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**Effect of steroid receptor antagonist RU486 (mifepristone) on *Chlamydophila pneumoniae* in IFN γ-induced persistent infection model with epithelial HEp-2 cells**

Kasumi Ishida[1], Tomohiro Yamazaki[1], Kazuki Motohashi[1], Miho Kobayashi[1], Junji Matsuo[1], Takako Osaki[3], Tomoko Hanawa[3], Shigeru Kamiya[3], Yoshimasa Yamamoto[2], Hiroyuki Yamaguchi[1]*

[1]*Department of Medical Laboratory Sciences, Faculty of Health Sciences, Hokkaido University, Sapporo, Japan*

[2]*Division of Microbiology, Department of Infectious Disease, Kyorin University, School of Medicine, Tokyo, Japan*

[3]*Laboratory of Molecular Microbiology, Department of Bioinformatics, Osaka University Graduate School of Medicine, Osaka, Japan*

*Corresponding author: Hiroyuki Yamaguchi

Department of Medical Laboratory Sciences, Faculty of Health Sciences Hokkaido University

Nishi-5 Kita-12 Jo, Kita-ku, Sapporo, Hokkaido 060-0812, Japan

Tel/Fax: +81-11-706-3326; E-mail: hiroyuki@med.hokudai.ac.jp

**Subject section and specified field**: Bacteriology and intracellular parasitology

**Short title**: Effect of RU486 on persistent infection with C. p

**List of abbreviations**: RT, reverse transcription; FITC, fluorescein isothiocyanate; IFU, inclusion-forming unit; LPS, lipopolysaccharide; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum

**Abstract**

We previously demonstrated that the steroid receptor antagonist mifepristone (RU486) causes growth inhibition of *Chlamydophila pneumoniae* by binding to and subsequently destroying bacteria during the normal developmental cycle of epithelial HEp-2 cells. In the present study, we assessed the efficacy of treatment with RU486 against persistent *C. pneumoniae* infection in interferon (IFN) γ-treated HEp-2 cells. Assessment of bacterial growth modification, the number of infectious progenies, the inclusion formation and the gene expressions of *C. pneumoniae* (*16S rRNA* and *hsp60*) were investigated in cells stimulated with or without IFN γ in the presence of RU486 using an inclusion-forming unit (IFU) assay, fluorescence microscopic analysis and RT-PCR, respectively. Our results indicate that RU486 treatment produced growth inhibition and an absence of gene expression for *C. pneumoniae* in normal HEp-2 cells and that it partially failed to inhibit the growth in HEp-2 cells stimulated with IFN γ. These results indicate that treatment with RU486 had a limited effect on *C. pneumoniae* growth only during the active developmental stage of bacteria, implying a possibility that the bacterial target molecule against RU486 is not enough expressed during persistent infection with an aberrant developmental cycle. Thus, our findings provide valuable insight into the complicated chlamydial biological processes involved in the recurrent cycling between normal and persistent infections.

**Key words**: *Chlamydophila pneumoniae*, mifepristone, steroid receptor antagonist, RU486, HEp-2, IFNγ, persistent infection

## Introduction

*Chlamydophila pneumoniae* is an obligate intracellular bacterium that has a unique developmental cycle involving infection of the elementary body (EB; an infectious form with metabolically inactive to cells) (early stage) and subsequent maturation of the reticulate body (RB; an intracellular metabolically active but non-infectious form) to form the EB (mid to late stages) [1]. This bacterial species is a well-known cause of common human respiratory infection [2, 3], with several studies also reporting that chronic chlamydial infection (persistent infection) can be an important clinical manifestation of persistent respiratory diseases such as asthma and atherosclerosis [4-6]. The presence of *C. pneumoniae* in persistent infection is characterized by an absence of culturability [7], in addition to altered bacterial RNA and protein levels [8-11], morphologically altered chlamydial bodies [8] and increased resistance to antimicrobials, such as azithromycin or clarithromycin, frequently used for the treatment of *C. pneumoniae* infection [12]. The mechanisms leading to persistent *C. pneumoniae* infection remain unclear; however, infection in reported cases occurred during an uncompleted developmental cycle with a lack of maturation of the EB, and an absence of bacterial metabolic activity caused a decrease in sensitivity to the antibiotics used. Consequently, many researchers have studied how intracellular bacterial persistence occurs using well-characterized epithelial HEp-2 cells and investigated which drug is most effective at eliminating bacteria that have developed to a persistent phase. Generally, persistent infection is induced by treatment with interferon (IFN) γ, as regulated by a broad range of bacterial genes encoding proteins associated with inclusion membranes [13], type III machinery [14], metabolism [15] and immunopathology [15-17]. However, an effective drug against persistent chlamydial

infection has yet to be discovered, and current therapy relies on potentially ineffective experimental drug treatments against chlamydial latent infection or reinfection, which are always characterized by refractory disorder.

