Chlamydia pneumoniae Growth Inhibition in Cells by the Steroid Receptor Antagonist RU486 (Mifepristone)\(^V\)

Hiroyuki Yamaguchi,1,6,7* Shigeru Kamiya,1 Tomonori Uruma,1,2 Takako Osaki,1 Haruhiko Taguchi,1 Tomoko Hanawa,4 Minoru Fukuda,3 Hayato Kawakami,4 Hajime Goto,2 Herman Friedman,5 and Yoshimasa Yamamoto5,6

Division of Microbiology, Department of Infectious Disease,1 Department of 1st Internal Medicine,2 Laboratory of Electron Microscopy,3 and Department of Anatomy,4 Kyorin University School of Medicine, Tokyo 181-8611, Japan; Department of Medical Microbiology and Immunology, University of South Florida College of Medicine, Tampa, Florida 33612; Division of Molecular Microbiology, Department of Basic Laboratory Sciences, Osaka University Graduate School of Medicine, Osaka 565-0871, Japan; and Department of Laboratory Sciences, College of Medical Technology, Hokkaido University, Nishi-5 Kita-12 Jo, Kita-ku, Sapporo, Hokkaido 060-0812, Japan

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Since steroids are powerful anti-inflammatory agents and increase susceptibility to a variety of infections, including Chlamydia (Chlamydiophila) pneumoniae respiratory tract infections, the effect of the steroid receptor antagonist RU486 (mifepristone) on C. pneumoniae growth in epithelial HEp-2 cells was examined. Treatment of HEp-2 cells with RU486 significantly inhibited the growth of C. pneumoniae in a dose-dependent manner. Electron microscopic studies also revealed that the treatment of infected cells with RU486 resulted in a marked destruction of infecting organisms. The addition of the host cell protein synthesis inhibitor cycloheximide to the infected cells did not alter the inhibition of C. pneumoniae growth by RU486. Pretreatment of C. pneumoniae organisms with RU486 before addition to culture also did not result in any modulation of bacterial growth in the cells. However, the binding of RU486 to C. pneumoniae organisms in cells at 24 h after infection was demonstrated by immune electron microscopy with anti-RU486 antibody. Incubation of cells with anti-RU486 antibody completely diminished the inhibition of C. pneumoniae growth by RU486. These results indicate that RU486 may directly bind to the bacteria within cells and cause the destruction of C. pneumoniae. This novel mode of regulation of C. pneumoniae growth in cells by RU486 might provide a new approach to understanding complicated aspects of C. pneumoniae infection.

Chlamydia (Chlamydiophila) pneumoniae is an obligate intracellular bacterium which causes a common human respiratory tract infection (13, 14). Current studies show that chlamydial infection has a tendency to induce chronic infections (24), which are important clinical manifestations associated with persistent respiratory diseases (15, 29). The mechanisms leading to persistent C. pneumoniae infection are not yet clear, but immunosuppression, which causes an incomplete resolution of the infection in the host, may be an important event. Steroid treatment is widely used in clinics as therapy for immunoreactive as well as inflammatory diseases. However, it is known that steroid treatment may induce susceptibility to a wide variety of infectious diseases due to its immunosuppressive activity. However, information on the effects of steroids on the growth of chlamydial organisms in cells is limited. Previous in vitro studies showed a significant increase in the number of inclusions produced from a constant inoculum of chlamydia in epithelial cells incubated with a steroid (6, 28). In this regard, experiments performed with a mouse model indicated the reactivation of infection and latent pulmonary infection with C. trachomatis in the presence of steroids (32). Recently, Malinverni et al. (18) also demonstrated the reactivation of C. pneumoniae infection of the lung in a mouse model following immunosuppression with cortisone. Clinically, seroepidemiological evidence from studies with primary care outpatients points to a role for C. pneumoniae infection in the pathogenesis of asthma (14). In addition, it has also been reported that the severity of asthma appears to be increased in C. pneumoniae-infected patients who have received prolonged therapy with corticosteroids (5, 14). Thus, such results suggest that steroids play an important role in accelerated C. pneumoniae growth in host cells. Therefore, treatment with steroid receptor antagonists may be a possible means of down-regulating bacterial growth during infection.

