MODELS OF INFECTION

Replication of *Chlamydia trachomatis* and *C. pneumoniae* in the human monocytic cell line U-937

K. NUMAZAKI, K. SUZUKI and S. CHIBA

Department of Pediatrics, School of Medicine, Sapporo Medical University, S. 1 W. 16 Chuo-Ku, Sapporo 060, Japan

Summary. To elucidate whether *Chlamydia trachomatis* and *C. pneumoniae* infections occur to a significant extent in monocytes-macrophages, the human monocytic cell line, U-937, was infected with *C. trachomatis* L or *C. pneumoniae* TW-183. Chlamydial DNA and genus-specific antigens of the lipopolysaccharides (LPS) in epitopes of the chlamydial cell wall were detected from *C. trachomatis* L-inoculated monocytes over a period of 150 days after inoculation and from the *C. pneumoniae* TW-183-inoculated cells during a period of 14 days. *C. trachomatis*-infected U-937 cells expressed significantly lower levels of CD4+, CD45RA+, CD11b+ and CD33+ cells, determined by flow cytometry, than control uninoculated cells on the seventh day after inoculation and they expressed a slightly increased level of CD4+ cells and lower levels of CD45RA+ and CD11b+ cells on the 14th day after inoculation. *C. pneumoniae*-infected U-937 cells expressed significantly lower levels of CD4+, CD45RA+, CD11b+ and CD33+ cells than controls on the seventh day after inoculation and an increased level of CD4+ and a lower level of CD45RA+ cells on the 14th day after inoculation. Unlike infection with *C. trachomatis* L strain, chronic persistent infection with *C. pneumoniae* appears not to occur in monocytes-macrophages.

Introduction

Chlamydiae are obligate intracellular bacteria that survive and grow in both epithelial and phagocytic cells. *Chlamydia trachomatis* serovars associated with endemic trachoma (A, B, Ba or C-complex) preferentially infect mucosal epithelial cells. In contrast, the lymphogranuloma venereum (LGV) serovars primarily infect lymph nodes, causing more systemic infections. The target cells for LGV strains are presumed to be monocyte-macrophages. *C. pneumoniae* is a common aetiological agent in respiratory infections, including pneumonia. Although elevated serum antibodies to *C. pneumoniae* and the presence of circulating chlamydia-specific immune complexes have been found in several chronic *C. pneumoniae* infections, the role of mononuclear phagocytes in the pathogenesis of *C. pneumoniae* infections has yet to be clarified. Despite the various pathogenic effects of chlamydiae, there is only limited direct evidence that chlamydial infections occur to a significant extent in monocytes and macrophages. It is likely that mononuclear phagocytes also play an important role in the persistence of chronic chlamydial infections and act as reservoirs and vehicles for chlamydial dissemination in the infected hosts. The present study was designed to determine the effects of *C. trachomatis* and *C. pneumoniae* on monocytes-macrophages and the mechanisms of persistence in these cells. The use of the human monocytic cell line of U-937 cells was necessary because of the difficulty of growing primary human monocytic cells in sufficient quantity for analysis. The activation or differentiation of mononuclear phagocytes may be associated with chlamydial infections. The changes in U-937 cells associated with chlamydial infections were analysed with antibodies directed against different surface molecules (CD markers).

Materials and methods

Cell line and propagation of *C. trachomatis* and *C. pneumoniae*

The U-937 monocytic cell line, established from malignant cells originally gained from a patient with diffuse histiocytic lymphoma, was obtained from the American Type Culture Collection (ATCC CRL 1593). We confirmed the absence of contamination with mycoplasmas, human cytomegalovirus (HCMV), human immunodeficiency virus type-1 (HIV-1), and
human T lymphotropic virus type-1 (HTLV-1). These cells were maintained as a cell suspension (2 x 10^6–2 x 10^9) in RPMI 1640 medium (Gibco Products) supplemented with heat-inactivated fetal calf serum 10%, 2 mM l-glutamine, penicillin 250 U/ml and streptomycin 250 μg/ml. C. trachomatis strain Lp/434/Bu was grown in cycloheximide-treated HeLa 229 cell cultures at 35°C for 48 h in a CO₂-incubator according to conventional techniques. C. pneumoniae TWAR strain (TW-183 obtained from the Washington Research Foundation) was grown in cycloheximide-treated HEp-2 cells at 35°C for 48 h in a CO₂-incubator. Infected cells were harvested and pooled. The resuspended pellet was layered over a 38-60% Renografin linear gradient and centrifuged. Purified organisms were resuspended in a sucrose phosphate-glutamate (SPG) medium and then frozen at -70°C. The infectivity titres were determined by titration in HeLa 229 cells or HEp-2 cells and expressed as multiplicity of infection (MOI). For the purpose of infection, a suspension containing 5 x 10⁶ U-937 cells was pre-treated with polybrene 2 μg/ml for 20 min in RPMI medium. The cells were washed by centrifugation and resuspended in 0.5 ml of suspensions of C. trachomatis Lp or C. pneumoniae TW-183 at 1:0 MOI for 3 h. In each case, the cells were centrifuged again to remove unbound chlamydiae, rinsed three times with fresh medium by centrifugation, and resuspended in fresh medium (10⁶ cells/ml). At intervals of 72 h, the cells were pelleted and resuspended in fresh medium. Cells and medium were also harvested at the same intervals and were centrifuged. The resulting pellet and supernate were stored separately.