Steroid treatment is widely used to treat immunoreactive and inflammatory diseases. However, the immunosuppressive activity of steroid treatment can also result in an increased susceptibility to a wide variety of infectious diseases. Previous *in vitro* studies have suggested that a significant increase in the number of inclusions was produced by a constant inoculum of chlamydia in epithelial cells incubated with steroid [18-20]. Furthermore, experiments in a mouse model indicated that chlamydial reactivation and latent pulmonary infection were also stimulated by the presence of steroids [20, 21]. Thus, these data strongly suggest that steroids increase the incidence of *C. pneumoniae* in the persistent phase in host cells, and that, in contrast, steroid antagonists may modify persistent infection by potentially inhibiting bacterial growth or directly killing bacteria.

The steroid receptor antagonist mifepristone (RU486), which is effective for termination of early pregnancy, has remarkable anti-steroid activity [22]. In the biopharmacological field, this drug is well characterized and used as a tool for analyzing steroid receptor signaling in cellular homeostasis [22]. We previously reported that RU486 directly binds to and kills *C. pneumoniae* within cells, indicating that the regulation of *C. pneumoniae* growth in cells by RU486 provides a new potential approach to drug therapy [23]. Furthermore, the fact implies that RU486 can kill *C. pneumoniae* regardless of the persistent infection with refractory disorder. To therefore investigate this approach, our current study aimed to examine whether treatment of host cells with steroid receptor antagonist RU486 inhibits *C. pneumoniae* growth during persistent infection induced by treatment with IFN γ. Although contrary to our

expectation RU486 treatment did not work on *C. pneumoniae* persistent infection at all, we show the data indicating that the expression of target molecule of RU486 on the bacteria obviously differs between normal developmental cycles and persistent stage, providing valuable insight into the complicated chlamydial biology.

## Materials and methods

### Cell line

Human epithelial cell line HEp-2 was kindly provided by R. Widen, Tampa General Hospital, Tampa, FL, USA. Cells were cultured in DMEM containing 10 % heat-inactivated FCS and antibiotics (gentamicin sulfate, 10 μg/ml; vancomycin, 10 μg/ml; amphotericin B, 1 μg/ml) (Sigma, St. Louis, MO) at 37 °C in 5 % $CO_2$. The absence of *Mycoplasma* in bacterial suspension and cell cultures were confirmed by touch-down PCR as reported previously [24].

### Bacteria

*C. pneumoniae* TW183 used in this study was kindly provided by G. Byrne, University of Wisconsin, Madison, WI. Bacteria were propagated in HEp-2 cell culture according to methods described previously [25].

### Drugs

RU486 (Sigma) was dissolved in ethanol to a stock concentration of 25 mM. The reagent was diluted to working concentrations using culture medium.

**Infection**

Cultured cells were infected with *C. pneumoniae* at a multiplicity of infection of 10 for 1 h at room temperature by centrifugation at 800 × g. After washing twice with medium, cells were resuspended in medium, and for immune staining, cells were placed on coverslips (diameter, 12 mm). Then infected cells were incubated for up to 72 h in the presence or absence of RU486 (10 or 25 μM), with or without IFNγ (5–40 ng/ml) (Sigma). Infected cells treated with ethanol at concentrations equivalent to those in RU486 solutions were also prepared as a vehicle control (ethanol of 2500-fold diluted solution). After infection, the both drugs (RU486 and IFNγ) were simultaneously added in the cultures. Bacterial growth modification was assessed by IFU assay, fluorescence microscopic analysis and RT-PCR as shown below. Because it is well known that ofloxacin (OFLX) or clarithromycin (CAM) are ineffective against bacteria in the persistent phase [12], these antibiotics were used as an indicator to confirm whether persistent infection occurred due to treatment with IFNγ.