It is known that RU486 (mifepristone), which is effective for the termination of early pregnancy, has remarkable antisteroid activity (8). In the biopharmacological field, this drug is widely utilized as a useful tool for the analysis of the interaction between cellular homeostasis and molecular signals via steroid receptors (8). Therefore, in the present study, in order to determine the effect of steroid receptor antagonists on C. pneumoniae growth in epithelial cells, the major target cells for this bacterium in vivo, RU486 was utilized as a representative antisteroid agent.

**MATERIALS AND METHODS**

**Bacteria.** C. pneumoniae TW183, kindly provided by G. Byrne, University of Wisconsin, Madison, was used in this study. The bacteria were propagated in the HEp-2 cell culture system by previously described methods (22). The number of infectious C. pneumoniae organisms was determined as the number of inclusion-
forming units (IFUs) by counting the numbers of chlamydial inclusions formed in HEp-2 cells with fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-Chlamydia antibody specific for Chlamydia lipopolysaccharide (Denka Seiken Co. Ltd., Tokyo, Japan) (22, 27). The bacterial suspensions were confirmed to be free of Mycoplasma by PCR, as reported previously (21).

**Cells.** The human epithelial cell line HEp-2 was kindly provided by R. Widen, Tampa General Hospital, Tampa, FL. The human breast cancer cell line MCF-7, which expresses a progesterone receptor, was also used as a positive control for reverse transcriptase (RT) PCR (20). The cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal calf serum and antibiotics (gentamicin sulfate, 10 μg/ml; vancomycin, 10 μg/ml; amphotericin B, 1 μg/ml; Sigma Chemical Co., St. Louis, MO) at 37°C in 5% CO₂.

**Chemicals.** RU486 and cycloheximide were purchased from Sigma Chemical Co. RU486 was dissolved in ethanol at a stock concentration of 25 mM. Cycloheximide was dissolved in pyrogen-free water at a stock concentration of 100 μg/ml and was sterilized by filtration through a membrane. The reagents were diluted to achieve the working concentration to be used with the medium.

**Antibodies.** Specific-pathogen-free female ICR mice (n = 20; age, 10 weeks; Nihon Clea Co. Ltd., Tokyo, Japan) were subcutaneously immunized with emulsified RU486 plus complete Freund adjuvant (100 μg/mouse). At 1 week after the first injection, the mice were also intraperitoneally immunized seven times on a weekly schedule with emulsified RU486 plus incomplete Freund adjuvant (100 μg/mouse). At 3 days after the last immunization, mouse sera were collected and the immunoglobulin G (IgG) fraction was purified with a Hitrap protein G column (Amersham Pharmacia Biotech Inc., Piscataway, NJ), according to the manufacturer’s instructions.

**C. pneumoniae infection.** The 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 two washings with medium, the cultures were resuspended in the medium and dispensed into wells as follows: 5 × 10⁴ cells/well (96-well plates) for cell viability count determination and the IFU assay, 5 × 10⁵ cells/well (24-well plates) for RNA isolation, and 1.5 × 10⁶ cells/well (6-well plates) for the electron microscopy study. They were incubated for up to 72 h in the presence or absence of RU486 (0.02 to 2 μM) and/or cycloheximide (0.1 to 10 μg/ml). In some experiments, anti-RU486 antibody (5 μg/ml) was added to the cells 4 h before infection with the bacteria. Pretreatment of the bacteria with RU486 (2 μM) for 1 h before infection of the cells was also performed. Infected cells that were not treated or that were treated with ethanol (vehicle) at concentrations equivalent to those in the RU486 preparation were prepared as controls. The effect of RU486 or cycloheximide on the viability of C. pneumoniae-infected cells was determined by the viable-cell-count method. In brief, 2, 24, 48, and 72 h after the treatment of C. pneumoniae-infected cells with RU486 and/or cycloheximide, the cells were washed with phosphate-buffered saline (PBS; Sigma Chemical Co.) and then were detached by treatment with trypsin-EDTA (Sigma Chemical Co.). The cells were then suspended in the medium. The number of viable cells was determined by the trypan blue exclusion method in a hemocytometer. None of the working concentrations of the drugs showed any significant cytotoxicity to the HEp-2 cells.