Detection of C. trachomatis and C. pneumoniae DNA by the polymerase chain reaction

Polymerase chain reaction (PCR) and endonuclease analysis were used to detect and distinguish genomes of C. trachomatis. From pellets of harvested U-937 cells, DNA was extracted in lysis buffer (10 mM Tris-HCl, Tween 20 0.5% and 1 mM EDTA) containing proteinase K 0.1 mg/ml, followed by phenol-chloroform extraction. The primers used in the PCR study were based on a conserved region of the DNA sequence, omp4 (or omp1) of major outer-membrane protein (MOMP) of C. trachomatis. The following oligonucleotides were designed as primers for PCR: CGS-2 (5’AGGGATCCATGAAAAAAACTCTTGA-AATCGG3’), CAT-3 (5’AATGGTACCGAAG-CGGAATTGTGCATTTACGTGAGC3’). Synthetic oligonucleotides used as primers were prepared with a DNA synthesizer Model 7500 (Milligan Corp., Redford, MA, USA) and purified by high-performance liquid chromatography. The c (complementary) DNA, primers and reagents were heated at 94°C for 5 min before addition of 2 Units of Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA) to give a final volume of 100 μl. The protocol of cycles was as follows: cycles 1-40 were performed by a DNA Thermal Cycler (Perkin-Elmer Cetus), at 94°C for 1 min (denaturation), 50°C for 2 min (annealing), and 72°C for 3 min (extension). The PCR reaction mixture was analysed by electrophoresis on agarose gel (Seakem agarose 1.2%, 40 mM Tris-acetate and 1 mM EDTA) containing ethidium bromide. Amplified DNA was visualised by ultraviolet fluorescence and recorded by Polaroid photography. Genotyping was performed by HinfI, HindIII and HhaI restriction endonuclease analysis of amplified ompA, according to the procedure described previously. Samples of amplified DNA were digested with a mixture of restriction endonucleases and extracted once with phenol-chloroform. The resulting fragments were examined by agarose gel electrophoresis. Two different sets of C. pneumoniae-specific primers that amplify distinct target sequences were also used. The set, HL-1 (5’GTTGTTCATGAAAGGCGCTACT3’) and HR-1 (5’TGCATAACCTACGGTGTCT3’), results in amplification of a 437-bp C. pneumoniae-specific DNA sequence and this was performed for 40 cycles. The protocol of cycles was as follows: 94°C for 1 min (denaturation), 50°C for 2 min (annealing), and 72°C for 3 min (extension). The set of primers was initially chosen by DNA sequence analysis of a cloned C. pneumoniae-specific 474-bp PstI restriction fragment. These primers do not amplify C. trachomatis or C. psittaci DNA. The parameters for amplification used in the assay were those described by Campbell et al. The specificity and sensitivity of primers used in this study were established in this laboratory. Amplification products were visualised by agarose gel electrophoresis. The results were presumed to be positive if the product was of the expected molecular mass. Confirmation of the identity of the 437-bp product was by Southern hybridisation. For Southern blot hybridisation assay, DNA was transferred to a nylon membrane filter, incubated with C. pneumoniae DNA labelled with digoxigenin, and detected with the Nucleic Acid Detection Kit (Boehringer Mannheim Yamanouchi Co. Ltd, Japan).

Isolation of C. trachomatis or C. pneumoniae and detection of chlamydial antigens

DEAE-dextran- and cycloheximide-treated HeLa 229 cells were used to isolate C. trachomatis from harvested culture media as reported previously. Cycloheximide-treated HEp-2 cells were used to isolate C. pneumoniae. A commercially available EIA kit (IDEIA Chlamydia, Dako Diagnostics Co.) was also used to detect genus-specific chlamydial cell-wall lipopolysaccharide (LPS) antigens in the media. Testing and interpretation of the results were performed according to the manufacturer’s instructions.

Flow cytometric analysis

The expression of surface antigens on the U-937 cells was assessed by flow cytometry (FACStar;
Table. Expression of surface antigens on C. trachomatis- or C. pneumoniae-inoculated U-937 cells by flow cytometry

<table>
<thead>
<tr>
<th>MAb</th>
<th>Uninoculated (control)</th>
<th>C. trachomatis-inoculated</th>
<th>C. pneumoniae-inoculated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>7 days</td>
<td>14 days</td>
</tr>
<tr>
<td>CD3</td>
<td>0.2</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>CD4</td>
<td>41.0</td>
<td>23.5</td>
<td>55.0</td>
</tr>
<tr>
<td>CD8</td>
<td>0.5</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>CD45RA</td>
<td>71.2</td>
<td>45.0</td>
<td>63.6</td>
</tr>
<tr>
<td>CD11b</td>
<td>28.2</td>
<td>1.7</td>
<td>20.7</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>0.8</td>
<td>1.0</td>
<td>0.4</td>
</tr>
<tr>
<td>CD33</td>
<td>56.0</td>
<td>49.3</td>
<td>51.5</td>
</tr>
</tbody>
</table>