**Cell viability**

The effect of RU486 on the viability of *C. pneumoniae*-infected cells with or without IFNγ was measured by counting viable cells. In brief, 2, 24, 48 and 72 h after

treatment of *C. pneumoniae*-infected cells with RU486, cells were washed with phosphate-buffered saline (PBS; Sigma), detached with trypsin-EDTA (Sigma), and then suspended in medium. Cell viability was determined as a measure of trypan blue exclusion, and viable cells were counted using a hemocytometer. As a result, HEp-2 cells incubated for up to 72 h with working concentrations of RU486 and IFNγ did not show any significant cytotoxicity (data not shown).

**IFU assay**

*C. pneumoniae* infectivity was measured by counting chlamydial inclusions formed in HEp-2 cells that were visualized with FITC-conjugated monoclonal anti-*Chlamydia* antibody specific to *Chlamydia* LPS (Denka Seiken, Tokyo, Japan) [inclusion-forming unit (IFU) assay] [25, 26]. The detection limit for the IFU assay was 1 IFU per culture.

**Fluorescence microscopic analysis**

To determine inclusion formation, the infected cells fixed with ethanol were stained with FITC-conjugated monoclonal anti-*Chlamydia* antibody specific to *Chlamydia* LPS (Denka Seiken) and then assessed using fluorescence microscopy.

**RT-PCR**

Total RNA was extracted from cultures using a RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol for bacterial cells. Extracted RNA was treated with DNase I (DNA-free; Ambion, Austin, TX) to eliminate contaminating

DNA. The resulting RNA preparations were confirmed to be DNA-free when a negative result was produced using PCR without the reverse transcription step. Reverse transcription of 2 μg of total RNA by avian myeloblastosis virus reverse transcriptase was performed with random primers in a commercial reaction mixture (Reverse Transcription System; Promega, Madison, WI). Resulting cDNAs were then subjected to PCR with pairs of primers specific for *C. pneumoniae 16S rRNA* [20] and *hsp60* [20]. As a control, PCR using primers specific to human *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was performed as described previously [27]. Thermal cycler parameters included a denaturation step at 95°C for 10 min, followed by 25–40 cycles of 95 °C for 15 s, 53-59 °C for 1 min and 72°C for 20 s. Aerosol-resistant tips were used to prevent carryover contamination.

**Statistical analysis**

Statistical analysis was performed with the unpaired Student's t test.

**Results**

**Establishment of *C. pneumoniae* persistent form in IFNγ-treated HEp-2 cells**

The persistent form of *C. pneumoniae* in human epithelial HEp-2 cells was induced by treatment with IFNγ according to the method described previously [28]. Development to the persistent stage was confirmed using IFU assay, fluorescence

microscopic analysis and RT-PCR. As shown in Fig 1B, a significant decease in the number of infectious progenies was observed between IFNγ treated and non-treated cells. A minimal decease in infectious progenies appeared to be IFNγ concentration dependent. In contrast, a decrease in inclusion formation using fluorescence microscopic analysis was minimal and no difference in bacterial gene expression was observed regardless of the presence or absence of IFNγ; the size of inclusions in cells were relatively smaller than those observed for untreated cells (Fig 1A and C). Also, a concentration of IFNγ (20ng / ml) with an optimal inhibitory effect on an increase of the bacterial progenies was selected and used for below experiments.

**Effect of RU486 on persistent *C. pneumoniae* infection in IFNγ-treated HEp-2 cells**

Initially, we confirmed the effect of RU486 treatment on the infective progenies of *C. pneumoniae* in normal HEp-2 cells cultivated over different time periods. RU486 treatment of *C. pneumoniae*-infected cells cultured without the addition of IFNγ resulted in a significant decrease of *C. pneumoniae* growth at 72 h after infection depending on drug concentration (Fig. 2A). These results were identical to our previously reported findings [23] and ensured that the experimental conditions used for the present study were optimal for obtaining reproducible results. In contrast, treatment with RU486 slightly decreased the number of infectious progenies for persistent infections induced using IFNγ (Fig. 2B). Similar results were observed when cells were treated with antibiotics such as OFLX or CAM, indicating that the effect of RU486 on persistent *C. pneumoniae* infection was minimal. To confirm this hypothesis, the presence of cellular inclusion bodies of *C. pneumoniae* was determined using fluorescence microscopic

analysis. Immunostaining revealed that treatment with RU486 clearly inhibited the formation of *C. pneumoniae*-induced inclusion bodies in normal culture of HEp-2 cells at 72 h after infection and did not inhibit formation in cells treated with IFNγ (Fig. 3A). As unexpected, RU486 treatment diminished the bacterial gene expressions (*16SrRNA* and *hsp60*) depending on the drug concentration regardless of the presence or absence of IFNγ (Fig. 3B). This finding is of contradiction, however it is possible that these gene expressions were less affected by the treatment with IFNγ. Taken together, these findings suggest that the effect of RU486 treatment on persistent *C. pneumoniae* infection is minimal, implying that the bacterial target molecule of RU486 is more strongly expressed during the normal developmental cycle for this bacterial species.