**Assessment of infective progeny (IFU assay).** To assess the infective progeny in the culture for different cultivation times, the cultures were frozen and thawed, as described previously (22, 27). The lysates in 10-fold serial dilutions were inoculated on HEp-2 cell monolayers in 96-well plates, centrifuged at 800 × g for 1 h, and then incubated in the medium with cycloheximide (1 μg/ml) for 72 h at 37°C. The number of IFUs in the cells was then assessed by staining with FITC-conjugated anti-Chlamydia antibody (22, 27).

**RNA extraction and real-time or conventional RT-PCR.** Total RNA was extracted from cultures by using an RNAeasy mini kit (Qiagen, Valencia, CA), according to the manufacturer’s instructions for the protocol with bacterial cells. Extracted RNAs were treated with DNase (DNA-free; Ambion, Austin, TX) to eliminate the contaminating DNA. The resulting RNAs were confirmed to be free of DNA by PCR without RT. The reverse transcription of 2 μg of RNA was performed with avian myeloblastosis virus RT with random primers in a commercial reaction mixture (reverse transcription system; Promega, Madison, WI). The resulting cDNAs were then subjected to real-time PCR with the master mixture (QuantiTect SYBR green PCR kit; Qiagen) containing primers specific for C. pneumoniae 16S rRNA (sense primer, 5’-CGA CCT TAG GTG ACA TGT-3’; antisense primer, 5’-CCA TGC ACG ACC TGT GTA TCT G-3’) (4). The results of a search with the BLAST program showed that the primers used for the real-time RT-PCR were specific for C. pneumoniae detection (data not shown). The thermal cycling conditions were 95°C for 10 min and 30 cycles of 95°C for 15 s, 60°C for 1 min, and 72°C for 20 s. The melting temperatures were assessed for each PCR run for confirmation of the specificities of the PCR products. As a standard for C. pneumoniae 16S rRNA, a series of diluted C. pneumoniae DNA extracted from C. pneumoniae-infected HEp-2 cells was used. The relative concentrations of C. pneumoniae 16S rRNA (number of copies per PCR mixture) were calculated from the standard curve.

**Electron microscopic study.** For transmission electron microscopy, the cells were harvested at each time point and fixed with 2.5% glutaraldehyde in PBS. The cell pellets were dehydrated and embedded in Epon 813 embedding solution (Chemische Werke Lowi GmbH, Waldkraiburg, Germany). For immune staining with mouse anti-RU486 IgG antibodies, the grids untreated with Lowicryl K4M embedding solution were floated for 10 min on a drop (100 μl) of 1% bovine serum albumin in PBS and incubated with mouse anti-RU486 IgG antibodies (10 μg/ml) diluted in 0.1% bovine serum albumin–PBS. IgG antibodies obtained from unimmunized mice were also used as a negative control. After the sections were washed with PBS, they were reacted with anti-mouse IgG antibodies conjugated with monodisperse colloidal gold particles (diameter, 12 nm; Jackson Immune Research Laboratories, Inc., West Grove, PA). The sections were stained with a saturated aqueous solution of uranyl acetate and citrate solution. The specimens were observed with a JEM-100 electron microscope (Joel, Tokyo, Japan).

