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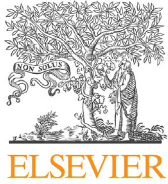

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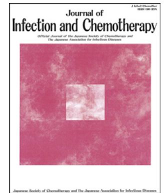

Original article

# Effect of *Ureaplasma parvum* co-incubation on *Chlamydia trachomatis* maturation in human epithelial HeLa cells treated with interferon-γ

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ABSTRACT

*Chlamydia trachomatis* is an obligate intracellular bacterium that causes a sexually transmitted disease. *Ureaplasma parvum* is commensal in the human genital tract, with a minimal contribution to urogenital infection. We have recently found that *U. parvum* has a significant effect on the presence of *C. trachomatis* in the genital tract of healthy women. We therefore assessed the effect of *U. parvum* co-incubation on *C. trachomatis* maturation from reticulate bodies (RBs) to elementary bodies (EBs) in HeLa cells in the absence or presence of interferon (IFN)-γ, which is a critical host defense factor. IFN-γ stimulation of viable *U. parvum* significantly prompted chlamydial growth with an increase in infectious particles, EBs, in HeLa cells. IFN-γ treatment of killed *U. parvum* had a similar effect on *C. trachomatis* maturation in HeLa cells. There was no change in expression of indoleamine 2,3-dioxygenase (IDO) in cultures of viable or killed *U. parvum*. We concluded that *U. parvum* co-incubation by IFN-γ helped *C. trachomatis* to mature from RBs to EBs in HeLa cells, independent of IDO expression. This suggests a novel survival strategy of *C. trachomatis* against IFN-γ exposure, prompting secondary infection of the genital mucosa, with possible clinical implications.

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## 1. Introduction

Obligate intracellular bacterium *Chlamydia trachomatis*, which infects the human genital tract and causes cervicitis, is the leading cause of bacterial sexually transmitted diseases (STDs), and its annual incidence is estimated at 100 million worldwide [1–4]. It is also predicted that the prevalence might be >10% of healthy women [2–4]. Furthermore, at least 70% of the women with *C. trachomatis* infections are usually asymptomatic for prolonged periods of time, without appropriate therapy [5]. They become exacerbated with more serious diseases, including pelvic inflammatory disease (PID) and fibrosis with fallopian tube scarring, subsequently causing infertility and ectopic pregnancy [6–10]. Thus, controlling genital chlamydial infection is an important public health issue, and development of an effective protective program, backed up by a chlamydial survival mechanism in the genital tract, is eagerly anticipated.

The commensal bacteria that inhabit the female genital tract are a complicated ecosystem that also provides metabolic benefits [11,12]. However, whether commensal bacteria provide some advantages for *C. trachomatis* survival in the genital tract remains unclear. *Ureaplasma parvum*, which belongs to the family Mycoplasmataceae, is a representative commensal bacterium in the genital tract [13–17]. Recently, we found a high prevalence of mixed infection of *C. trachomatis* and *U. parvum* in the genital tract of healthy women attending their first prenatal visit in a community hospital in Japan [18], suggesting a possible mutual interaction between the bacteria. However, the exact reason why *C. trachomatis* preferentially co-infects with *U. parvum* in the genital tract remains uninvestigated.

C. *trachomatis* has a unique biphasic developmental cycle consisting of elementary bodies (EBs), which are infectious in host cells, and reticulate bodies (RBs), which are a replicating form, which takes place in membrane vesicles called inclusions [1].

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Interferon (IFN)-γ is a crucial defense factor against *C. trachomatis* infection in the human genital tract, through inhibition of bacterial maturation from RB to EB in the inclusions [19–23]. The mechanism has been thoroughly investigated, and depletion of tryptophan in IFN-γ-exposed cells, through activation of indoleamine 2,3-dioxygenase (IDO), which is an essential enzyme in the kynurenine metabolic pathway, is a key event for eliminating the bacteria from infected cells [20–23]. However, whether *U. parvum* alters the effect of IFN-γ on *C. trachomatis* elimination remains unknown.

In the present study, we investigated the effect of *U. parvum* co-incubation on *C. trachomatis* growth in HeLa cells in the absence or presence of IFN-γ, which is a critical host defense factor for eliminating *C. trachomatis*.

## 2. Methods

### 2.1. Human cell lines

The epithelial cell lines HeLa and HEp-2 were purchased from ATCC (Manassas, VA, USA) and Riken Cell Bank (Tsukuba, Japan), respectively. Both cell lines were cultured at 37 °C in 5% $CO_2$ in Dulbecco's Modified Eagle's Medium (DMEM; Sigma, St. Louis, MO, USA) containing 10% heat-inactivated fetal calf serum (FCS), 10 μg/ml vancomycin and 1 μg/ml amphotericin B (Sigma) [24].