**Discussion**

Persistent infection of *C. pneumoniae* in HEp-2 cells was induced by the treatment with IFNγ, since accumulating evidence from studies using *in vitro* persistent infection models suggested that IFNγ was the most effective reagent for inducing persistent *C. pneumoniae* infection with an incomplete developmental cycle [1, 26, 28]. As a result, treatment with IFNγ had an obvious effect on the growth of *C. pneumoniae*, resulting in a decrease in number of infectious progenies and a reduction of inclusion size. Gene expression of the *16S rRNA*, which is critical for *C. pneumoniae* survival in cells, remained unchanged. Thus, persistent *C. pneumoniae* infection in HEp-2 cells was stably induced using IFNγ and supported the persistent infection models described previously [26, 28].

Since the presence of bacterial transcripts serves as a marker for viable and bacterial metabolic activity, the modification of *C. pneumoniae* gene expression by RU486 in persistent infection could be interpreted as evidence of bacterial non-viability. Therefore, whether the drug could modify bacterial gene expression of *16S rRNA* and *hsp60*, which are essential for all metabolically active stages of *C. pneumoniae*, including non-infectious RBs in infected cells [15, 20, 29], was assessed by RT-PCR analysis, with determining inclusion formation and the number of infectious progenies. However, interestingly, our data indicated a possibility that these gene expression were unlikely to enough reflect bacterial viability in persistent stage induced by IFNγ, although further study for seeking more appropriate gene makers reflecting bacterial viability in the stage should be needed.

Previous works suggested that persistent chlamydial infection might be associated with incomplete chlamydial development with sporadic production of EBs refractory to treatment with antibiotics, although its reason remains unknown [26, 28]. Therefore, we assumed that a decrease of the sporadic EBs with a drug might be a significant indicator to evaluate the elimination of *C. pneumoniae* in persistent infection, and assessed changes of the numbers of infectious progenies (by IFU assay) in persistent infection. As compared with the control treated with IFNγ, a slight difference was observed in the number of infectious progenies and the inclusion formation for persistent infection induced by the treatment with IFNγ and treated with RU486. Similar results were observed using common antibiotics such as CAM and indicated that RU486 treatment could not enough eliminate bacteria that had developed to a persistent stage infection. While this result was contrary to our expectation, it is noteworthy that RU486 may not access bacteria that have developed to the persistent phase. As reported previously,

RU486 treatment eliminates bacteria from cells as a consequence of direct binding of the drug to the bacteria as they undergo maturation in inclusion bodies. Therefore, it is most likely that the target molecule of RU486 is expressed strongly in bacteria undergoing the normal developmental cycle and not enough in bacteria undergoing the abnormal cycle associated with persistent infection. This is the first report to show that expression of the bacterial target molecule against a possible therapeutic drug differs between bacterial stages undergoing different developmental cycles. Although the target molecule of RU486 remains undetermined, it is possible that the molecule is strongly expressed in bacteria in the normal developmental cycle. As there is no effect of the drug on EB before cells become infected [23], some of the molecules expressed during the mid to late stages of the active developmental cycle are thought to be candidates for the target molecule of RU486. The expression level for this target molecule appears to be arrested in persistent infection, implying that the molecule is directly associated with bacterial maturation through acceleration of the developmental cycle. The alteration of chlamydial gene expression in the persistent infection was well documented [16, 17, 26, 29]. In particular, the persistent stage of the bacteria was characterized by the down-regulation of a broad range of genes, associating with metabolism and cell division; in contrast the genes regulating DNA repair and replication were normally expressing even in the persistent stage [29]. Therefore, it is possible that these genes regarding metabolism and/or cell division are attractive candidates as target molecule of RU486, although further study should be needed. RU486 is a well-known antiprogestin with a high affinity for the progesterone receptor and causes competitive binding to intracellular progesterone receptor [22]. However, BLAST searching revealed an absence of DNA sequence homology to the progesterone receptor on the *C. pneumoniae*

whole genome, and no expression of progesterone receptor in HEp-2 cells with or without *C. pneumoniae* infection in the presence or absence of RU486 was observed [23]. This indicated that the bacterial receptor of RU486 might not be the progesterone receptor homolog.