**Statistical analysis.** Statistical analysis was performed by use of the unpaired Student t test.

**RESULTS**

**Effect of RU486 on C. pneumoniae growth in cells.** In order to determine the effect of RU486 on C. pneumoniae growth in cells, the bacterial growth in HEp-2 cells in the presence or the absence of RU486 was assessed by the IFU assay. As shown in Fig. 1, treatment of C. pneumoniae-infected cells with RU486
resulted in a significant decrease in \textit{C. pneumoniae} growth at 72 h after infection. The inhibition of the bacterial growth by RU486 was dose dependent, and the lowest concentration required for significant inhibition was 0.2 \(\mu\text{M}\). When a higher concentration, such as 20 \(\mu\text{M}\), was used for treatment of the cells, no infective chlamydial organisms were observed but obvious cytotoxicity to the HEp-2 cells was observed (data not shown). Therefore, concentrations up to 2 \(\mu\text{M}\) of RU486 were selected for use in the following experiments. A time course analysis with 2 \(\mu\text{M}\) was used for treatment of the cells at each time point after infection was determined by the IFU assay. The data represent the means \(\pm\) standard deviations for three experiments. *\*, the results are significantly different \((P < 0.05)\) from those for the control group.

**Electron microscopic analysis.** In order to analyze the inhibitory effect of RU486 on \textit{C. pneumoniae}, the morphology of \textit{C. pneumoniae} in cells was examined by transmission electron microscopy. Figure 3 shows representative electron micrographs of chlamydial inclusion bodies. At 24 h after infection, there were reticulate bodies (Fig. 3A and D), but no morphological differences between RU486-treated and untreated cells were observed. However, at 48 h after infection, the bacterial cell walls were obviously detached from the microorganisms in treated cells and no dividing bacteria were observed in RU486-treated cells (Fig. 3E). In contrast, a large inclusion with many chlamydial organisms was seen in control nontreated cells (Fig. 3B). At 72 h after infection, the bacteria in the inclusions in RU486-treated cells were mostly destroyed, as shown in Fig. 3F. There were many \textit{C. pneumoniae} organisms in untreated control cells, and they appeared to have a shape that indicated that they were active (Fig. 3C).

**Effect of cycloheximide on RU486-induced anti-Chlamydia activity.** In order to clarify whether the inhibition of \textit{C. pneumoniae} growth in cells by RU486 was caused by a host response via host cellular metabolic pathways, the effect of cycloheximide on RU486-induced \textit{C. pneumoniae} growth inhibition was examined. As evident in Fig. 4, treatment of the infected cells with cycloheximide did not cause any alteration of the RU486-induced inhibition, and furthermore, cycloheximide accelerated bacterial growth in the cells without RU486.

These results indicate that the RU486-induced inhibition of \textit{C. pneumoniae} growth might not be mediated by host metabolism-related events.

Since it is known that the \textit{C. pneumoniae} aberrant body, which is thought to be a persistent form, lacks infectivity for host cells and, therefore, is not detected by the standard IFU assay (26), it may be possible that aberrant bodies are induced by RU486 treatment. Quantitation of bacterial transcripts in infected cells by real-time RT-PCR is appropriate for the analysis of not only actively growing \textit{C. pneumoniae} organisms but also persistent \textit{C. pneumoniae} organisms in cells. Analysis of infected cells treated with RU486 and/or cycloheximide or untreated infected cells for bacterial transcripts, such as \textit{C. pneumoniae} 16S rRNA, showed a marked reduction in bacterial transcript levels in RU486-treated cells at both 24 and 72 h after infection (Fig. 5). The treatment of RU486-treated cells with cycloheximide did not abolish the reduction caused by RU486 treatment, as was also seen in the IFU assay study. Thus, it is obvious by the assessment of infective progeny (IFU assay) as well as bacterial transcripts that host cell metabolic inhibition by cycloheximide did not modify the RU486-induced inhibition of \textit{C. pneumoniae} growth in cells.

**Expression of progesterone receptor mRNA.** It is well known that RU486 is an antiprogestin with a high affinity for the progesterone receptor, and the major action of RU486 is caused by its competitive binding to the intracellular progesterone receptor (8). Therefore, in order to determine whether the progesterone receptor was present in HEp-2 cells, transcripts of the receptor were assessed by RT-PCR. However, as shown in Fig. 6, no expression of the progesterone receptor in HEp-2 cells with or without \textit{C. pneumoniae} infection in the presence or absence of RU486 was observed.

