is typically included in the growth medium to inhibit host cell protein synthesis, thereby enhancing organism growth and decreasing the length of the developmental cycle.

3. Discussion

In a previous study in endothelial cells, *C. pneumoniae* was shown to utilize the M6P/IGF2 receptor for infectivity based on hapten inhibition experiments with ligands known to bind to the M6P/IGF2, including retinoic acid. In contrast, TTNPB, an RAR agonist that does not bind to the M6P/IGF2 receptor, had no effect on infectivity of endothelial cells. The identification of ligands that affect infectivity *in vitro* raises the possibility of potential intervention strategies to prevent infection *in vivo* through inhibition of infection *in vivo* or in the case, of retinoic acid, which has antioxidant properties, to affect *C. pneumoniae* accelerated atherosclerosis. Because the primary site of infection is the respiratory epithelium, we determined whether infection of epithelial cells was similarly inhibited by ATRA, and if so, whether the effect occurred through inhibition of attachment of the organism to epithelial cells.

In this study, both ATRA and TTNPB were found to inhibit inclusion formation in HL cells when the cells were incubated with these ligands for 1 hour prior to infection, suggesting that this inhibitory effect for HL cells was due to binding to nuclear RAR receptor and not to the M6P/IGF2. This was further confirmed by demonstrating that neither attachment to nor internalization into HL cells of metabolically labeled organisms was affected by inclusion of ATRA in the assay. In contrast, attachment of metabolically labeled *C. pneumoniae* organisms to endothelial cells is significantly inhibited by ATRA. The lack of an effect of ATRA on attachment of *C. pneumoniae* to HL cells is also consistent with a previous report indicating that IGF-2, which binds to the M6P/IGF2 receptor at a separate site from ATRA, affects infectivity of endothelial cells, but not epithelial cells [16]. This suggests that alternative modes of entry are preferentially used in this cell line or that receptor abundance or exposure of binding sites on HL cells differ from those on endothelial cells.

In the hapten inhibition experiments, the ligand was removed from the host cell prior to infection with the organism. Inclusion of ATRA throughout the growth cycle was shown to inhibit infectivity (based on the number of inclusion counts in the first passage) and growth (based on determination of infectious progeny in the second passage). These effects were mediated through the RAR receptor as TTNPB was also inhibitory in both endothelial cells and HL cells. Similar inhibitory effects have been reported with herpes simplex virus and *Mycobacterium tuberculosis*, although the mechanism(s) responsible for the inhibition are unknown [15,17]. Two of the ATRA binding sites potentially mediating this inhibition are: 1) the M6P/IGF-2 receptor on the cell surface and 2) the nuclear RAR receptor. The fact that TTNPB, a RAR agonist, which is known to interact only with the RAR nuclear receptor [18], had the same inhibitory effect suggests that inhibition was not due to binding to the M6P/IGF-2 surface receptor, but at the intracellular level. This study also showed that ATRA inhibited the growth of *C. pneumoniae* in HL cells, but higher concentrations were required for inhibition than in endothelial cells.

ATRA exhibits pleiotropic effects that affect a variety of biological functions including growth, cellular differentiation, metabolism, morphogenesis and homeostasis [13,14]. Upon ligand activation of RAR and RXR, these proteins function as transcription factors controlling expression of genes containing RA response elements [14,19]. ATRA also disrupts the Golgi apparatus, which is independent of RAR/RXR activation [20]. Although activation of RAR is indicated by the effect of TTNPB on chlamydial growth, an additional effect due to vacuolization of the Golgi apparatus can not be excluded because the structure of the Golgi apparatus appears altered in the electron microscopic pictures of ATRA treated infected cells (Fig. 2, panels C and D). Interestingly, ATRA has also been reported to increase the intracellular ceramide concentration, by increasing the production of acid sphingomyelinase, which results in hydrolysis of sphingomyelin to ceramide [21]. It is known that Chlamydia utilize host cell derived sphingolipids for synthesis of the inclusion membrane [22]. Therefore, one possible

mechanism by which ATRA might inhibit chlamydial growth would be by interfering with utilization of sphingolipids by chlamydiae. This hypothesis is also consistent with the electron microscopic findings showing alterations in EB morphology and the inclusion membrane structure in retinoic acid treated culture.

