Analysis of Chlamydia pneumoniae Growth in Cells by Reverse Transcription-PCR Targeted to Bacterial Gene Transcripts

Shusaku Haranaga, Hideaki Ikejima, Hiroyuki Yamaguchi, Herman Friedman, and Yoshimasa Yamamoto*

Department of Medical Microbiology and Immunology, University of South Florida College of Medicine, Tampa, Florida 33612

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Chlamydia pneumoniae, an obligate intracellular bacterium and has a unique development cycle consisting of an elementary body (EB) and reticular body (RB). EBs survive in extracellular environments as well as infect susceptible host cells. However, EBs display no measurable metabolic activity. In contrast, RBs are metabolically active and can replicate in a host cell but are noninfectious. Therefore, analysis of C. pneumoniae growth in infected cells by conventional bacterial culture may not permit sufficient information about growth of the bacteria in cells. In this study, therefore, we examined the usefulness of the reverse transcription (RT)-PCR method for analysis of bacterial transcripts to evaluate C. pneumoniae growth in HEP-2 cells because the levels of bacterial gene transcripts are known to show the metabolic activity of bacteria. The transcripts for the C. pneumoniae hsp60 gene and 16S rRNA in the cells were easily detected just after infection, followed by a marked increase. In contrast, pyk and omcB transcripts slowly increased after a latent period. The hydrocortisone treatment of C. pneumoniae-infected cells induced an increase of all bacterial transcripts tested compared with the control group. The treatment of the infected cells with the antibiotic minocycline showed a selective inhibition of bacterial gene transcripts, even though the complete inhibition of EB production determined by the bacterial culture assay was evident. These results indicate that the determination of bacterial gene transcripts by RT-PCR might be a powerful method to analyze in detail growth of C. pneumoniae in host cells, particularly altered bacterial growth caused by agents such as antimicrobials.

Chlamydia pneumoniae, an obligate intracellular bacterium, causes a wide spectrum of respiratory tract infections (10, 11, 14). Current studies indicate that this pathogen is associated with not only respiratory diseases but also chronic inflammatory diseases, such as atherosclerosis, endocarditis, asthma, and arthritis (9, 12, 20, 24). Similar to other chlamydia species, it is known that the infection of cells with C. pneumoniae is initiated by an environmentally resistant electron-dense form termed the elementary body (EB). The transition from EB to the metabolically active replication cell, called the reticulate body (RB), begins within the first few hours after infection. The RBs multiply by binary fission until the late phase of infection and begin to convert back to EBs (7, 17). In general, the growth of this organism can be monitored by passage of cultures on appropriate freshly prepared cells, such as HEP-2 cells. However, since RB is not infectious, this bacterial culture system reflects only the EB number in infected cells. Although an electron microscopic study could demonstrate the detail of morphological changes of this pathogen, it cannot show viability or metabolic activity of the bacteria. In particular, it can be conjectured that if some agents, such as antibiotics, alter the development cycle of C. pneumoniae and interfere with the conversion to EBs but leave viable forms in cells, the presence of such bacteria may not be assessed by ordinary bacterial culture.

Since some structure proteins of EBs and RBs are known to be different (13, 18), analysis of transcript levels of such protein genes in infected cells by reverse transcription (RT)-PCR may be a suitable method to assess C. pneumoniae infection. Therefore, in the present study we examined the usefulness of the detection of C. pneumoniae transcripts by RT-PCR specific for C. pneumoniae 16S rRNA and several other genes during the infection in cells.

MATERIALS AND METHODS

Cells. HEP-2 cells were purchased from the American Type Culture Collection, Manassas, Va. The cells were maintained in Dulbecco's modified Eagle's medium (Sigma Chemical Co., St. Louis, Mo.) containing 10% fetal calf serum (HyClone Laboratories, Logan, Utah) and antibiotics (gentamicin, 10 μg/ml; vancomycin, 10 mg/ml; amphotericin B, 1 μg/ml).

Organisms. C. pneumoniae (AR-39) was obtained from the American Type Culture Collection and propagated in HEP-2 cell cultures (23, 29). Chlamydial EBs were purified by density gradient centrifugation with urographin (Schering Japan, Osaka, Japan) as previously described (4). Purified EBs were suspended in sucrose-phosphate-glutamic acid buffer (0.2 M sucrose, 3.8 mM KH2PO4, 6.7 mM Na2HPO4, 5 mM L-glutamic acid [pH 7.4]) and then stored at −70°C until used. Inclusion-forming units (IFU) of the prepared EBs were determined by counting chlamydial inclusions in HEP-2 cell monolayers.

