In Vitro Susceptibility of Human Vascular Wall Cells to Infection with *Chlamydia pneumoniae*

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Chlamydia pneumoniae is a common respiratory pathogen. Recent studies have demonstrated the presence of *C. pneumoniae* in coronary and aortic atherosclerotic lesions. To study the role of *C. pneumoniae* in atherosclerosis, we investigated the susceptibilities of three different cells of the human vascular wall to infection with *C. pneumoniae* AR-39. These cell types were endothelial cells, smooth muscle cells, and macrophages derived from peripheral blood monocytes. Infection was assessed by using a direct fluorescent antibody to assess inclusion counts. Duplicate cell samples were harvested 3 days postinfection and were passed in HL cells, a susceptible human epithelial cell line, to determine if infectious organisms were produced. Endothelial cells, smooth muscle cells, and macrophages were capable of supporting *C. pneumoniae* growth in vitro. These results showed that three different cell types known to be important in atherogenesis are susceptible to infection with *C. pneumoniae*.

Chlamydia pneumoniae is a common respiratory pathogen and is the cause of 10% of community-acquired pneumonia and 5% of bronchitis (3). Epidemiological data have shown an association between coronary artery disease and the presence of serum antibodies against *C. pneumoniae* (18, 20). These epidemiological studies have been supported by the detection of *C. pneumoniae* organisms in atherosclerotic lesions by electron microscopy, immunocytochemistry, and PCR (11).

Viruses in the human herpesvirus family have also been associated with atherosclerosis, and their ability to alter smooth muscle cell gene expression has been demonstrated (4). No single etiologic event has been assigned for atherogenesis. Rather, multiple factors are expected to contribute to lesion formation, and pathogen-induced deviation from normal gene expression may be one of these (16). The role of *C. pneumoniae* in atheroma formation has not been determined. To substantiate previous findings in situ, we assessed the in vitro susceptibilities of human arterial endothelial cells, smooth muscle cells, and monocyte-derived macrophages to infection with *C. pneumoniae*.

MATERIALS AND METHODS

Sources of cells and cultures. Transformed human arterial endothelial cells (HMEC-1) were obtained from E. W. Ades at the Centers for Disease Control and Prevention (1). Cells were cultured in endothelial cell basal medium (Clenetics, San Diego, Calif.) supplemented with 10% fetal calf serum, 10 ng of epidermal growth factor per ml, 1 µg of hydrocortisone per ml, 100 µg each of streptomycin and vancomycin per ml, and 25 U of mycostatin per ml. Cells were grown in 75-cm² flasks (Costar, Cambridge, Mass.) at 37°C with 5% CO₂ and saturated humidity.

Restenotic plaque smooth muscle cells were derived from human femoral atherectomy tissue. Smooth muscle cells derived by atherectomy of a primary stenotic aortic lesion were obtained from S. M. Schwartz, Department of Pathology, University of Washington. Immunostaining with antiactin antibody SM-1 (Sigma, St. Louis, Mo.) ascertained that the cells were smooth muscle cells. Primary cultures were maintained in smooth muscle cell growth medium (Clo-

netics) in 75-cm² flasks in a humidified incubator with 5% CO₂ at 37°C. They were at the 4th to 10th passages when they were used for inoculation.

Peripheral blood monocytes were separated from heparinized blood from healthy human volunteers by centrifugation through Histopaque (Sigma). Following separation the cells were grown on 12-mm-diameter glass coverslips in 24-well culture plates (Falcon Labware, Becton Dickinson, Lincoln Park, N.J.) in RPMI 1640 medium (GIBCO, Grand Island, N.Y.) supplemented with 15% fetal bovine serum, 0.4% glutamine, and 100 µg each of streptomycin and vancomycin per ml. The cells were kept in a humidified incubator with 5% CO₂ at 37°C.

HL cells, a continuous human epithelial cell line, were grown in 32-oz. (960-ml) glass prescription bottles in Eagle's minimal essential medium supplemented with 10% fetal calf serum and 100 µg each of streptomycin and vancomycin per ml (MEM) at 37°C. HL cells were used as a standard of cell susceptibility since it is a susceptible cell line that is conventionally used to grow *C. pneumoniae* (10).

Chlamydial organisms. *C. pneumoniae* organisms (strain AR-39) were propagated in cell cultures and were purified by Hypaque gradient centrifugation. Purified organisms were resuspended in chlamydial transport medium, sucrose-phosphate-glutamic acid (SPG), and were stored at -70°C in small aliquots until use. This strain is the first respiratory isolate for *C. pneumoniae* obtained from a university student in Seattle with pharyngitis in 1983 (3). The sequence of the PCR-amplified rRNA gene product from coronary atheromatous plaques was identical to that of AR-39 (11).