In conclusion, the steroid receptor antagonist RU486 inhibited growth of *C. pneumoniae* during the normal developmental cycle in epithelial HEp-2 cells yet unsuccessful eliminated bacteria during persistent infection. These findings provide novel evidence that expression of the target molecule of RU486 on *C. pneumoniae* is different between the normal and abnormal developmental cycles of persistent infection and further explains the variation in drug therapy success. The exact mechanism causing the changes in expression of the target molecule remains undetermined and further investigation is necessary to better understand the complicated chlamydial biological processes involved in the recurrent cycling between normal and persistent infections.

**Acknowledgments**

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**Figure legends**

**Fig. 1** Establishment of *C. pneumoniae* persistent form in IFN γ-treated HEp-2 cells. **A** Fluorescence micrographs with FITC-labeled anti-*Chlamydia* antibody of *C. pneumoniae*-infected HEp-2 cells in the presence or absence of IFNγ. Magnification, × 200. **B** Number of infectious progenies of *C. pneumoniae* per culture in the presence or absence of IFN γ. The bacterial numbers were assessed by IFU assay. Further details are provided in the text. The data shown represent the means + standard deviation (error bars) for at least three experiments. **P*<0.05, indicates statistically significant vs. control (without IFN γ). **C** Bacterial (*16S rRNA*) and host cellular (*GAPDH*) gene expressions were assessed by RT-PCR.

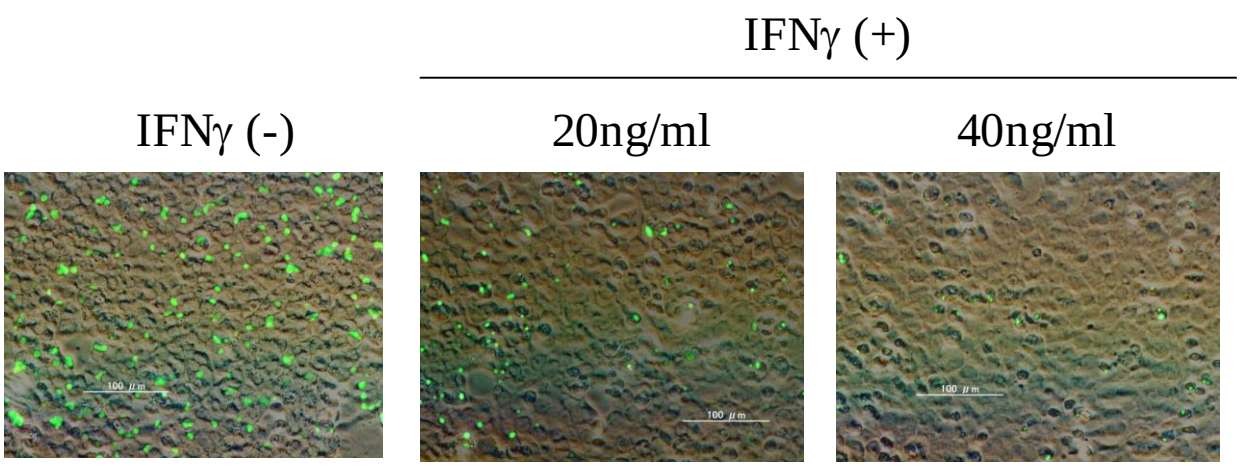
**Fig. 2** Effect of RU486 treatment on the infective progenies of *C. pneumoniae* in HEp-2 cells in the presence (B) or absence (A) of IFNγ at different time points. The data shown represent the means + standard deviation (error bars) for at least three experiments. **P*<0.05, indicates statistically significant vs. control (without IFNγ).

