Pre-exposure of infected human endometrial epithelial cells to penicillin in vitro renders Chlamydia trachomatis refractory to azithromycin

Priscilla B. Wyrick1,2* and Stephen T. Knight1

1Department of Microbiology and Immunology, University of North Carolina School of Medicine, Chapel Hill, North Carolina; 2Department of Microbiology, J. H. Quillen College of Medicine, East Tennessee State University, Johnson City, TN, USA

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Objective: The clinical significance of the potential for persistent human chlamydial infections in vivo is being actively reassessed because of the increased frequency of recurrent infection with the same serovar despite compliance with an effective antibiotic regimen. The ability to extend the length of time of in vitro cultivation of polarized human endometrial epithelial cells (HEC-1B) provided the opportunity to establish a model system to determine if a persistent form of Chlamydia trachomatis had the same susceptibility as the actively growing form to a cidal concentration of azithromycin.

Methods: Polarized HEC-1B cells cultivated on extracellular matrix were infected with C. trachomatis serovar E and exposed to penicillin at 24 h post-infection (hpi) to induce a persistent infection characterized by slowly metabolizing but non-dividing, ultrastructurally aberrant reticulate bodies within the chlamydial inclusion; at 48 hpi, infected cultures were exposed to a bactericidal concentration of azithromycin for 72 h.

Results: Persistent chlamydiae were phenotypically resistant to azithromycin; the number of chlamydial inclusions on subpassage of progeny from persistent chlamydiae following removal of penicillin and recovery was essentially the same as from progeny from persistent chlamydiae following removal of penicillin and azithromycin and recovery. Neutrophils were attracted in vitro to persistently infected HEC-1B cells that had been exposed to penicillin and azithromycin.

Conclusions: Thus, this study provides evidence at the cellular microbiology level in vitro for mechanisms that could exist in vivo to create sustained, but perhaps clinically inapparent inflammation, which might eventually lead to conditions such as silent pelvic inflammatory disease.

Keywords: C. trachomatis, chlamydial persistence, polarized HEC-1B cells, pelvic inflammatory disease

Introduction

It is well recognized that Chlamydia trachomatis serological variants (serovars) D–K continue to be the most common infectious bacterial agents of sexually transmitted disease worldwide, with an estimated 89 million new genital infections occurring annually.1 It is also appreciated that a difficulty in detecting such infections is that the signs of active genital chlamydia infection are often mild or inapparent. The current recommended treatment for chlamydial genital infections is azithromycin.2,3 If patients go undiagnosed and are left untreated, C. trachomatis can progress from the lower to upper reproductive tract and induce destructive sequelae such as pelvic inflammatory disease (PID), infertility and ectopic pregnancy. In males, destructive consequences include prostatitis and epididymitis.1 Recent studies reveal that exposure of sperm to C. trachomatis serovar E results in significantly reduced sperm motility and viability, which may be contributing to subfertility in males.4

C. trachomatis is reportedly responsible for ~25% of the >1 million cases of PID diagnosed per year in the USA. Further, there are new indications that one out of four women infected with C. trachomatis have unrecognized or silent PID, with damage to fallopian tubes due to an inflammatory process that is clinically inapparent.5 The clinical significance of the potential for persistent human chlamydial infections in vivo is being actively reassessed because of such correlations and because of

*Corresponding author. Tel: +1-423-439-6294; Fax: +1-423-439-8044; E-mail: pbwyrick@mail.etsu.edu

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the increasing frequency of recurrent infection with the same serovar despite compliance with an effective antibiotic regimen. In an important study by Patton et al., evidence was provided that ~79% of women with post-infectious tubal infertility, despite appropriate antimicrobial therapy, appeared to have persistent chlamydial infection. A similar trend of possible persistent infection, of 1–3 months, in chlamydia-positive female adolescents following the recommended treatment regimen was reported by Katz et al. However, either reinfection from an untreated partner or simply temporary microbial suppression following treatment and subsequent reemergent infection are equally plausible. The microbiological rationale for implicating retention of viable, persistent chlamydiae in vivo, coupled with the practical difficulties of obtaining convincing documentation in patients, are discussed in an elegant review by Darville. There is clear evidence that Chlamydia exposed to penicillin, interferon-gamma (IFN-γ), or amino acid or iron deprivation can produce a persistent infection in eukaryotic host cell cultures in vitro and each of these triggers can occur in vivo.