In conclusion, this study showed differential interactions of ATRA with host cell receptors depending on the exposure conditions and the cell line used; however, both affected the outcome of infection. For endothelial cells, ATRA exhibited two effects, one that occurred at the level of attachment through the M6P/IGF2 receptor and the other that affected intracellular growth through the RAR receptor. In contrast, the effects of ATRA effects in HL cells occurred through activation of the RAR receptor. These results suggest potential therapeutic benefits of ATRA on disruption of chlamydial growth.

4. Materials and Methods

4.1. Chlamydial organisms

C. pneumoniae AR-39 was propagated in human epithelial (HL) cells. The elementary bodies were purified by hypaque gradient centrifugation, resuspended in chlamydia transport medium, sucrose-phosphate-glutamic acid, and stored in aliquots at -70°C until used.

4.2. Cell lines

HL cells were maintained in α -minimal essential medium (α -MEM) containing 10% heat inactivated fetal calf serum (FCS). Human transformed arterial endothelial cell line (HMEC-1) [23] was maintained in supplemented endothelial cell culture medium (Clonetics EGM[®], Walkersville MD).

4.3. Infection of the cells with chlamydiae

HL and HMEC-1 cells were trypsinized from stock cultures and plated at 2×10^5 cells per well onto glass cover slips in 24-well plates. Once confluent, the cells were washed twice with Hanks' balanced salt solution (HBSS) and infected with *C. pneumoniae* at an MOI resulting in tens of inclusions per x 400 field (MOI of 1–2 for HL cells and 10 for HMEC). The plates were incubated for 2 hrs at 37°C on a rocking plate. After three days of incubation at 35°C , cell monolayers were fixed with methanol and stained with *C. pneumoniae*-specific monoclonal antibody (TT-401) conjugated to FITC or with a Chlamydia genus-specific antibody (Pathfinder, Chlamydia culture confirmation monoclonal antibody, Bio-Rad, Redmond, WA)..

The inclusions were counted in 30 random fields at a magnification of 400 x. The infectivity titers were expressed as inclusion-forming units per milliliter. All experiments were performed 2–5 times.

4.4. Effects of retinoids

The effect of retinoids on chlamydial infection in HL cells and in HMEC cells was studied by treating the cells with 0–25 μM all-trans-retinoic acid (ATRA) or 0–12.5 μM 4-[(E)-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid (TTNPB) (Sigma, St. Louis, MO) for 1 hr prior to inoculation (haptens inhibition) followed by washing cells with PBS or during inoculation and in the growth medium. Control cultures were similarly incubated with media without ligands. Growth of *C. pneumoniae* was evaluated in the primary culture (containing retinoids) and production of infectious progeny during retinoid treatment was evaluated after passage into fresh HL cells (cultured in the absence of retinoids, but in the presence of cycloheximide, 0.5 $\mu\text{g}/\text{ml}$). In experiments to measure the effect of different incubation times in growth medium containing ATRA on production of infectious particles,

the infected cells were first grown in medium containing ATRA for 24 to 72 hours and replaced with α -MEM at various times post-infection. To determine the fold increase or decrease in growth of *C. pneumoniae* following incubation with retinoids, the IFUs/ml in treated cells were divided by the IFUs/ml in untreated cells as described above. To evaluate the effect of retinoids on production of infectious progeny, the burst size was determined following passage from primary culture in endothelial cells to HL cells (infectivity titers in HL cells divided by infectivity titers in the primary culture (endothelial cells). Toxicity of retinoids to host cells was monitored by microscopically determining cell growth and monolayer formation, detachment of cells, and normal/abnormal cell morphology. To determine that the concentrations of retinoids used were non-toxic to *C. pneumoniae*, organisms were incubated with the retinoid for 30 min at 37°C prior to infection and compared to organisms incubated in the appropriate buffer under the same conditions prior to inoculation of cells and determination of inclusion counts.

For binding assays, *C. pneumoniae* organisms were metabolically labeled with ³⁵S-methionine as described previously [24] and incubated with cells for 2 hr at 4°C (organisms attach but do not enter host cells) or at 37°C (organisms attach and enter) in the presence of retinoids or buffer. After washing to remove unbound label, cell lysates were air-dried on glass fiber filter papers. Filters and scintillation fluid (Formula-989, Packard Instrument Co., Meriden, CT) were added to scintillation vials and the radioactivity of bound or internalized elementary bodies (EBs) was counted on the scintillation counter.

