A Chlamydia pneumoniae infection model using established human lymphocyte cell lines

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Received 2 August 2002; received in revised form 18 September 2002; accepted 19 September 2002
First published online 18 October 2002

Abstract

Since current studies indicate possible infection of human lymphocytes with Chlamydia (Chlamydophila) pneumoniae, establishment of an in vitro C. pneumoniae infection model using lymphocyte cell lines was demonstrated. Human lymphoid cell lines (Molt4 [T-cell] and P3HR1 [B-cell]) were utilized for this purpose besides human monocyte cell line (THP-1) and human epithelial cell line (HEp-2), as a reference of monocyte/macrophage cells and a positive control for support of C. pneumoniae growth, respectively. Both lymphoid cells (Molt 4 and P3HR1) supported the growth of C. pneumoniae as demonstrated by Chlamydia inclusion formation, detection of increased infective progenies and increased bacterial antigen levels. Similar data were obtained using monocyte THP-1 cells. However, the bacterial growth in these cells was less than that in HEp-2 cells. The electron microscopic study showed typical inclusions with many Chlamydia elementary bodies in lymphoid cells tested, similar to that seen in HEp-2 cells. These results indicate that C. pneumoniae can infect cells with lymphocyte properties and this infection model with lymphoid cell line cells could be valuable to study details of lymphocyte–C. pneumoniae interaction.

Keywords: Lymphocyte; Infection model; Established cell line; Chlamydia pneumoniae

1. Introduction

Chlamydia (Chlamydophila) pneumoniae is an obligate intracellular bacterium associated with respiratory tract infections. Current studies indicate that this bacterial infection may be involved in the pathogenesis of atherosclerosis [1]. It has been widely recognized that atherosclerosis is a chronic inflammatory disease [2] and the formation of the atherosclerotic plaque, which is known to be caused by excessive inflammatory fibroproliferation with damage of endothelial and smooth muscle cells [3,4], is critical in coronary artery disease. Vasoregulatory molecules, growth factors and cytokines following host immunomodulation have been implicated in this process [5]. In this regard, macrophages and lymphocytes which migrate to inflammatory sites, such as atherosclerosis lesions, are thought to be the important cells associated with this process [3,6]. C. pneumoniae preferentially infects respiratory tract epithelial cells as well as macrophages [7–9]. Moreover, in recent years, the findings that DNA from this bacterium is detected in the inflammatory tissues, such as an atherosclerosis lesion, have been reported [10]. This raises the possibility that the development of atherosclerosis is related to immunomodulation due to C. pneumoniae infection of immune cells. Besides epithelial and monocyte/macrophage cells, it is known that this bacterium multiplies in vitro in endothelial cells and aortic smooth muscle cells [8,11,12]. Furthermore, our current study indicates that C. pneumoniae can infect and multiply in lymphocytes in vitro [13]. In this regard, Kaul et al. [14] showed that chlamydia DNA could be recovered from CD3+ peripheral blood leukocytes obtained from the patients attending a cardiology clinic. These findings suggest the possibility
that lymphocytes may be a host cell for *C. pneumoniae*. Since lymphocytes are another major immune cell type besides macrophages in the development of atherosclerosis [2], interaction between lymphocytes and this pathogen may contribute to the pathogenesis of atherosclerosis as well as other chronic inflammatory diseases associated with *C. pneumoniae*. For instance, it can be conjectured that activation of lymphocytes by infection with *C. pneumoniae* may lead to up-regulation of receptors for adhesion molecules on their surfaces as well as production of cytokines, which may contribute to the establishment of inflammation. In fact, abundant lymphocytes in an activated state are observed in atherosclerotic lesions [15]. Therefore, in order to understand details of lymphocyte–*C. pneumoniae* interaction, we attempted to establish an in vitro *C. pneumoniae* infection model using defined lymphocyte cell line cells and analyzed the growth of this bacterium in lymphocytes by electron microscopy.

2. Materials and methods

2.1. Bacteria

*C. pneumoniae* TW183 strain was kindly provided by G. Byrne, University of Wisconsin, Madison, WI, USA. The bacterial cultures were confirmed Mycoplasma-free by PCR, as described previously [16]. The bacteria were propagated in the HEp-2 cell culture system according to methods described previously [17]. *C. pneumoniae* elementary bodies (EBs) were purified by density gradient centrifugation with Percoll (Sigma Chemical, St. Louis, MO, USA) [8]. 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 (IFUs) of prepared EBs were determined by counting chlamydial inclusions in HEp-2 cell monolayers [17,18]. UV-treated bacteria prepared by placing cultures counting chlamydial inclusions in HEp-2 cell monolayers in units (IFUs) of prepared EBs were determined by methods described previously [17]. Centrifuged and washed bacteria were used as a control. The viability of the UV-treated bacteria determined by specific inclusion formation showed that no viable bacteria were detected.