**Fig. 3** Effect of RU486 on persistent *C. pneumoniae* infection in IFN γ-treated HEp-2 cells. **A** Fluorescence micrographs with FITC-labeled anti-*Chlamydia* antibody of *C. pneumoniae*-infected HEp-2 cells with or without RU486 (10μM, 25μM) in the presence or absence of IFNγ. Magnification, × 200. **B** For cultured normally or

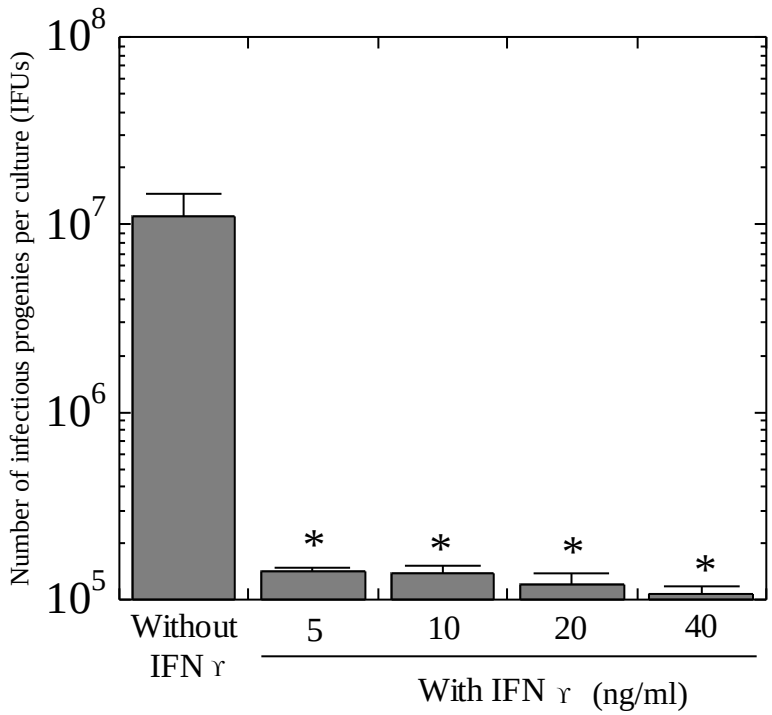
persistent *C. pneumoniae* infection, bacterial (*16S rRNA* and *hsp60*) and host cellular (*GAPDH*) gene expressions were assessed by RT-PCR.

FIG.1

A


IFNγ (+)
IFNγ (-)
20ng/ml
40ng/ml

B


Number of infectious progenies per culture (IFUs)
10^8
10^7
10^6
10^5
*
*
*
*
Without IFNγ
5
10
20
40
With IFNγ (ng/ml)

C

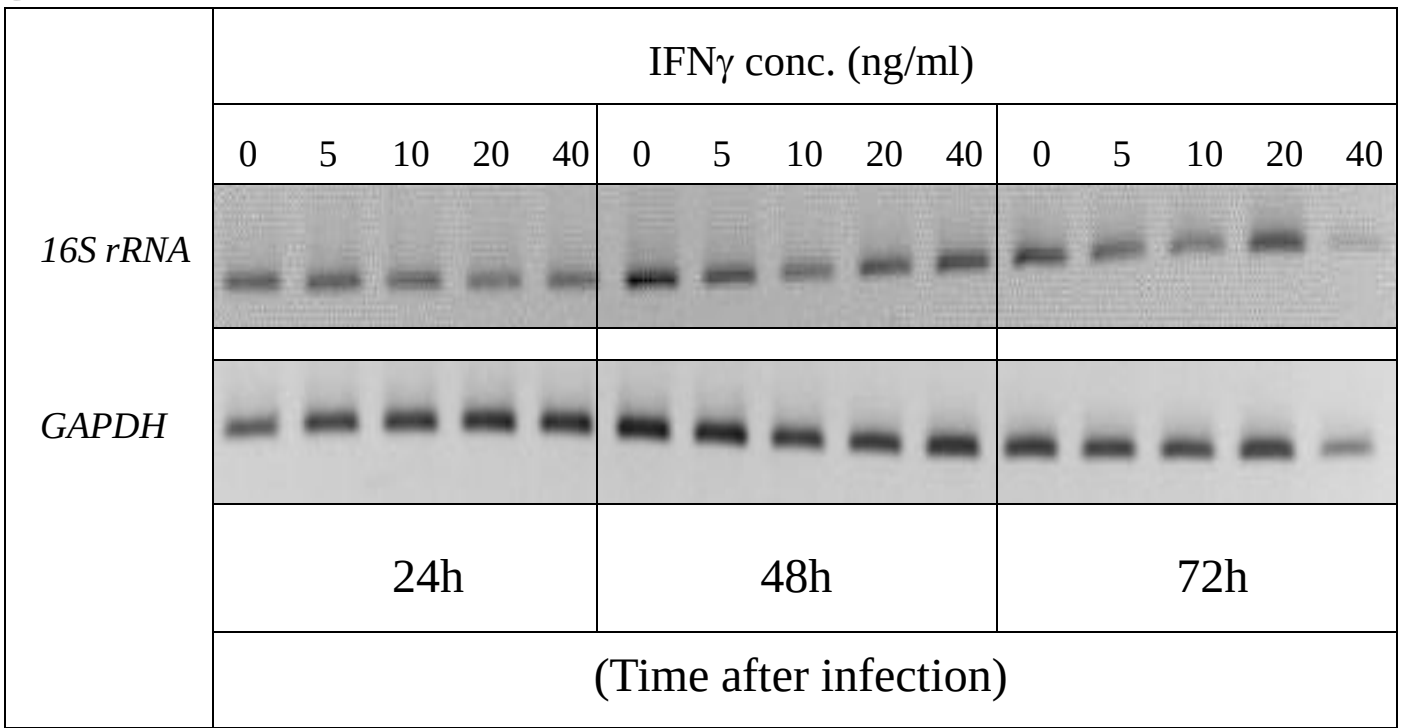
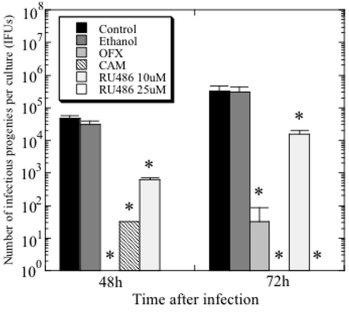