**Direct effect of RU486 on \textit{C. pneumoniae} organisms.** In order to examine whether RU486 has a direct effect on \textit{C. pneumoniae} growth, pretreatment of bacteria with RU486 was performed, followed by infection and assessment of the growth of the bacteria in the cells by the IFU assay. As shown in Fig. 7, pretreatment of the bacteria with RU486 did not cause any alteration of \textit{C. pneumoniae} growth in the cells. However, addition of RU486 to infected cells, as a positive control, resulted in the marked inhibition of \textit{C. pneumoniae} growth, determined at 72 h after infection.

**Binding of RU486 to \textit{C. pneumoniae} organisms.** To determine whether RU486 binds to \textit{C. pneumoniae} microorganisms in cells, the bacteria were assayed for the presence of RU486 by an immune electron microscopy assay with anti-RU486 antibody. The cells were infected with bacteria and then incubated for 24 h in the presence or the absence of RU486. The presence of RU486 in cells was visualized by electron microscopy with anti-RU486 antibody and anti-mouse IgG antibody conjugated with colloidal gold particles. Gold signals were observed in both the cytoplasm and the nucleus, indicating that RU486 possibly binds to unknown cellular molecules like steroid receptors in cells (Fig. 8B). At 24 h after infection, the presence of RU486 on a microbe was demonstrated by immune electron microscopy (Fig. 8C). Control mouse IgG did not show any nonspecific binding of gold to microorganisms, as shown in Fig. 8A.

**Anti-RU486 antibody diminishes RU486-induced \textit{C. pneumoniae} growth inhibition.** To confirm whether the binding of RU486 to bacteria is essential for \textit{C. pneumoniae} growth inhi-
bition, the effect of anti-RU486 antibody on RU486-induced C. pneumoniae growth inhibition in infected cells was examined. As shown in Fig. 9, the anti-RU486 antibody completely abolished the RU486-induced C. pneumoniae growth inhibition in cells. In contrast, control normal mouse IgG did not show any significant alteration of RU486-induced inhibition. Although the mechanism by which RU486-induced C. pneumoniae growth inhibition was abolished by anti-RU486 antibody remains to be established, it seems likely that the antibody raised may prevent the drug from entering cells.

FIG. 3. Transmission electron micrographs of C. pneumoniae in HEp-2 cells cultured in the absence (A, B, and C) or the presence (D, E, and F) of 2 µM RU486 for 72 h. The samples were prepared at 24 h (A and D), 48 h (B and E), and 72 h (C and F) after infection. Bars, 1 µm (A, D, E, and F) and 0.5 µm (B and C).
DISCUSSION

The present study clearly showed that the steroid receptor antagonist RU486 has the ability to control *C. pneumoniae* growth in cells by directly binding to bacteria following bacterial destruction. The concentration of RU486 required to be effective against *C. pneumoniae* growth without cytotoxicity to the target cells was 2 μM. The pharmacokinetics and clinical efficacy of RU486 for the termination of early pregnancy have been examined previously (8). That is, following oral administration of RU486 at up to 800 mg, the drug is rapidly absorbed from the gastrointestinal tract and the average maximum plasma concentration of RU486 eventually reached is 2.5 mg/liter (5.8 μM) (2, 26). Therefore, the concentrations effective for *C. pneumoniae* growth inhibition of RU486 revealed in this study can readily be reached in vivo. The possible involvement of steroids in the inhibition of *C. pneumoniae* growth by RU486 revealed in this study is unlikely, because the experimental systems utilized in this study did not receive any steroids.