Results

The expression of surface antigens in the U-937 cells, measured by flow cytometry, before and after infection with C. trachomatis or C. pneumoniae, is shown in the table. The initially high levels of CD4⁺, CD45RA⁺, CD11b(Mac1)⁺ and CD33(Leu-M9)⁺ expression in uninoculated U-937 cells showed some changes after chlamydial infection. C. trachomatis-infected U-937 cells expressed significantly lower levels of CD4⁺, CD45RA⁺, CD11b⁺ and CD33⁺ than did the uninfected-control cells on the seventh day after inoculation and expressed slightly increased levels of CD4⁺ cells and lower levels of CD45RA⁺ and CD11b⁻ cells on the 14th day after inoculation. C. pneumoniae-infected U-937 cells expressed significantly lower levels of CD4⁺, CD45RA⁺, CD11b⁺ and CD33⁺ cells (particularly CD4⁺ cells) than did the control cells on the seventh day after inoculation and an increased level of CD4⁺ and a lower level of CD45RA⁺ cells on the 14th day after inoculation. CD3⁺, CD8⁺, HLA-DR⁺ cells showed little change, except for HLA-DR⁺ cells at 7 days but not at 14 days after inoculation with C. pneumoniae. At 30 days after infection (results not
shown), there was little difference in detectable values for any of the surface markers between inoculated and uninoculated U-937 cells.

The figure shows representative results from HindIII, HindIII and HhaI digestion products of the PCR-amplified chlamydial DNA of ompA from U-937 cells infected with C. trachomatis L2 on the 90th day after inoculation. The availability of complete sequences for the ompA gene of serovar L2 allowed the construction of endonuclease cleavage site maps that confirmed the fragment-size patterns observed by electrophoresis with reference serovars (unpublished results). The C. trachomatis L2 serovar from inoculated U-937 cells was identified on the basis of digestion with restriction endonucleases. Chlamydial DNA which codes ompA of MOMP was detected from C. trachomatis L2-inoculated U-937 cells over a period of 150 days. Chlamydial genus-specific LPS antigens were also detected by the IDEIA-Chlamydia method in the medium from C. trachomatis L2-inoculated U-937 cell cultures over a period of 150 days. During the same period, C. trachomatis had been isolated from such medium by tissue culture. However, chlamydial antigens were detected from the medium from C. pneumoniae-inoculated U-937 cells during only the first 14 days after inoculation. C. pneumoniae was also isolated from the medium by tissue culture until 14 days but not thereafter, in contrast to the 150 days for C. trachomatis.

The amplification of chlamydial DNA from U-937 cells infected with C. pneumoniae TW-183 was done by PCR assay (results not shown). The resulting 437-bp amplification products of C. pneumoniae DNA were detected in harvested U-937 cells over a period of 14 days.

Discussion

Pneumonia due to C. trachomatis is a disease limited for the most part to infants under 6 months of age. 1 C. pneumoniae usually causes pneumonia and other respiratory infections in children, adolescents and adults. 10 Chlamydial infections induce inflammatory changes that might modulate secretion of cytokines. 11 Alveolar macrophages are thought to be the major immune response-regulating cells of the lung. The limitation of occurrence of C. trachomatis pneumonia to early infancy and of C. pneumoniae pneumonia to children > 2 years old, adolescents and adults might be due in part to the possible maturational or functional differences between alveolar and peripheral blood macrophages of infants and adults. 12 Theoretically, chlamydiae might enter mononuclear phagocytes in three ways: non-specific phagocytosis, specific receptor-mediated binding of chlamydiae to the cell membrane and subsequent fusion, or receptor-mediated endocytosis of antibodies complexed with chlamydiae. C. trachomatis and C. pneumoniae can be utilised as intracellular microbial targets to charac-
MOMP gene sequences for detection of *C. trachomatis* and that of the species-specific PCR assay for detection of *C. pneumoniae*. Although the sensitivity of species-specific PCR assay was better than that of digestion patterns of MOMP gene sequences (results not shown), this did not influence the results and conclusions obtained in this study.

U-937 cells share several functional characteristics with monoblasts and immature monocytes and they can be induced to differentiate into phagocytic cells. From the results of the flow cytometry study, changes shown were seen mainly in surface antigen markers with *C. trachomatis*- or *C. pneumoniae*-infected U-937 monocytes. Changed proportions of CD4<sup>+</sup>, CD45RA<sup>+</sup>, CD11b<sup>+</sup> and CD3<sup>+</sup> cells in U-937 cells were associated with chlamydial infections in this study. Some degree of differentiation may be necessary for permissive infection of U-937 cells with chlamydiae.

Finally, it is likely that specific cellular interactions as well as secretion of cytokines are important for the pathogenesis of chlamydial infections. Further studies need to include clarification of the roles of monocytes-macrophages and other cells of the immune system in persistent chlamydial infections.

We thank Michihide Mikami, R.T., Ogyu Laboratory, Sapporo, Japan, for technical assistance.

References