IFNγ conc. (ng/ml)
0 5 10 20 40 0 5 10 20 40 0 5 10 20 40
16S rRNA
GAPDH
24h
48h
72h
(Time after infection)

Fig. 2

A


Control
Ethanol
OFX
CAM
RU486 10uM
RU486 25uM
Number of infectious progenies per culture (IFUs)
48h
72h
Time after infection

B

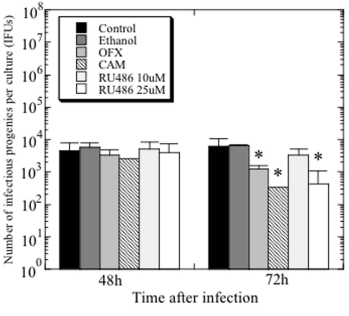
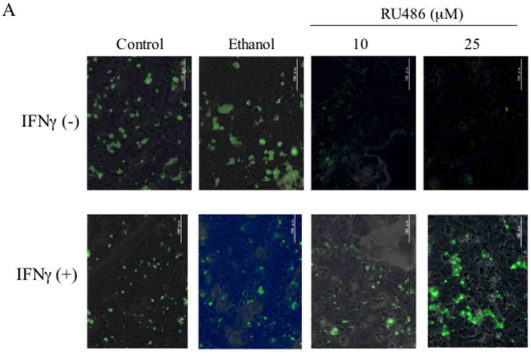
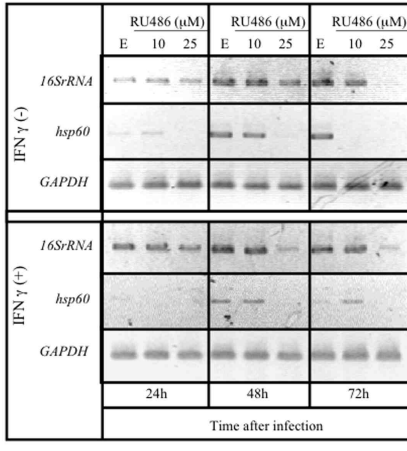

Control
Ethanol
OFX
CAM
RU486 10uM
RU486 25uM
Number of infectious progenies per culture (IFUs)
48h
72h
Time after infection

Fig. 3

A


RU486 (μM)
Control
Ethanol
10
25
IFNγ (-)
IFNγ (+)

B


RU486 (μM)
RU486 (μM)
RU486 (μM)
E 10 25
E 10 25
E 10 25
IFN γ (-)
16SrRNA
hsp60
GAPDH
IFN γ (+)
16SrRNA
hsp60
GAPDH
24h
48h
72h
Time after infection