Infection with C. pneumoniae. The HEP-2 cells were dispensed to 6-well or 24-well (with or without glass coverslips) culture plates and incubated for 2 h in 5% CO2 at 37°C. The cells were then infected with EBs at a multiplicity of infection (MOI) of 5 by centrifugation at 900 × g for 1 h, washed two times with Hanks' balanced salt solution, and incubated in the medium at 37°C in 5% CO2 for up to 72 h. Uninfected HEP-2 cells were also prepared as a control culture.

IFU assay. The infected cells were harvested at appropriate time points (6 to 72 h) after infection and then disrupted by sonication (Sonics Disembrator 60; Fisher Scientific, Pittsburgh, Pa.). The resulting cell lysates were serially diluted and centrifuged on the HEP-2 cell monolayers. After 72 h of cultivation with cycloheximide (1 μg/ml), the cells were fixed with ethanol and stained with fluorescein isothiocyanate (FITC)-conjugated anti-Chlamydia lipopolysaccharide monoclonal antibody (Research Diagnostics Inc., Flanders, N.J.). The chlamydial inclusion bodies were counted under a fluorescence microscope.
performed in a separate room. An aerosol-resistant tip was used in all steps. Preparation of the PCR mixture was optimized before being applied to samples. The PCR cycle consisted of a 5-min denaturation at 95°C followed by 25 to 40 cycles each of 45 s of denaturation at 94°C; 45 s of annealing at 60°C; and 1 min of extension at 72°C. The PCR products were visualized by electrophoresis with an ethidium bromide-stained 2% agarose gel. The levels of chlamydia mRNA transcript were normalized relative to the hsp60 level using densitometry readings. To prevent carryover contamination, the primers for hsp60 were designed from GenBank cDNA sequences using the Web site program Primer 3 (http://www.path.cam.ac.uk/cgi-bin/primer3cgi). The sequences of all primers used in this study are shown in Table 1. The total RNA was extracted from infected cells using an RNaseasy Mini Kit (Qiagen, Valencia, Calif.) according to the manufacturer’s instructions with the protocol for bacterial cells. The concentration of RNA was quantified by spectrophotometry, and RNA was stored at −70°C until used. The extracted RNAs were treated with DNase (DNA-free; Ambion, Austin, Tex.) to eliminate the contaminating DNA. The resulting DNA-free RNAs were confirmed by PCR without RT. The RT of 2 μg of RNA was performed with avian myeloblastosis virus reverse transcriptase (Promega, Madison, Wis.) with random primers in a commercial reaction mixture (20 μl; Reverse Transcription System; Promega). The resulting cDNAs (2 μl) were then subjected to PCR with primers specific for C. pneumoniae 16S rRNA, hsp60, pyk, and omcB. The primers for pyk and omcB were designed from GenBank cDNA sequences using the Web site program Primer 3 (http://www.path.cam.ac.uk/cgi-bin/primer3cgi). The sequences of all primers used in this study are shown in Table 1. As a control, the PCR for human β-actin was also performed. The primer sequences for β-actin were described previously (31). Each PCR condition was optimized before being applied to samples. The PCR cycle consisted of a 5-min denaturation at 95°C followed by 25 to 40 cycles each of 45 s of denaturation at 94°C; 45 s of annealing at 60°C for β-actin, 55°C for 16S-rRNA and omcB, 58°C for hsp60, and 53°C for pyk; and 1 min of extension at 72°C. The PCR products were visualized by electrophoresis with an ethidium bromide-stained 2% agarose gel. The levels of chlamydia mRNA transcript were normalized relative to the β-actin level using densitometry readings. To prevent carryover contamination, an aerosol-resistant tip was used in all steps. Preparation of the PCR mixture was performed in a separate room.

Treatment with HC and minocycline. The effect of the immunomodulatory agent hydrocortisone (HC) and the antimicrobial agent minocycline on the growth of C. pneumoniae in cells was analyzed by RT-PCR for bacterial mRNAs. The cells were infected with C. pneumoniae (MOL 5) and then treated with or without HC (0.1 to 10 μg/ml; Sigma) or minocycline (0.1 to 10 μg/ml; Sigma). At 24 and 48 h after incubation, the growth of C. pneumoniae in the cells was assessed by bacterial culture (IFU assay) and RT-PCR.