The mechanism of inhibition of *C. pneumoniae* growth by RU486 is unclear, but the inhibitory activity of RU486 was not affected by treatment with cycloheximide (up to 10 μg/ml),
which is a host cellular metabolic inhibitor. It is well known that the major action of RU486, which has a high affinity for the progesterone receptor, is caused by its competitive binding to the intracellular progesterone receptor (8). However, no expression of this receptor in the cells was observed by RT-PCR. It is also known that the intracellular growth of *C. pneumoniae* is regulated by the modulation of activity of indoleamine 2,3-dioxygenase, which cleaves the essential amino acid 5-tryptophan (23). However, addition of an excess (up to 100 μg/ml) of tryptophan to RU486-treated cells infected with *C. pneumoniae* showed no alteration of the inhibition of bacterial growth induced by RU486 (data not shown). Thus, these results indicate that host metabolism, including the activity of indoleamine 2,3-dioxygenase, which is one of the major mechanisms responsible for the anti-*C. pneumoniae* activity induced by gamma interferon (2, 7, 26), is not likely involved in the inhibition of *C. pneumoniae* growth induced by RU486.

*Chlamydia* aberrant bodies lacking infectivity as well as growth potential with metabolic activity are often observed in cells treated with antimicrobial agents and cytokines (23). This form is involved in persistent infection, and the detection of this form is therefore not possible by a general microbiological method based on the IFU assay. In this regard, the detection of *C. pneumoniae* transcripts by real-time RT-PCR may be a useful method for the detection of bacteria, including such aberrant bodies (9, 11). As assessed by RT-PCR, the treatment of infected cells with RU486 showed a significant decrease in bacterial transcripts similar to that observed by the IFU assay. These results indicate that treatment with RU486 may inhibit *C. pneumoniae* growth in cells without the formation of such aberrant bodies.

The immune electron microscopic study clearly showed the binding of RU486 to the bacteria in cells 24 h after infection. The pretreatment of bacteria with RU486 before infection of the cells did not result in any bacterial growth inhibition. These results indicate that RU486 may bind to bacteria which are in a specific stage, like reticulate bodies of the chlamydial developmental cycle in cells, but not to elementary bodies, which are infective but not metabolically active. However, since the present data are limited, further studies need to be performed to determine whether the target of RU486 is definitely reticulate bodies and not elementary bodies. In this regard, Shaw et al. (25) showed that chlamydial gene expression in cells depends on chlamydial developmental cycles. In addition, a recent study by analysis with a DNA microarray indicates that the genomic transcriptional levels in a reticulate body of *C.
Chlamydia pneumoniae growth inhibition by RU486. The cells were infected with the bacteria and were then incubated with or without RU486 (2 μM) in the presence or the absence of anti-RU486 antibody (5 μg/ml) for 72 h. At 72 h after infection, the numbers of infective progeny in the cell lysates were assessed by the IFU assay. The data are presented as the means ± standard deviations for three experiments. (−), without antibodies; Abs, antibodies; *, the results are significantly different (P < 0.05) from those for the control group.

Chlamydia pneumoniae is preferentially infects respiratory tract epithelial cells but also infects endothelial cells, aortic smooth muscle cells, and monocytes as well as lymphocytes (1, 10, 16, 17, 31). Since the results of current studies indicate that C. pneumoniae in blood cells, such as monocytes as well as lymphocytes, is resistant to antibiotic treatment, whereas C. pneumoniae in epithelial cells is not (12, 30, 31), another approach to the mechanism of such resistance is the preferential infection of other bacteria to antibiotics in immune cells in vitro (data not shown). However, the experiments were performed with only a limited number of bacteria, and therefore, any conclusion from the study regarding specificity is not yet unequivocal.

In conclusion, the steroid receptor antagonist RU486 inhibits C. pneumoniae growth in epithelial HEP-2 cells by directly binding to the bacteria. Even though the mechanism of inhibition is not clear, this novel mechanism of regulation of C. pneumoniae growth in cells by RU486 might be useful for obtaining an understanding of the complicated aspects of C. pneumoniae infection. Moreover, it might also be helpful in the development of a novel type of antimicrobial agent that inhibits C. pneumoniae growth in host cells.

REFERENCES