Chlamydia have long been known to be reactive to penicillin in vitro, with the resulting production of abnormally enlarged, aberrant reticulate bodies (RB). These RB can persist for weeks and even months under continued antibiotic exposure yet can still reorganize and mature into infectious elementary bodies (EB) once the penicillin is removed. Barbour et al. demonstrated the presence of penicillin-binding proteins (PBPs) in C. trachomatis, and the C. trachomatis serovar D genome sequence revealed protein homologues for transpeptidase (D682, D270) and carboxypeptidase (D551), the PBPs that are exposed to penicillin, interferon-gamma (IFN-γ), or amino acid or iron deprivation can produce a persistent infection in eukaryotic host cell cultures in vitro and each of these triggers can occur in vivo.

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In any event, RB do change morphologically and physiologically, with a dramatically slowed metabolism, in the presence of penicillin. In IFN-γ-induced persistence, synthesis of chlamydial outer membrane components, such as the major outer membrane protein and lipopolysaccharide, is measurably reduced. In the past, penicillins have been used for treatment of chlamydial infections, and in patients with gonococcal urethritis or cervicitis who were doubly infected with chlamydiae. Thus, the use of penicillin to induce an in vitro model of chlamydial persistence is a valid scenario.

The question precipitating this study was that if latent genital chlamydial infections do occur, does a persistent form of C. trachomatis have the same susceptibility to antibiotics as compared with actively growing chlamydiae? Whereas IFNγ is considered currently to be a more relevant inducer of chlamydial persistence both in vitro and in vivo, the human endometrial (carcinoma) cell line, subclone 1B (HEC-1B) used in this study does not respond to IFN-γ; the target IFN-γ up-regulated enzyme, indoleamine 2,3-dioxigenase—which catalyses oxidative decylation of the essential amino acid tryptophan—is missing. Therefore, to answer this question, we have used an in vitro model of polarized endometrial epithelial cells in conjunction with penicillin to induce a persistent C. trachomatis serovar E infection, followed by exposure to a concentration of azithromycin previously determined to be bactericidal for serovar E in polarized HEC-1B cells.

Materials and methods

Growth of Chlamydia and host cell lines

A human urogenital isolate of C. trachomatis E/UW-5/CX was originally obtained from S.P. Wang and C.-C. Kuo (University of Washington, Seattle, WA, USA) and has been maintained in our laboratory for several years. The same standardized inocula of C. trachomatis serovar E elementary bodies (EB), prepared from chlamydiae grown in McCoy cells cultured on microcarrier beads, was used for all experiments.

The HEC-1B cell line (HTB-113, American Type Culture Collection, Rockville, MD, USA), derived from an endometrial carcinoma, was used throughout this study. Stock cultures of epithelial cells were propagated in flasks containing Dulbecco’s Modified Eagle medium (MEM; Life Technologies GIBCO-BRL, Grand Island, NY, USA) supplemented with 10% fetal calf serum (HyClone, Logan, UT, USA) and 10mM HEPES buffer, pH 7.3 (DMEM). The cell cultures were routinely monitored and shown to be free of mycoplasma contamination.

The effect of azithromycin on penicillin-induced persistent chlamydial infections

HEC-1B cells (1 × 10^5) were grown in cell culture chamber inserts as polarized monolayers on permeable filters (Falcon, Franklin Lakes, NJ, USA) coated with extracellular matrix (ECM; Bio-Technologies Incorporated, Staufton, MA, USA). The apical surfaces of host cells were then inoculated with C. trachomatis EB (50 µL) by passive adsorption with a titre of crude stock diluted such that at least 50% of the epithelial cells were infected. At 24 h post-infection (hpi), the infected cells were exposed to 25 U/mL of penicillin added exogenously to the growth medium for 48 h to induce persistent chlamydiae, followed by the addition of medium containing penicillin and a bactericidal concentration of azithromycin (0.5 mg/L ≥ MBC90; Pfizer, Groton, CT, USA; Figure 1). Both antibiotics were removed at 144 hpi and the cultures were
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reincubated for an additional 72 h to allow viable chlamydiae to recover and form infectious EB. These cultures were then harvested, subpassaged onto HEC-1B cell monolayers grown on glass coverslips, and examined for the presence of chlamydial inclusions, identified by immunofluorescence microscopy. The infected host cells were fixed with cold methanol and stained with a pool of fluorescein isothiocyanate (FITC)-labelled mouse monoclonal antibodies which recognized chlamydial major outer membrane protein (Syva MicroTrak; Wampole Lab., Cranbury, NJ, USA). The percentage of infected cells in each monolayer was calculated from the number of fluorescent antibody-labelled inclusions counted in 10 representative areas from duplicate or triplicate samples at 400 times magnification. In the longest term cultures (216 h) where some infected cells began to be released from the coverslips, especially during methanol fixation, the number of inclusions per 4–5 microscopic grid fields per patch were counted in a minimum of five patches per coverslip. These experiments were repeated twice on separate occasions.