Table 1. Primer sequences used for PCR

| Target gene | Primer direction | Sequence | Fragment size (bp) |
|-------------|------------------|----------|-------------------|
| 16S rRNA    | Sense            | 5'-TGA CAA CTG TAG AAA TAC AGC-3' | 463 |
|             | AntiSense        | 5'-CGC CTC TCT CCT ATA AAT-3'    |      |
| hsp60       | Sense            | 5'-ACG TCA CGT AGT TAT AGA TAA GAG-3' | 507 |
|             | AntiSense        | 5'-AAG TAG CTG GAG AGG TAT CCA CGG-3' |      |
| pyk         | Sense            | 5'-TGT GGA TGT GGT TGC TGC AT-3' | 411 |
|             | AntiSense        | 5'-GCA TTT TCC CTT CCT GAC AAC AT-3' |      |
| omcB        | Sense            | 5'-GGA TCT GTG ATT GCC CTG AA-3' | 217 |
|             | AntiSense        | 5'-CAG AAC ATT TGT GTC CAC CG-3' |      |

Statistical analysis. Statistical analysis was performed with Student’s t test.

RESULTS

Analysis of C. pneumoniae growth by RT-PCR. In order to determine the usefulness of bacterial transcript assay (RT-PCR) for assessment of C. pneumoniae infection in cells, HEp-2 cells, which are widely utilized for propagation of this pathogen (23, 29), were utilized in this study as a host cell. The growth of bacteria in the cells was assessed by detection of chlamydial inclusions and measurement of infectious progeny by repassage on freshly prepared HEp-2 cells (IFU assay). As seen in Fig. 1, C. pneumoniae-infected HEp-2 cells showed the typical apple green when stained with FITC-labeled anti-Chlamydia antibody but small inclusion bodies at 6 h after infection. The size of inclusions was then increased until 60 h after infection. At the late phase of infection, such as 72 h after infection, many ruptured inclusions were observed. These morphological observations were well matched to the results of infectious progeny assay (IFU assay) (Fig. 2). That is, the number of infectious progeny (EBs) at the beginning of the infection (zero time) was limited and may reflect the number of initial EBs in infected cells. At 12 h after infection, the formation of infectious progeny was decreased and then underwent a marked increase. At 48 h after infection, the number of infectious bacteria in infected cells reached a plateau. The

![FIG. 1. Fluorescence micrographs of C. pneumoniae-infected HEp-2 cells stained with FITC-labeled anti-Chlamydia antibody. The numbers indicated represent the time after infection. Magnification, ×1,000.](http://cvi.asm.org/Downloaded from http://cvi.asm.org/ on April 28, 2019 by guest)
reproduction profile of infectious chlamydia organisms during the infection in HEp-2 cells observed in this study was similar to that found in a previous study in HeLa cells infected with C. trachomatis (25).

The assessment of C. pneumoniae growth by RT-PCR specific for four different C. pneumoniae genes—16S rRNA, hsp60, pyk, and omcB—was performed. Both 16S rRNA and hsp60 transcripts are known to be expressed in both EBs and RBs (25). pyk, which encodes pyruvate kinase, and omcB, which encodes the 60-kDa cysteine-rich outer membrane protein, are known to be transcribed during the mid- and the later developmental cycles, respectively (25). Particularly, omcB transcripts are recognized to be expressed at a point when RBs begin to differentiate back to EBs in the case of C. trachomatis infection in HeLa cells (25). Figure 3 shows the representative RT-PCR result of the RNA isolated from the infected cells at different time points. Although all gene transcripts tested were expressed during the infection, the levels of each transcript depended upon the phase of infection.

In order to quantify the transcription level of the gene, the relative expression levels of each gene to the host cell housekeeping gene (β-actin) expression level were measured. As shown in Fig. 4, both 16S rRNA and hsp60 transcripts were rapidly expressed at the beginning of infection, followed by a consistent increase until the late phase of infection, such as 72 h after infection. The levels of pyk gene transcripts were minimum at the beginning of the infection and relatively low during the midphase of infection, such as 6 and 12 h after infection, but reached a plateau at 24 h after infection. In contrast, omcB transcripts were relatively high at the beginning of infection and decreased during the midphase of infection, followed by a sharp increase at the late phase of infection. The fluctuation of omcB transcripts observed was well matched to the reproduction profile of infectious EBs assessed by IFU assay.