### 2.2. Bacteria

*C. trachomatis* D/UW3 Cx strain (VR-885) was purchased from ATCC, and propagated in HEp-2 cells as described previously [25]. The infected cells were collected at 2 days after infection, and then disrupted by freezing–thawing. After centrifugation to remove cell debris, bacteria were concentrated by high-speed centrifugation. Bacterial pellets were resuspended in sucrose–phosphate–glutamic acid buffer, and stored at −80 °C until use. The numbers of infectious progenies for *C. trachomatis* were determined as IFU by counting chlamydial inclusions formed in HEp-2 cells using fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-*Chlamydia* antibody specific for *Chlamydia* lipopolysaccharide (LPS) (with Evans Blue) (Denka Seiken, Tokyo, Japan) [25]. *U. parvum* (ATCC27813) was also purchased from ATCC. The bacteria were propagated in PPLO (Pleuro pneumonia-like organism) medium [1.5% (w/v) PPLO powder, 20% (v/v) horse serum, 2.5% (w/v) yeast extract, 0.1% (w/v) urea, 0.005% (w/v) phenol red, 10 μg/ml vancomycin, and 1 μg/ml amphotericin B, pH 6.0] [26–28]. The numbers of *U. parvum* were determined by counting colonies formed on the PPLO medium containing 1% agar at 14 days after cultured under a phase-contrast microscope. Heat-killed (85 °C for 30 min) and UV-treated (UV irradiator; UVP, Upland, CA, USA; 46 800 $\mu J/cm^2$) *U. parvum* were also used.

### 2.3. Infection

HeLa cells were adjusted to a concentration of $1 \times 10^5$ cells/well and simultaneously infected with *C. trachomatis* at multiplicity of infection (MOI) 1 and *U. parvum* at MOI 0.01–10 by centrifugation for 450 × g at room temperature. HeLa cells were pretreated with IFN-γ (5 ng/ml) for 1 day before infection. After washing to remove noninfecting bacteria with DMEM, the cells at a concentration of $1 \times 10^5$ cells/well were incubated with 10% FCS–DMEM containing vancomycin (10 μg/ml) and amphotericin B (0.5 μg/ml) for 2 days at 37 °C in 5% $CO_2$ in the presence or absence of IFN-γ (5 ng/ml). Cells were harvested and used to determine EB numbers by IFU assay (see above) and assessment of changes in IDO expression (see below). The working concentration of IFN-γ (5 ng/ml) was determined with reference to previous studies showing a maximal effect of IFN-γ on *C. trachomatis* growth [29,30]. We also confirmed by using Trypan Blue exclusion assay that each of the drugs (antibiotics and IFN-γ) or PPLO medium at working concentration had no cytotoxic effect on the cells.

### 2.4. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Total RNA was extracted from infected or uninfected cells using High Pure RNA Isolation Kit (Roche, Indianapolis, IN, USA) according to the manufacturer's protocol. Extracted RNA was treated by DNase I (DNA-free; Ambion, Austin, TX, USA). Reverse transcription of 1 μg total RNA by avian myeloblastosis virus RT was performed with random primers in a commercial reaction mixture (ReverTra Ace qPCR RT Master Mix; Toyobo, Osaka, Japan). Synthesized cDNAs were used for PCR amplification with primers specific for *ido1* (forward: 5′-CCT GAG GAG CTA CCA TCT GC-3′, reverse: 5′-TCA GTG CCT CCA GTT CCT TT-3′) [31], and *gapdh* (forward; 5′-AAC GGG AAG CTC ACT GGC ATG-3′, reverse: 5′-TCC ACC ACC CTG TTG CTG TAG -3′) [32]. PCR conditions consisted of 10 min denaturation at 95 °C, followed by 40 cycles, each of 30 s denaturation at 95 °C, 30 s annealing at 60 °C, and 30 s extension at 72 °C. The amount of *ido1* PCR products was expressed as a ration to that of *gapdh*.

### 2.5. Western blotting

HeLa cells collected from each culture were boiled for 5 min at 100 °C in a reducing sample buffer containing 2-mercaptoethanol. Samples were loaded and separated by 10% (w/v) SDS-PAGE (20 mA, 80 min). Separated proteins were transferred to polyvinylidene difluoride membranes by semi-dry electroblotting. Membranes were blocked with 5% (w/v) skimmed milk in Tris-buffered saline and incubated with an anti-IDO antibody (Epitomics, Burlingame, CA) or anti-human α-tubulin antibody (Sigma) for 1 h at room temperature, followed by a horseradish-peroxidase-conjugated goat anti-rabbit IgG for 1 h at room temperature. Labeled proteins were visualized with Pierce® Western Blotting Substrate (Thermo, Rockford, IL).

### 2.6. Statistical analysis

Comparison of data was performed using an unpaired Student *t* test. A value of $p < 0.05$ was considered significant.

## 3. Results and discussion

### 3.1. U. parvum co-incubation with IFN-γ recovered C. trachomatis maturation from the developmental cycle suppressed in HeLa cells

IFN-γ is a critical host defense factor directly associating with intracellular chlamydial elimination [20–23]. The effect of *U. parvum* stimulation on *C. trachomatis* growth in the presence of IFN-γ was investigated. Fluorescence staining revealed that although IFN-γ exposure drastically restricted the expansion of chlamydial inclusion size (Fig. 1(A) and (B)), the inclusion size recovered in the presence of *U. parvum* (Fig. 1(C)). There was also possibly an increase in the number of bacterial EBs, suggesting that *U. parvum* co-incubation with IFN-γ partially inhibited host defense. To confirm this, we assessed using the inclusion forming units (IFU) assay the changes in the number of *C. trachomatis* infectious EBs in HeLa cells with or without *U. parvum* co-incubation. *U. parvum* co-incubation with IFN-γ significantly increased the number of infectious particles, depending on MOI of *U. parvum* (Fig. 2). Meanwhile, there was no significant difference in the number of chlamydial infectious particles (IFU values/ml ± standard deviation) between viable (MOI 1: $2.7 \times 10^5 \pm 4.9 \times 10^4$) and heat killed *U. parvum* co-incubation

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