Chlamydia pneumoniae Resists Antibiotics in Lymphocytes

Hiroyuki Yamaguchi,[1] Herman Friedman,[1] Mayumi Yamamoto,[2]
Keigo Yasuda,[2] and Yoshimasa Yamamoto[1,3]*

*Department of Medical Microbiology and Immunology, College of Medicine, University of South Florida,
Tampa, Florida 33612,[1] and Third Department of Internal Medicine, School of Medicine,
Gifu University, Gifu,[2] and Division of Molecular Microbiology, Department of
Basic Laboratory Sciences, School of Allied Health Sciences,
Faculty of Medicine, Osaka University, Osaka,[3] Japan*

Chlamydia pneumoniae infection of lymphocytes in blood has been well documented, and it is apparent that control of this pathogen in these cells may be critical in the development of chronic inflammatory diseases associated with infection by this bacterium. The activity of antibiotics against *C. pneumoniae* in lymphocytes was assessed in this study by utilizing an in vitro infection model with lymphoid cells. The results obtained indicated that although all of the antibiotics tested showed remarkable activity against bacterial growth in epithelial cells, *C. pneumoniae* in lymphocytes was less susceptible to antibiotics than was bacterial growth in epithelial cells, which are widely used for the evaluation of anti-*C. pneumoniae* antibiotics.

Mounting evidence supports the contention that atherosclerosis is a chronic inflammatory process that develops in response to a variety of injuries (25). A number of microbial organisms have been implicated in such pathogenesis. The strongest evidence to date for an association between an infectious agent and atherosclerosis is that for *Chlamydia* (*Chlamydophila*) *pneumoniae* (14), an obligate intracellular bacterium which is a causative agent of respiratory tract infections. However, there is still controversy regarding the possible involvement of *C. pneumoniae* in the pathogenesis of atherosclerosis. Nevertheless, it has been shown seroepidemiologically that 50 to 80% of the adult population has had prior exposure to this pathogen (2, 24).

C. pneumoniae preferentially infects respiratory tract epithelial cells. While the pathogenic potential of this pathogen in the respiratory system is well established, several current studies suggest that the organism may disseminate from this site, probably via circulating leukocytes, including monocytes and lymphocytes (6, 17). In this regard, the detection of *C. pneumoniae* antigen in blood obtained from patients with or without coronary artery disease (CAD) has been demonstrated (7, 8, 15, 20). Furthermore, it has been shown that *C. pneumoniae* DNA can be recovered from $CD3^+$ peripheral blood leukocytes obtained from patients with CAD (9). These findings indicate that peripheral blood leukocytes, particularly lymphocytes, may be host cells for *C. pneumoniae* and may play a critical role in the development of atherosclerosis (6).

The susceptibility of *C. pneumoniae* to antibiotics after invasion of monocytes and lymphocytes is not well known but likely is critical in controlling the spread of the organism from the lungs to possible sites of chronic infection (e.g., atheroma). Therefore, it is important to determine the antibiotic suscep-

tibility of *C. pneumoniae* in monocytes and lymphocytes to provide information for improved treatment of individuals who carry *C. pneumoniae* in their blood. In this regard, a recent study showed that antibiotics do not inhibit chlamydial growth within monocytes (3).

C. pneumoniae TW183, kindly provided by G. Byrne, University of Wisconsin, Madison, was used in this study. The bacteria were propagated in HEp-2 cells as described previously (21). Human epithelial HEp-2, monocytic THP-1, T-lymphoid Molt 4, and B-lymphoid P3HR1 cells were kindly provided by R. Widen, Tampa General Hospital, Tampa, Fla. HEp-2 cells were cultured in Dulbecco's minimal essential medium (Sigma, St. Louis, Mo.) with 10% heat-inactivated fetal calf serum (FCS; HyClone, Logan, Utah). Other cells were cultured in RPMI 1640 medium with 10% FCS.