**Chemotaxis of polymorphonuclear leucocytes (PMN)**

The upper compartments of the cell culture chamber inserts were seeded with 10^5 HEC-1B cells and polarized cell sheets formed in ~4–5 days of incubation at 35°C. The polarized HEC-1B cell cultures were inoculated with 50 μL of *C. trachomatis* EB diluted to infect ~50% of the host cells, and the infected cultures were incubated at 35°C. DMEM containing penicillin was added at 24 hpi and the cultures were incubated for 48 h. At 72 hpi, DMEM containing azithromycin in combination with penicillin was added to the infected cultures and maintained for an additional 24 h. At 96 hpi, the medium was removed from the culture chambers, the inserts were inverted and the bottom side of the filters layered with 1% agarose (SeaPlaque, FMC BioProducts, Rockland, MD, USA), prepared in DMEM containing 5% heat-inactivated human serum, and allowed to solidify for 5 min. A 3 mm diameter well was excised from the centre of the agarose layer and 50 μL containing 5 × 10^8 PMN, pre-loaded with azithromycin, was added to the agarose well. A drop of fresh agarose was applied to the well to prevent drying and the inserts were reincubated, right side up, at 35°C for an additional 3 h to allow for PMN chemotaxis, which progressed through the ECM and from the basal to apical domain of the infected, polarized HEC-1B cells. Multiple control studies performed in previous experiments using calcein-loaded PMN for quantification of migrating PMN reproducibly indicated that the number of PMN undergoing directed chemotaxis to chlamydiae-infected cells was 100-fold greater than random PMN migration to uninfected cells. Human neutrophils were isolated from heparinized whole blood of healthy donors by a one-step Ficoll-Hypaque separation procedure followed by a 3% Dextran sedimentation step to enrich the PMN fraction. The PMN were washed, resuspended in DMEM and counted microscopically. Loading the PMN with azithromycin was accomplished by incubating the leucocytes in DMEM containing 25 mg/L of antibiotic for 1 h at 35°C. The PMN were then washed three times to remove exogenous azithromycin and resuspended in DMEM; the protocol for azithromycin loading has previously been shown not to affect the viability or chemotaxis of the PMN.

**Assessing infected polarized HEC-1B cells by microscopy**

The effect of the antibiotics on chlamydiae and PMN migration was assessed by light and transmission electron microscopy. Cultures for light microscopy were embedded in JB-4 Plus, a glycol methacrylate histology resin (PolySciences, Warrenton, PA, USA). Microtome sections, 2–5 microns thick, were cut to provide a sagittal view of the infected polarized cell sheet and stained with toluidine blue to improve the contrast for high magnification light microscopy. Duplicate samples were processed for transmission electron microscopy. Briefly, infected monolayers were fixed in situ with 2% glutaraldehyde–0.5% paraformaldehyde in 0.1 M cacodylate buffer for 60 min at 25°C. The filters were cut out of the insert supports, cut into strips and enrobod by dipping into 5% molten agarose. Following two washes in cacodylate buffer, the samples were post-fixed with 1% osmium tetroxide prepared in cacodylate buffer for 1 h at 25°C and washed again in buffer. After two brief washes in 70% ethanol, the samples were exposed to 5% uranyl acetate in 70% ethanol for 10 min, dehydrated in 95% and 100% ethanol and finally propylene oxide. Infiltration and curing were performed in Epon–Araldite resin. Gold thin sections were cut with a diamond knife on a Reichart Ultracut S microtome (Leica Microsystems, Inc., Bannock, IL, USA), counterstained with uranyl acetate in 70% ethanol for 10 min, dehydrated in 95% and 100% ethanol and finally propylene oxide. Infiltration and curing were performed in Epon–Araldite resin, and examined in a Philips 201 transmission electron microscope operating at 60 kV.