4.5. Electron microscopy

Cell pellets were fixed for 30 minutes with 1% formaldehyde and 2% glutaraldehyde in PBS at pH 7.4, washed twice with PBS, post-fixed with 1% osmium tetroxide, dehydrated, embedded in Epon, sectioned and viewed in a JEOL electron microscope by standard methods.

4.6. Statistical analysis

Statistical analysis was done using the Student's t-test.

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Figure 1.

The effect of all-trans-retinoic acid (ATRA) and 4-[(E)-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid (TTNPB) on infectivity, attachment and internalization of *C. pneumoniae*. In all panels, error bars indicate 1 standard deviation from the means of inclusion counts from triplicate coverslips. * $P < 0.05$ (Student's t-test) for culture with indicated ligands vs. culture without a ligand. In Panel A, HL cells were incubated for 1 hr prior to inoculation with ATRA or TTNPB and washed with PBS prior to inoculation with *C. pneumoniae* (AR-39). Infectivity of *C. pneumoniae* is expressed as mean inclusion counts per well at 72 h postinfection (as percent of control). Both ATRA and TTNPB inhibit *C. pneumoniae* infectivity of epithelial cells. In panel B, binding assays were done by inoculating HL cells or endothelial cells with [35 S]-methionine-labeled purified *C. pneumoniae* organisms in the presence of ATRA for 2 h at 4°C (organisms attach, but are not internalized) or 37°C (organisms attach and are internalized), and washed. Radioactivity in cell lysates was counted and expressed as mean of cpm per well (as percent of control). ATRA inhibited both attachment to and internalization of *C. pneumoniae* into HMEC-1 cells, whereas no statistically significant differences were observed in HL cells. Panel C demonstrates the effect of ATRA and TTNPB on infectivity of *C. pneumoniae* in HL and HMEC-1 cells. ATRA and TTNPB were included in the growth medium after inoculation. Infectivity of *C. pneumoniae* is expressed as mean inclusion counts per well at 72 h post infection (as percent of control). Both ATRA and TTNPB inhibited *C. pneumoniae* infectivity of epithelial cells and endothelial cells, when included in the growth medium. In panel D, the time-dependent effect of ATRA and TTNPB on infectivity of HL cells is shown. ATRA and TTNPB were included in the growth medium after inoculation for indicated time periods. Subsequently, the cultures were fed with medium without the ligand. Infectivity of *C. pneumoniae* could be recovered if the cultures were treated with low concentrations of retinoids for 24 h, but with higher concentrations and longer duration of retinoid treatment, the effects became irreversible.

Figure 2.

All-trans-retinoic acid (ATRA) affects inclusion formation in HL cells. HL cells were infected with *C. pneumoniae* without ATRA in the growth medium (Panels A and C) or with 12.5 μM ATRA (Panels B and D). Cultures were incubated for three days at 35°C. For panels A and B, monolayers were fixed with methanol and stained with a Chlamydia genus-specific monoclonal antibody conjugated to FITC (Pathfinder, Chlamydia culture confirmation monoclonal antibody, Bio-Rad, Redmond WA). The image was obtained using a Leica DM 4000 B microscope at a magnification of 400 fold and a Leica DFC490 camera. Panel A demonstrates a normal inclusion. Panel B illustrates a reduced inclusion size in cells grown in medium containing ATRA. For Panels C and D, cell pellets were fixed for 30 minutes with 1% formaldehyde and 2% glutaraldehyde in PBS at pH 7.4, washed twice with PBS, post-fixed with 1% osmium tetroxide, dehydrated, embedded in Epon, sectioned and viewed in a JEOL electron microscope by standard methods. Panel C demonstrates a chlamydial inclusion in cells grown in growth medium for 72 hrs without ATRA and Panel D shows a disrupted inclusion membrane with aberrant particles. In Panel C, the arrow labeled EB points to an elementary body while the arrow labeled RB points to a reticulate body. In panel D, white arrows point to a disrupted inclusion membrane and black arrows demonstrate aberrant particles.