Alteration of bacterial growth by minocycline and HC. In order to determine the usefulness of the bacterial transcript assay for assessment of altered bacterial growth, infection was modulated by two agents, i.e., the antibiotic minocycline and immunosuppressant HC (30). The HEp-2 cells were infected with C. pneumoniae and then treated with several concentrations of minocycline or HC for 48 h. The altered bacterial growth in HEp-2 cells treated with these agents was then assessed by IFU as well as bacterial transcript assays. As shown in Fig. 5, the antibiotic minocycline markedly inhibited the reproduction of infectious EBs at all concentrations tested.
contrast, HC significantly enhanced the reproduction of infectious EBs 48 h after infection at concentrations (1.0 and 10.0 μg/ml) which can be reached in blood after administration of 100 to 500 mg of HC (22, 28).

The assessment of C. pneumoniae growth by bacterial transcript assay also revealed alteration by these agents. As apparent in Fig. 6, the treatment of the cells with minocycline induced a marked suppression of all bacterial gene transcripts at the highest concentration of 10 μg/ml, which is more than 150 times the MIC (0.06 μg/ml) of minocycline, at both 24 and 48 h after infection. However, when the concentration of minocycline was reduced to a low level, such as 1.0 or 0.1 μg/ml, the suppression profile of bacterial gene transcripts was still notable. That is, the low concentration (1.0 μg/ml) of minocycline selectively inhibited the expression of C. pneumoniae genes, such as hsp60 and omcB. Particularly, the hsp60 gene transcript was the most sensitive to the minocycline treatment among the bacterial genes tested. In contrast, both 16S rRNA and pyk transcripts were not affected by minocycline at the 0.1-μg/ml dose, even though such a concentration showed a marked inhibition of reproduction of infectious EBs (Fig. 5). In the case of HC-treated cells, all bacterial gene transcripts tested were increased at the high HC concentrations, such as 1 and 10 μg/ml, at 48 h after infection (Fig. 7). Some bacterial gene transcripts, such as 16S rRNA and omcB, were significantly increased at the low concentration (0.1 μg/ml) of HC, even
though such a concentration did not result in any significant increase of reproduction of infectious EBs as determined by IFU assay.

DISCUSSION

The detection of bacterial transcripts as a marker for viable and metabolically active bacteria has been utilized for a wide variety of bacteria, including *Escherichia coli* (26), *Legionella pneumophila* (2), *Vibrio cholerae* (3), *Mycobacterium leprae* (21), and *Enterococcus faecalis* (5). Because mRNA is turned over rapidly in living bacterial cells, with most mRNA species having a half-life of only a few minutes (1), the presence of certain bacterial mRNAs can be regarded as a valid and convincing criterion for assessing cell viability (3, 21, 26). In this regard, determination of viable *C. pneumoniae* by RT-PCR targeting bacterial mRNAs in clinical specimens has been examined for demonstration of viable and metabolically active bacteria (6, 8).

A recent study by Shaw et al. (25) showed that the expression of *C. trachomatis* genes during the developmental cycle in cells may be classified in three groups: genes expressed at the early cycle, which are detected by 2 h after infection during the germination of EBs to RBs; at the midcycle, which occurs between 6 and 12 h after infection and represents transcripts expressed during the growth and multiplication of RBs; and at the late cycle, which occurs between 12 and 20 h after infection and represents those genes transcribed during the terminal differentiation of RBs to EBs. Since *C. pneumoniae* has a developmental cycle in cells similar to that of *C. trachomatis*, differential bacterial gene expressions during the bacterial growth in infected cells seems likely.