**Results**

**Ultrastructural analysis of penicillin-induced persistent C. trachomatis**

At 48 hpi, *C. trachomatis* serovar E inclusions are filled with normal metabolically active RB (Figure 2a). On exposure of these control, infected cells to azithromycin alone for 48 h, an early effect of inhibition of protein synthesis is manifested ultrastructurally by extensive RB outer membrane blebbing (Figure 2b), by 72 h of azithromycin exposure, empty RB envelope ghosts dominate (Figure 2c), confirming the efficacy of azithromycin on growing chlamydiae.

The presence of penicillin in the culture medium did not reduce the number of chlamydial inclusions in polarized HEC-1B cells over the course of 144 h, as shown by light microscopy; however, the sizes and contents of the inclusions varied considerably within the same culture. Transmission electron microscopy confirmed that penicillin did induce a persistent chlamydial infection in endometrial epithelial cells, which was maintained for up to 120 h of exposure to penicillin (Figures 1 and 2). When compared with chlamydiae in cultures that had not been exposed to antibiotics at 48 hpi and which contained metabolically active and dividing RB (Figure 2a), the penicillin-induced persistent RB were notably enlarged (Figure 2d–f). Abundant chlamydial outer membrane blebs and chlamydial envelope ghosts were also present within the inclusions. In addition, some intact as well as irregular EB could be found within the same inclusions (Figure 2d, arrows). As incubation of the infected HEC-1B cells progressed through 120 h exposure to penicillin, the altered chlamydial ultrastructure persisted (Figure 2f).

**Ultrastructural analysis of the effect of azithromycin on penicillin-induced persistent C. trachomatis**

Infected polarized HEC-1B exposed to penicillin for 48 h followed by the addition of azithromycin appeared misshapen,
more so than when the cultures were exposed to penicillin alone. The contents of these enlarged inclusions contained mostly aberrant RB, a few RB in various stages of apparent destruction as well as RB envelopes and membrane blebs (Figure 2g–i). A surprising number of EB were found within the same inclusions despite the combined presence of penicillin and azithromycin for up to 120 h (Figure 2i, arrows).

Recovery of infectious chlamydiae after exposure of infected endometrial cells to penicillin and azithromycin

Infected polarized HEC-1B cell cultures, which had been exposed to penicillin or the combination of penicillin and azithromycin, were allowed to recover for 72 h in the absence of antibiotics, and chlamydiae were harvested for subpassage directly onto fresh HEC-1B monolayers grown on glass coverslips (Figure 1). Surviving C. trachomatis serovar E exposed to a combination of penicillin and azithromycin were able to produce inclusions in the majority of HEC-1B cells (Figure 3d) qualitatively equivalent in number to those chlamydiae which had been exposed to penicillin alone (Figure 3c) or no antibiotic at all (Figure 3a); reproducible quantitative counts were extremely difficult to obtain because after 216 h in culture, only patches of infected cells remained on the coverslips. However, there were $\sim 1.5 \times 10^3$ inclusions per four microscopic grid fields per patch in the penicillin-only exposed, infected cells and $\sim 1.2 \times 10^3$ inclusions in the same number of infected cells exposed to both penicillin and azithromycin. It is not entirely
clear why the inclusions in the penicillin only-exposed infected cells (Figure 3c) were considerably larger than the inclusions in infected cells exposed to penicillin and azithromycin (Figure 3d); essentially nothing is known regarding the osmotic/turgor pressures inside the inclusion, but isotonic, or even slightly hypertonic, conditions would probably support maintenance of spheroplast-like forms of these Gram-negative bacteria. These data demonstrated that exposure of a persistent chlamydial infection to a bactericidal concentration of azithromycin was not lethal to *C. trachomatis* in *vitro*. In contrast, on subpassage of progeny from control, infected HEC-1B samples containing actively growing chlamydiae exposed to azithromycin for 72 h in the absence of penicillin, no/rare chlamydial inclusions developed (Figure 3b).  

**Neutrophil chemotaxis to infected endometrial epithelial cells containing penicillin-induced persistent chlamydiae in inclusions exposed to azithromycin**

Acutely infected HEC-1B cells produce early *in vitro* signature endometrial mRNA for the pro-inflammatory chemokines ENA-78 and GCP-2, probably in differentiation between infection and organ-induced physiologic-like inflammatory processes.  