Azithromycin (AZM; Pfizer Inc., New York, N.Y.), clarithromycin (CLR; Taisho Pharmaceutical Co., Ltd., Tokyo, Japan), tosufloxacin (TFLX; Toyama Chemical Co., Ltd., Tokyo, Japan), and minocycline (MINO; Sigma) were used in this study. MICs and minimal bactericidal concentrations (MBCs) of antibiotics against *C. pneumoniae* were determined by a method recommended by the Japan Society of Chemotherapy (10, 11). In brief, HEp-2, THP-1, Molt 4, or P3HR1 cells (2 × 10^5 cells/well) were dispensed into 24-well plates 24 h prior to infection with bacteria. The cells were infected with *C. pneumoniae* at infectivity ratios of 0.1 (HEp-2 cells) to 1 (THP-1, Molt 4, and P3HR1 cells) organism per cell by centrifugation at 700 × g for 1 h. These infectivity ratios were designed to develop 10^4 chlamydial inclusions per well at 3 days after infection. The infected cells were then cultured with cycloheximide (HEp-2 and THP-1 cells, 1 μg/ml; Molt 4 and P3HR1 cells, 0.5 μg/ml) in the presence or absence of serially diluted antibiotics at 37°C for 72 h in 5% CO_2. After incubation, the cells were stained with fluorescein isothiocyanate-conjugated anti-*Chlamydia* genus-specific monoclonal antibody (Research Diagnostics, Flanders, N.J.) for the detection of chlamydial inclusions. The MIC was the lowest antibiotic concentration at which no inclusions were seen. The MBC was the lowest anti-

* Corresponding author. Mailing address: Division of Molecular Microbiology, Department of Basic Laboratory Sciences, School of Allied Health Sciences, Faculty of Medicine, Osaka University, 1-7 Yamadaoka, Suita, Osaka 565-0871, Japan. Phone: 81-6-6879-2580. Fax: 81-6-6879-2499. E-mail: yyamamot@sahs.med.osaka-u.ac.jp.

TABLE 1. MICs and MBCs of AZM, TFLX, CLR, and MINO
against *C. pneumoniae* in different cell types

Antibiotic	MIC/MCC (μg/ml) for *C. pneumoniae* in the following cells:			
	HEp-2	THP-1	Molt 4	P3HR1
AZM	0.25/0.5	0.5/8	>32/>32	>32/>32
TFLX	0.25/0.5	0.25/8	0.25/1	>32/>32
CLR	0.125/0.125	>32/>32	0.125/0.5	>32/>32
MINO	0.0625/0.0625	>32/>32	>32/>32	>32/>32

biotic concentration which resulted in no inclusions after passage. Since the MBC assay used in this study did not have any passage step in antibiotic-free HEp-2 cells before the assessment of the MBC, it can be conjectured that some bacteria might still be present and waiting for a chance to grow.

For assessment of the activity of antibiotics against bacteria infecting primary lymphocytes, mouse lymphocytes prepared from spleens of 10- to 12-week-old BALB/c female mice (Jackson Laboratory, Bar Harbor, Maine) were used. Spleen cell suspensions in RPMI 1640 medium containing 10% FCS were cultured on tissue culture dishes to promote adherence of macrophages for 2 h at 37°C. Nonadherent lymphocytes (10^6 cells/well, 24-well plates) were collected and then infected with bacteria (one organism per cell). HEp-2 cells were used as reference cells. The infected cells were then cultured with cycloheximide (HEp-2 cells, 1 μg/ml; lymphocytes, 0.5 μg/ml) in the presence or absence of antibiotics at the following final concentrations (16 times the MICs): AZM and TFLX, 4 μg/ml; CLR, 2 μg/ml; and MINO, 1 μg/ml.

Bacterial growth and viability in cultures were assessed by measurement of bacterial transcripts as described previously (5). The extracted RNAs were subjected to reverse transcription (RT)-PCR specific for *C. pneumoniae* 16S rRNA as described previously (5). The PCR products were determined by densitometric reading after electrophoresis in an ethidium bromide-stained 2% agarose gel. Statistical analysis was performed with the unpaired Student *t* test.