Four *C. pneumoniae*-specific genes, 16S rRNA, hsp60, *pyk*, and *omeB*, were selected in this study as a possible marker for each stage of the developmental cycle in cells. Both 16S rRNA and hsp60 transcripts have been detected in the early cycle of *C. trachomatis* infection, but *pyk* and *omeB* transcripts were observed in the midcycle and the late cycle of this pathogen, respectively (25). The detection of these transcripts during the infection of HEp-2 cells with *C. pneumoniae* in this study showed similar results as seen in *C. trachomatis*-infected HeLa cells (25), but the present study revealed semiquantitative fluctuation of these gene transcripts during the infection. Since detection of bacterial transcripts by RT-PCR only may not provide sufficient information regarding bacterial growth due to heterogenous bacterial growth stages occurring simultaneously in infected cells, the quantitative assay for transcripts may be essential for analysis of *C. pneumoniae* growth in cells. Both 16S rRNA and hsp60 transcripts were detected in the early phase of infection and increased rapidly, even though the IFU assay, which detects only infectious EBs, did not show any increase during the early phase of infection, such as 6 to 24 h.

![Graphs showing the effect of minocycline (MINO) on the levels of bacterial transcripts in infected cells determined by RT-PCR.](http://cvl.asm.org/ "Downloaded from http://cvl.asm.org/)
after infection. These results indicate that both 16S rRNA and hsp60 transcripts are expressed in all metabolically active stages of C. pneumoniae growth, including noninfectious RBs in infected cells. In contrast, both pyk and omcB transcripts were detected at significant levels until the mid- to late phase of infection. In particular, omcB transcripts reached a significant level only at the late phase of infection, such as 48 h after infection. This relatively slow increase of omcB transcripts paralleled the IFU results. Therefore, the expression levels of omcB may be a good marker for the presence of EBs. The omcB encodes the 60 kDa cysteine-rich outer membrane protein, which is known to be solely expressed in EBs but not in RBs of C. trachomatis (19). The results obtained in this study with C. pneumoniae were consistent with such previous results of C. trachomatis-infected cells. The reason for the decline of some bacterial gene transcript levels at the late stage of infection, such as 72 h after infection, is not clear. However, it seems likely that the limited microenvironment for the growth of the bacteria may affect the expression levels of some bacterial genes due to overgrowth.

The analysis of C. pneumoniae growth in HEp-2 cells treated with the antibiotic minocycline or immunosuppressant HC by the bacterial transcript assay revealed some detail of the altered bacterial growth. The minocycline treatment of cells markedly inhibited the production of infectious EBs determined by IFU assay at all concentrations tested. However, the lowest concentration of minocycline tested inhibited only hsp60 and omcB transcripts at 24 (hsp60) and 48 h (hsp60 and omcB) after infection. The 16S rRNA and pyk transcripts were not affected by the minocycline treatment at the concentration of 0.1 μg/ml, which induced an almost-complete inhibition of EB production. These results indicate that the low concentration (0.1 μg/ml) of minocycline may block the conversion of RBs to EBs but may not be sufficient to inhibit the metabolically active form, such as RBs. The inhibition of hsp60 expression by the low minocycline concentration may be due to, but not only, the result of inhibition of protein synthesis, which is the common antimicrobial mechanism of tetracycline antibiotics, including minocycline.

Steroid treatment is widely utilized in clinics as a therapy to treat immunoreactive as well as inflammatory diseases. However, it is known that the steroid treatment may induce susceptibility to a wide variety of infections due to its immunosuppressive activity (30). Recent studies show that HC enhances the growth of C. pneumoniae as well as C. trachomatis in vitro (15, 16, 27). Furthermore, it has also been demonstrated that cortisone treatment induces a reactivation of persistent C. pneumoniae in a mouse model (27). In the present study, the HC treatment induced the enhancement of C. pneumoniae growth in cells demonstrated by both the bacterial transcript assay and bacterial culture (IFU assay). Specifically, treatment with the lowest concentration of HC, such as 0.1 μg/ml, enhanced both 16S rRNA and omcB transcripts, even though the IFU assay did not show any significant increase of EB numbers at this concentration. These results indicate that the HC treatment induces the growth of C. pneumoniae, including both RBs and EBs, and furthermore, the bacterial transcript assay using RT-PCR is more sensitive than the IFU...
assay to determine the effect of HC on the growth of *C. pneumoniae*.

Thus, the results in this study indicate that the bacterial transcript assay using RT-PCR may be a powerful tool to analyze the growth of *C. pneumoniae* in cells; in particular, altered *C. pneumoniae* growth caused by antibiotics or immunomodulatory agents, which may not be assessed sufficiently by the conventional bacterial culture methods such as the IFU assay, is the object.

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