2.2. Cell lines

The human epithelial cell line HEp-2 [19], human monocytic cell line THP-1 [20] and human lymphocyte cell lines Molt 4 (mature T-cell) [21] and P3HR1 (mature B-cell) [22] were kindly provided by R. Widen, Tampa General Hospital, Tampa, FL, USA. The lymphocyte and monocytic cell lines were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (FCS) and antibiotics (gentamicin sulfate, 10 μg ml⁻¹; vancomycin, 10 μg ml⁻¹; amphotericin B, 1 μg ml⁻¹) (Sigma) at 37°C in 5% CO₂. The epithelial HEp-2 cells were cultured in DMEM medium (Sigma) with 10% FCS and antibiotics.

2.3. *C. pneumoniae* infection

Cultures of each cell line (1×10⁶ cells) were infected with either viable (1×10⁷ IFUs) or UV-treated bacteria (1×10⁷ organisms) or cultured without bacteria for 3 h at 37°C in 5% CO₂ with gently shaking. After two washings with medium by centrifugation at 1000 rpm for 10 min, the cultures were re-suspended in medium and then incubated for 72 h in the presence of cycloheximide (Sigma), which was adjusted to specific concentrations as follows: HEp-2, 1 μg ml⁻¹; THP1, 1 μg ml⁻¹; Molt 4, 0.5 μg ml⁻¹; P3HR1, 0.5 μg ml⁻¹. The concentrations of cycloheximide used were confirmed to show the best chlamydia growth in the cultures in a preliminary study.

2.4. Assessment of Chlamydia inclusions

After 3 (immediately after the washing) to 72 h, the cells were collected for determination of the number and morphology of chlamydia inclusions, as described previously [13]. In brief, the cells were centrifuged on a microscopic slide by a Cytospin (Shandon, Sewickley, PA, USA). After fixing with ethanol, cells were stained with *Chlamydia* genus-specific fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody (specific to *Chlamydia* lipopolysaccharide; Research Diagnostics, Flanders, NJ, USA). The presence of chlamydia inclusions in the sample was then determined with a fluorescence microscope.

2.5. Assessment of infective progeny

To assess the infective progeny in the culture for different time cultivation periods, the cultures were frozen and thawed, as described previously [17]. The lysates in 10-fold serial dilutions were inoculated on HEp-2 cell monolayers in 96-well plates, centrifuged at 800×g for 60 min, and then incubated in the medium with cycloheximide (1 μg ml⁻¹) for 72 h at 37°C. The number of IFUs in cells was then assessed by staining with FITC-conjugated anti-*Chlamydia* antibody [8].

2.6. Enzyme-linked immunosorbent assay (ELISA)

*Chlamydia* lipopolysaccharide (LPS) antigen in cultures was detected by an ELISA kit (IDEIA PCE Chlamydia; Dako Ltd., Ely, UK), as described previously [13,23]. Purified *C. pneumoniae* EBs were used for preparation of a standard curve. The standard curve permitted quantification of the number of bacteria from 5×10⁴ to 5×10⁸ IFUs ml⁻¹. The results of the ELISA assay were expressed as relative number of bacteria converted from the standard curve.

2.7. Electron microscopy

For transmission electron microscopy, the cells were
immersed in a fixative containing 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.4, for 24 h at 4°C and then rinsed in 2.5% sucrose in PB. After a brief rinse in 2.5% sucrose-PB, they were processed for alcohol dehydration and embedding in Epon 813, as described previously [24]. Ultrathin sections of the cells were stained with lead citrate and uranium acetate before viewing on an electron microscope.

### 2.8. Statistical analysis

All experiments were repeated at least three times and the statistical significance of difference was assessed by unpaired Student’s *t*-test.

### 3. Results

#### 3.1. Chlamydia inclusion formation

In order to determine whether *C. pneumoniae* infects and grows in the cells of lymphocyte cell lines, both T-lymphocyte Molt 4 and B-lymphocyte P3HR1 cells were infected with bacteria and assessed for *Chlamydia* inclusion formation during the cultivation. The monocyte cell line THP-1 was used as a reference for mononucic cells. The epithelial cell line HEp-2 was also used as a control. Fig. 1 shows representative fluorescence microscopic images of infected lymphocytes as well as monocytic cells at 72 h after infection. Obvious *Chlamydia* inclusions were observed in all cells tested. Control cells, which were not infected and cells treated with UV-killed bacteria did not show any inclusions stained with FITC-conjugated anti-*Chlamydia* antibody. Magnification, ×1000.