The model of established lymphoid cell lines infected with *C. pneumoniae* used in this study was established by Yamaguchi et al. (27). The growth of *C. pneumoniae* in all of the cells used in this study was well supported, with more than 10-fold increases in infective progenies in THP-1, Molt 4, and P3HR1 cells and more than a 100-fold increase in infective progenies in HEp-2 cells during culturing for 3 days. As shown in Table 1, all antibiotics used were very effective against *C. pneumoniae* growth in HEp-2 epithelial cells. Bacterial growth in monocytes (THP-1) was also markedly suppressed by either AZM or TFLX at the same concentrations as those that were effective in epithelial cells. However, CLR and MINO did not show any inhibitory activity against chlamydial growth in monocytes. In contrast, *C. pneumoniae* growth in T lymphocytes (Molt 4) was inhibited by TFLX and CLR, with MICs comparable to those in HEp-2 cells, but not by AZM or MINO. Surprisingly, all antibiotics tested failed to inhibit the growth of *C. pneumoniae* in B lymphocytes (P3HR1), even at a concentration as high as 32 μg/ml, which was more than 10 times the concentration in serum.

In order to confirm the effect of antibiotics on the growth of *C. pneumoniae* in lymphocytes, primary cultures of mouse
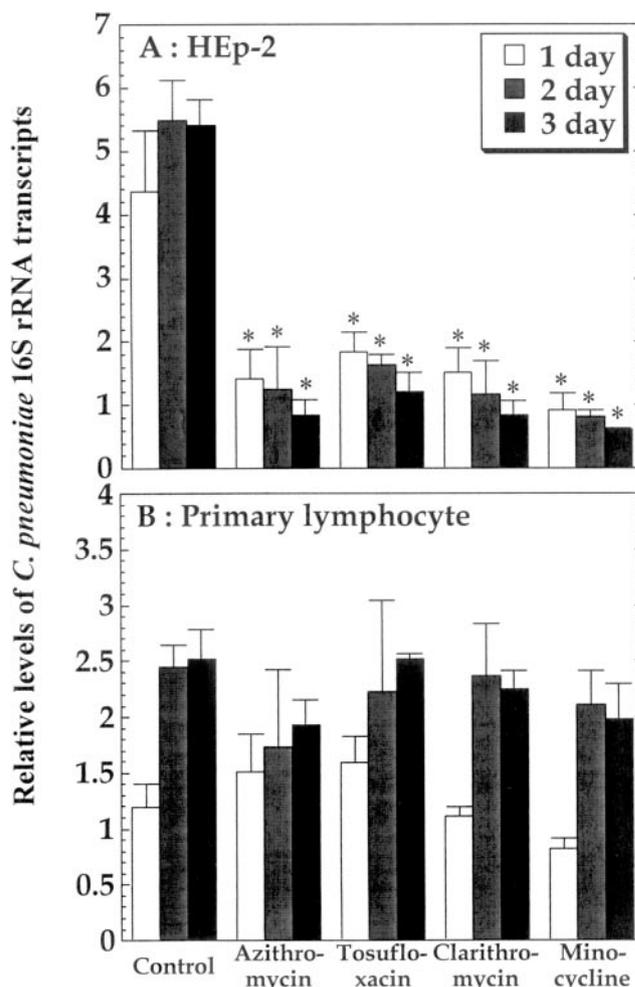

FIG. 1. Effect of antibiotics on the growth of *C. pneumoniae* in primary lymphocyte cultures versus established HEp-2 epithelial cell cultures. Relative levels of *C. pneumoniae*-specific 16S rRNA transcripts in infected cells at the indicated days after infection were determined by RT-PCR. Data are shown as arbitrary reading units and represent the mean and standard deviation for three experiments. An asterisk indicates a *P* value of <0.05 for treated cultures compared to control cultures without antibiotics.

spleen lymphocytes were used for *C. pneumoniae* infection, since immortalized cells may be different from primary cells with regard to pharmacological as well as cell biological aspects. The assessment of bacterial growth in lymphocytes by RT-PCR revealed a significant increase in the levels of *C. pneumoniae* 16S rRNA transcripts during culturing for 3 days (data not shown). All antibiotics used failed to inhibit bacterial growth in primary lymphocyte cultures (Fig. 1B) at concentrations which completely inhibited *C. pneumoniae* growth in HEp-2 reference cells (Fig. 1A).