PMN underwent directed chemotaxis to these chlamydial-infected cells, both in the absence as well as in the presence of azithromycin exposure. The PMN chemotaxis assay was performed in the current experiments to determine if specific targeted migration also occurred to persistently infected HEC-1B cells.

PMN were observed by light microscopy to move freely through the ECM barrier and transmigrate to close association with infected HEC-1B cells exposed to penicillin and azithromycin for 72 h, for a total of 96 hpi (Figure 4). Examination of numerous cytological sections from different locations in the specimens revealed no discernible difference in the numbers of chemotactic PMN to infected cells exposed to penicillin alone or the combination of penicillin and azithromycin, even when the incubation period for chemotaxis was extended from 3 to 12 h (data not shown).

**Discussion**

The clinical significance of persistent human chlamydial infections *in vivo* has been brought to the forefront of sexually transmitted disease research, primarily because of accumulating data on the increasing frequency of recurrent infection with the same serovar despite compliance with an effective antibiotic therapy.  

Although reinfection cannot be discounted in the clinical studies described, some evidence supports the possibility that recurrent infections arose from the survival and reactivation
of *C. trachomatis* in genital tissue long after antibiotics had been cleared from the body.25

The capability for maintaining and significantly extending the cultivation of polarized endometrial epithelial cells provided the opportunity to gain insight at the cellular level in *vitro* into the antimicrobial failure of a poky prokaryotic protein synthesis inhibitor, such as azithromycin,24 on persistently growing *C. trachomatis*. Aberrant RB, induced by exposure to penicillin in this study, were refractory to the killing influence of penicillin, shown by damaged RB and empty cell envelopes.

Importantly, our findings substantiate the results of Gieffers et al.,30 who reported that persistent *Chlamydia pneumoniae* in circulating monocytes from patients with coronary artery disease, as well as CD14+ human peripheral blood monocytes infected *ex vivo*, were also refractory to azithromycin; cultural recovery of viable *C. pneumoniae* progeny from azithromycin-exposed monocytes was demonstrated.

Whereas different mechanistic pathways exist for inducing aberrant forms of chlamydiae and recent DNA microarray37 and proteomics38 data have revealed different gene and protein expression profiles, significantly enlarged RB are a common finding. It is tempting to speculate that compartmentalized RB membrane functions, once juxtaposed and tightly regulated, are much delayed in distended RB via disruption of the rapid, orderly biochemical relay systems and there are probably some common consequences intracellularly, such as altered DNA replication, chromosome segregation and cell division.39 In addition, the presence of some intact EB, which would essentially be impervious to antibiotics effective only against metabolically active bacteria, is a reminder of the asynchrony of the chlamydial developmental cycle and, by extension, the importance of patient compliance with full therapeutic regimens. It is possible that some of these EB entered the host cell late and did not receive the necessary cues to convert to RB and, as such, remained unchanged. Whether or not such EB can be ‘reactivated’ after the removal of the antibiotic pressure is unknown at this time.

Exposure of polarized, *C. trachomatis* serovar E-infected HEC-1B cells to azithromycin alone for 72 h resulted in the formation of excessive amounts of chlamydial outer membrane blebs and large envelope ghost structures,32 also confirmed in this study. Previous studies from our laboratory had shown this residual chlamydial envelope material, while gradually reduced in amount over time, was retained for at least 4 weeks in *in vitro* culture, and PMN chemotaxis targeted to the endometrial cells containing envelope-filled inclusions occurred at weekly intervals throughout the 4 weeks. These data imply that the pro-inflammatory signals triggered in the infected epithelial cells were sustained over an extended period. In this *in vitro* two-stage co-culture model, in the absence of antibiotics, PMN chemotaxis does not begin until mid-developmental cycle, or ~30 hpi. However, mRNA expression for the endometrial infection-specific chemokines ENA-78 and GCP-2 was detected as early as 6 and 12 hpi, respectively.32 Thus, even though acute infection in the current studies was dampened by the presence of antibiotics, the innate response in these infected mucosal epithelial cells is triggered early and transmigrating PMN were still able specifically to target the persistently infected HEC-1B cells for killing. In summary, this study provides evidence at the cellular microbiology level in *vitro* for mechanisms that could exist *in vivo* to create sustained, but perhaps clinically inapparent inflammation, which might eventually lead to conditions such as silent PID.

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References


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