![Fluorescence microscopy images of *C. pneumoniae*-infected human monocyte cell line, THP-1 (A), and lymphoid cell lines, Molt 4 (B) and P3HR1 (C), 72 h after infection. The cells were stained with FITC-conjugated anti-*Chlamydia* antibody.](A B C)

Fig. 1. Fluorescence microscopy images of *C. pneumoniae*-infected human monocyte cell line, THP-1 (A), and lymphoid cell lines, Molt 4 (B) and P3HR1 (C), 72 h after infection. The cells were stained with FITC-conjugated anti-*Chlamydia* antibody. Magnification, ×1000.

#### 3.2. Inclusion number

![Graph A: The relative number of bacteria calculated from the amounts of *C. pneumoniae* LPS determined by ELISA in cultures after infection.](A)

Fig. 2. A: The relative number of bacteria calculated from the amounts of *C. pneumoniae* LPS determined by ELISA in cultures after infection. The cells (1×10⁶ cells/flask, 25-cm² flask) were infected with bacteria (infectivity ratio, 10 bacteria per cell) and incubated for up to 72 h. ELISA assay was performed at 3 h (open bar) and 72 h (closed bar) after infection. B: The number of infective progenies in the cultures after infection. The cells were infected with *C. pneumoniae* and incubated for up to 72 h. The cell lysates prepared at each time point were then inoculated on HEp-2 cell monolayers for assessment of infective progeny. Number of inclusions was determined by staining with FITC-conjugated anti-*Chlamydia* antibody. The data represent the mean ± S.D. for three experiments. *P < 0.05 compared to 3 h cultures of each cell.

![Graph B: Number of infective progenies / culture](B)
3.2. Growth of C. pneumoniae in lymphoid cells

In order to determine quantitatively the growth of C. pneumoniae in the cultures, the amount of bacterial antigen was assessed. As evident in Fig. 2A, the relative numbers of bacteria calculated from the concentrations of bacterial antigen recovered from all cell line cultures were significantly increased during cultivation for 72 h. The control HEp-2 cells showed the highest amount of bacterial growth, i.e., approximately a 500-fold increase, during the incubation. The P3HR1 showed a marked potency for bacterial growth, which was more than a 80-fold increase, at 72 h after infection. This potent support of C. pneumoniae growth was more than for the monocytic cell line THP-1, which showed approximately a 15-fold increase. The Molt 4 cells showed somewhat less potency for bacterial growth, but the growth was still obvious. These results corresponded to that of microscopic observations. The number of bacteria in cultures infected with UV-treated bacteria showed no increase during the incubation (data not shown).

Demonstration of an increased number of infective progenies in cell cultures is important to establish an in vitro infection model of specific cells with C. pneumoniae. As shown in Fig. 2B, all established cell lines tested showed a significant increase of infective progenies with a temporary decrease at 24 and 48 h after infection during the cultivation. There were more than 10-fold increases in monocytic as well as lymphoid cells tested at 24 and/or 48 h after infection. In contrast, control HEp-2 cells showed a marked increase of infective progenies during the cultivation, i.e., more than a 100-fold.

3.3. Morphological analysis of infected cells

EM studies of infected cells supported the findings that C. pneumoniae infected and multiplied in the cells of both monocytic and lymphoid cell lines. As shown in Fig. 3, typical EBs as well as reticulate bodies (RBs) in an inclusion were observed in all cells tested at 72 h after infection. A size of inclusions in lymphoid and monocytic cells was relatively small compared with that in control HEp-2 cells. It is noteworthy that small irregular bodies of chlamydiae in a small inclusion in monocytic and lymphoid cells were observed (Fig. 3E-G).

Fig. 3. Transmission electron micrographs of C. pneumoniae-infected epithelial HEp-2 (A), monocye THP-1 (B,E), lymphoid Molt 4 (C,F) and lymphoid P3HR1 (D,G) cells. The cells were infected with bacteria (infectivity ratio was 10 bacteria per cell) and incubated for 72 h. Arrows indicate aberrant body. N, nucleus. Bar, 1 μm.
4. Discussion

In order to understand *C. pneumoniae*-lymphocyte interaction, utilizing of stable established lymphoid cells for in vitro analysis of a relationship between *C. pneumoniae* and lymphocytes should be valuable. In the present study, both Molt 4 and P3HR1 lymphoid cells were shown to be valuable as an in vitro infection model of lymphocytes replicating *C. pneumoniae*. The Molt 4 cell line is widely used as a model for T lymphocytes and shares many features, like cytokine secretion [25] and receptor expression [26], with normal T cells. Another lymphoid cell line P3HR1 [22] is a model for mature B lymphocytes with surface B cell markers CD10 and CD19 [27].