The recovery of *C. pneumoniae* from atheromas of CAD patients has led to the initiation of pilot studies of ways to eradicate this pathogen from vascular tissue by antibiotic treatment. A number of trials of prospective antibiotic interventions targeted against *C. pneumoniae* infections in patients with CAD are now under way (1). Data from some intervention studies suggested that treatment with antibiotics decreases in-

flammatory markers, but adverse clinical events were not uniformly reduced in the trials (25). For instance, in the largest study published to date on antimicrobial treatment in CAD patients, AZT treatment had no significant effect on clinical events (18). In addition, the present study indicates that the pathogen may not be eliminated from its host by standard antichlamydial treatments (3). These reports leave unanswered questions regarding the effectiveness of antibiotics against *C. pneumoniae* in the blood vessel system.

Tetracyclines and macrolides have frequently been used for the treatment of chlamydial infections (4, 22, 23). However, all data published regarding the in vitro susceptibility of *C. pneumoniae* to antibiotics are limited because of the use of only established epithelial cells, such as HEp-2 cells. The susceptibility of *C. pneumoniae* to antibiotics in leukocytes, such as lymphocytes and monocytes, which may be important in the pathogenesis of atherosclerosis associated with this pathogen, has not been well studied.

The study reported here clearly demonstrated that the susceptibility of *C. pneumoniae* to antibiotics was dependent on host cells, even though the results were obtained under only limited experimental conditions. The growth of *C. pneumoniae* in HEp-2 epithelial cells was markedly suppressed by the antibiotics used, and MICs and MBCs were comparable to those in previous reports (11, 16, 26). In contrast, bacterial growth in THP-1 monocytes and Molt 4 T lymphocytes did not show uniform susceptibility to the antibiotics used. Furthermore, *C. pneumoniae* in P3HR1 B lymphocytes showed remarkable resistance to all of the antibiotics tested. The reason for the differences among host cells in susceptibility to antibiotics is not clear. Differences between epithelial cells and other cells, particularly lymphocytes, in the pharmacokinetics of antibiotics are a possible mechanism. In this regard, different levels of AZM uptake by cultured cells, including epithelial cells, neutrophils, and macrophages, have been demonstrated (19). However, there is limited information regarding the pharmacokinetics of antibiotics in lymphocytes. Differences among host cells in bacterial growth rates are also a possible mechanism for explaining why *C. pneumoniae* in lymphocytes was resistant to antibiotics, since actively growing bacteria are more susceptible to antibiotics. In this regard, recent studies reported by Kutlin et al. (12, 13) showed that antibiotics, including AZM and CLR, probably do not eliminate *C. pneumoniae* from HEp-2 cells because of its persistent state in these cells. Therefore, a persistent, nonreplicating state of this pathogen is also a possible reason for the resistance. However, this mechanism may not fully explain the resistance of bacteria in lymphocytes, since P3HR1 cells supported more bacterial growth than Molt 4 cells, although the susceptibility of bacteria in P3HR1 cells was minimal (data not shown).

The reason for the different antimicrobial activities of AZM and CLR, both of which are macrolide antibiotics, against bacteria in immune cells is not clear. The different pharmacokinetics of these antibiotics in immune cells may be a responsible factor.

The antibiotic susceptibility of bacteria in immortalized cells may not be directly comparable with that in vivo. Therefore, the effect of antibiotics on bacteria in primary lymphocytes was assessed. Since *C. pneumoniae* growth in primary lymphocyte cultures was inferior to that in HEp-2 cells (6), measurement of bacterial transcripts by RT-PCR, which has been established as an antibiotic susceptibility assay for this pathogen (10), was performed to assess bacterial growth. The results obtained showed that all drugs tested had no effect on bacterial growth in primary lymphocyte cultures.

Even though only one bacterial strain was used in this study for evaluation of the effects of antibiotics on bacterial growth in different host cells, differences among host cells in the susceptibilities of the bacteria were obvious. Particularly in lymphocytes, *C. pneumoniae* was quite resistant to antibiotics. Thus, the results obtained indicate the possibility that commonly available antibiotics may not eradicate *C. pneumoniae* from blood, particularly from lymphocytes.

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