MODELS OF INFECTION

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

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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* L₂ 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* L₂-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* L₂ 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.¹ *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.² 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.³ 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 (2×10^5 – 2×10^6) 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 L₂/434/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.⁴ 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 5×10^5 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* L₂ 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 (10^6 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, *ompA* (or *omp1*) of major outer-membrane protein (MOMP)^{5,6} of *C. trachomatis*. The following oligonucleotides were designed as primers for PCR: CGS-2 (5'AGGGATCCATGAAAACTCTTGAATCGG3'), CAT-3 (5'AATGGTACCGAAGCGGAATTGTGCATTACGTGAGC3'). 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 *Hinf*I, *Hind*III and *Hha*I restriction endonuclease analysis of amplified *ompA*, according to the procedure described previously.⁶ 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'GTTGTTTCATGAAGGCCTACT3') and HR-1 (5'TGCATAACCTACGGTGTGTT3'), results in amplification of a 437-bp *C. pneumoniae*-specific DNA sequence and this was performed for 40 cycles.⁷ 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 *Pst*I 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.*⁷ 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.⁸ 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.⁹ 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

MAb	Uninoculated (control)	Percentage of MAb-reactive cells			
		<i>C. trachomatis</i> - inoculated		<i>C. pneumoniae</i> - inoculated	
		7 days	14 days	7 days	14 days
CD3	0.2	0.1	0.1	0.5	0.1
CD4	41.0	23.5	55.0	8.7	53.6
CD8	0.5	0.1	0.1	0.5	0.1
CD45RA	71.2	45.0	63.6	13.3	59.8
CD11b	28.2	1.7	20.7	11.9	24.1
HLA-DR	0.8	1.0	0.4	4.5	1.6
CD33	56.0	49.3	51.5	46.7	59.9

Figure. Digestion patterns of the PCR-amplified *ompA* gene of MOMP—*Hinf*I, *Hind*III, and *Hha*I cleavage patterns of *ompA* from *C. trachomatis*-infected U-937 cells harvested on the 90th day after inoculation (lanes 1–3) and from *C. trachomatis* L₂ reference cultures (4–6); M, mol. wt markers. Fragment sizes (in bp) of several fragments are shown on the right.

Becton-Dickinson, Mountain View, CA, USA) as described previously.³ The results were recorded as the percentage of cells positive for each of the monoclonal antibodies (MAbs). A logarithmic scale of fluorescence intensity was used, and cells whose intensity exceeded the upper limit values for the negative control were considered to be positive. The cells were stained with the following MAbs: CD3, CD4, CD8, CD45RA, CD11b(Mac 1), CD33(Leu-M9), HLA-DR and Fc immunoglobulin (Becton Dickinson).

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 *Hinf*I, *Hind*III and *Hha*I 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-

terise the antimicrobial mechanisms of human monocytes and activated macrophages. Some investigators have speculated that interferon (IFN) also may play a role in producing or perpetuating persistent chlamydial infection by maintaining the organisms as immature forms within intracytoplasmic inclusions.

The infection and persistence of the LGV biovar of *C. trachomatis* in monocytes-macrophages may have critical roles in the pathogenesis and immunological reactions in systemic infections. It has been reported that macrophages derived from human peripheral blood mononuclear cells (PBMC) were not able to kill *C. trachomatis* L₂ and other LGV strains but were capable of killing trachoma serovars.^{13,14} From the results of the present study, it can be speculated that mononuclear phagocytes can also kill *C. pneumoniae* or have some role in restricting the persistence of *C. pneumoniae* infection to a short period. Although it is unclear whether or not *C. pneumoniae* causes persistent infection in man, unlike *C. trachomatis* L₂ strain, chronic persistent infection of *C. pneumoniae* in monocytes-macrophages was not observed experimentally in this study.

As cultures of lung biopsies from infants with *C. trachomatis* pneumonia have frequently failed to yield the organism, immunological reactions of the host to these agents appear to be more important than the direct effects of *C. trachomatis* or *C. pneumoniae* in the pathogenesis of chlamydial pneumonia.^{1,11} The 60-kDa heat-shock protein (hsp) may also have some role in inducing the non-specific hypergammaglobulinaemia, delayed-type hypersensitivity reaction, and auto-immune reaction associated with chlamydial infections.¹⁵ It was also postulated that activation of specific suppressor/cytotoxic CD8⁺ cells might play a part in the persistence of chlamydial infections.^{16,17}

The epitopes that distinguish serovars of *C. trachomatis* are located on MOMP. A PCR assay was used to amplify *ompA*, a large part of the MOMP gene, including four variable domains whose sequences have been determined for 15 serovars of *C. trachomatis*. The sequences for all 15 serovars allowed construction of restriction endonuclease cleavage-site maps that confirm the fragment size patterns observed by electrophoresis.⁶ This method for typing *C. trachomatis* serovars on the basis of restriction endonuclease digestion patterns of MOMP gene sequences also allows quick and objective identification of the organisms. As we were able to obtain only one TWAR strain (TW-183) of *C. pneumoniae*, the application of this method for identification and typing of *C. pneumoniae* serovars was not examined in this study. Theoretically, application of a similar approach to *C. pneumoniae* or *C. psittaci* serovars will probably prove fruitful. However, the choice of a gene with demonstrated heterogeneity, such as MOMP of *C. trachomatis*, will be necessary. The different PCR systems may have different sensitivities and influence the detection rates. We have determined the sensitivity of the restriction endonuclease digestion patterns of

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.¹⁸ From the results of the flow cytometry study, changes were seen mainly in surface antigen markers with *C. trachomatis*- or *C. pneumoniae*-infected U-937 mono-

cytes. Changed proportions of CD4⁺, CD45RA⁺, CD11b⁺ and CD33⁺ 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.

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