Fluorescence microscopic analysis of infected cells with FITC-conjugated anti-Chlamydia antibody revealed that obvious *Chlamydia* inclusions in cells (Molt 4, P3HR1 and THP-1) could be observed at 72 h after infection. However, the size of the inclusions was relatively small compared with that in epithelial HEp-2 cells, which are widely utilized for propagation of *C. pneumoniae*. In this regard, it has been shown that the formation of chlamydia inclusions is different depending on the type of host cells for *C. pneumoniae* growth, such as HEp-2 cells [17], HL cells [28], U937 cells [8], RAW cells [8], human endothelial cells [8,9], and aortic smooth muscle cells [8]. Therefore, the morphology, including size, of inclusions may reflect the nature of the host cell.

Since determination of *Chlamydia* inclusions in cells by staining does not provide much information concerning quantity of bacteria, the amount of bacterial antigen in cultures was measured by ELISA specific for *Chlamydia* LPS. The relative numbers of bacteria calculated from the concentrations of bacterial antigen recovered from all cell line cultures were significantly increased during the cultivation for 72 h. The P3HR1 cells showed marked bacterial growth during the incubation. This potent support of *C. pneumoniae* growth was more than for the monocytic cell line THP-1. The Molt 4 cells showed a little less potency for bacterial growth. In contrast, bacterial growth measured by detection of antigen in epithelial HEp-2 cells was remarkable and was approximately 10 times more than that in P3HR1 cells. These results indicate that all three immune cells tested may be less potent in regards to supporting *C. pneumoniae* growth in cells compared with that in HEp-2 cells.

The assessment of infective *Chlamydia* progenies in the cultures provides information as to whether a complete development cycle of *C. pneumoniae* is achieved in cells. The results indicate an obvious increase of infective progenies in all cells tested during the cultivation for 72 h. As observed in the measurement of bacterial antigen, HEp-2 cells showed a remarkable production of infective progenies during the culture. In contrast, all Molt 4, P3HR1 and THP-1 cells produced much fewer progenies than HEp-2 cells. The levels of progeny were similar among these immune cells. The numbers of bacteria determined by progeny assay were lower than that by the ELISA assay and this may be due to the counting of only infective EBs in the progeny assay. The ratio of the number of infective progenies (number of EBs)/number of bacteria determined by ELISA (number of EBs, RBs plus others) at 72 h after infection was similar between cells tested, including HEp-2 cells (data not shown). That is, the percentage of EBs per total number of bacteria was not much different between cells tested, indicating there may be no qualitative growth difference. There may be only limited growth in lymphoid and monocytic cells.

Ultrastructural analysis of *C. pneumoniae* infected lymphoid cells as well as monocytic cells by EM revealed that *Chlamydia* organisms were located in large inclusions formed in the cytoplasm of the cells at 72 h after infection. The majority of inclusions observed in both lymphoid and monocytic cells contained typical electron dense small EBs, relatively large RBs and some intermediates. These chlamydial particles were the same as that found in HEp-2 cells. However, the size of inclusions in monocytic and lymphoid cells was smaller than those in HEp-2 cells. In particular, many very small inclusions often found in monocytic and lymphoid cells contained a low electron dense particle with irregular shape. Spontaneous abnormal chlamydial bodies have been found in a continuous-infection model [29]. Moreover, chlamydia aberrant bodies were often observed in cells treated with antimicrobial agents and cytokines [29–32]. Even though the irregular shape of chlamydial bodies observed in this study was not similar to that in these previous reports, it seems likely that such irregular bodies in small inclusions observed in monocytic and lymphoid cells may lack a growth potential. These findings are consistent with the lower number of infective progenies as well as total number of bacteria in monocytic and lymphoid cells than that in HEp-2 cells. The reasons for many small inclusions in immune cells tested are not known, but it may be speculated that monocytic and lymphoid cells may respond to *C. pneumoniae* infection and produce cytokines, which may control in some degree bacterial growth in the cells. In fact, our current study using alveolar macrophages showed that TNFα production was induced by *C. pneumoniae* infection and neutralization of TNFα by antibody caused an enhancement of *Chlamydia* growth in the cells (manuscript in preparation).

There were some differences between B- and T-lymphocyte cell lines regarding support of *C. pneumoniae* growth, but such differences observed in this study may not be sufficient to distinguish differential susceptibility between T- and B-cells to this bacterial infection. Furthermore, since there may be some differences between immortalized cells vs. primary cells, any conclusion from the present results regarding differential susceptibility between T- and B-lymphocytes is not yet unequivocal. Nevertheless, the observations firmly established an in vitro *C. pneumoniae*...
infection model using human lymphoid cell lines. This model may be useful in further study regarding the host–parasite relationship between C. pneumoniae and lymphocytes, which might be essential for understanding the possible involvement of C. pneumoniae infection in the pathogenesis of some chronic inflammatory diseases, including atherosclerosis.

References