Inoculation of cell culture. HL cells and endothelial cells were trypsinized from stock cultures and were plated to achieve a cell monolayer in 24 or 48 h in glass shell vials and 24-well culture plates, respectively. Monocytes were plated at 0.5×10^6 cells per well, and smooth muscle cells were seeded at various cell densities and were allowed to grow to confluency before infection. All cells were grown on 12-mm-diameter glass coverslips for ease of staining and to retain a greater percentage of viable, attached cells in the case of macrophages. Medium was removed from the chambers before inoculation. Macrophages and smooth muscle cells were rinsed with Hanks' balanced salt solution (HBSS) prior to infection. Inocula were diluted with HBSS or SPG and were either centrifuged onto cell layers for 1 h at 700 to 800 $\times$ g at 25°C or spread over the cell layers on a rocker platform for 2 h at 37°C. An inoculum of 0.2 ml was used for arterial wall cells in wells, and 0.1 ml was used for HL cells in vials. Several dilutions of the organism were used for each cell type. The following inoculum size ranges were used: 2×10^3 to 3×10^4 inclusion-forming units (IFUs) for endothelial cells and smooth muscle cells, 2×10^4 to 3×10^5 IFUs for macrophages, and 3×10^3 to 2×10^4 IFUs for HL cells. After absorption, the inocula were removed and replaced with the appropriate medium and the cells were incubated under the same conditions used for cell growth. Infected HL cells were cultured in Eagle's MEM supplemented with 0.6 µg of cycloheximide, an inhibitor of eukaryotic protein synthesis, per ml, to enhance the growth of chlamydia. After determining that 0.6 µg of cycloheximide per ml enhanced the growth of *C. pneumoniae* in endothelial cells, cycloheximide was also incorporated into the media for culturing infected endothelial cells.

Quantification of infection. Three days after inoculation the cells were fixed for 10 min in absolute methanol or acetone, air dried, and stained with fluorescein isothiocyanate-conjugated monoclonal antibody. The murine monoclonal antibodies used were CF-2, a *Chlamydia* genus-specific antibody against lipopolysaccharide (LPS), and TT-401, a *C. pneumoniae* species-specific antibody (the target antigen is not yet characterized because of its sensitivity to physical

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FIG. 1. Morphology of *C. pneumoniae* inclusions in artery wall cells. Cells were stained with fluorescein-conjugated monoclonal antibody against chlamydia 3 days after inoculation. (a) Endothelial cells; (b) smooth muscle cells; (c) macrophages derived from peripheral blood monocytes.

and chemical treatment [15]). When infectivity assays were done with confluent cell monolayers such as endothelial, smooth muscle, and HL cells, inclusions were counted in 30 random fields at $\times 400$ magnification. Infectivity in macrophages was determined as the percentage of cells containing inclusions because confluent monolayers of macrophages were not obtained for infection. The infectivity titers in terms of IFUs per milliliter were adjusted for dilution factor and inoculum size and were used for comparison. Duplicate or triplicate coverslips were stained and counted for each dilution in each experiment.

Assessment of productive infection. To determine if *C. pneumoniae* is able to complete its growth cycle and produce infectious progeny, duplicate samples were stained as described above and were harvested on day 3 or 4 postinfection. Two wells of infected cells to be harvested were scraped off the coverslips and were suspended in SPG. These harvests were then diluted as necessary in SPG and were used to infect new HL cell monolayers.

Calculation of burst size. To obtain the burst size (number of infectious units in an inclusion), duplicate samples of first-passage cell cultures were harvested and stained. Harvests were diluted, if necessary, and were used to infect HL cell monolayers (second passage). The number of inclusions harvested from cells infected in the first passage was determined by counting the number of inclusions in the stained duplicate samples. The same method was used to calculate the number of inclusions resulting from the second passage. The number of inclusions observed in the second passage was divided by the number of inclusions in the amount of harvest used for the second-passage infection. This calculation gave the number of IFUs contained in each inclusion obtained in the first passage (burst size), which expresses the number of infectious particles formed during the first cycle of growth in each cell type. These are the bacteria available to potentially infect other cells.

RESULTS

Appearance of *C. pneumoniae* inclusions. The morphology of *C. pneumoniae* inclusions in endothelial cells was essentially the same as that in HL cells. They were usually single and round and filled most of the cytoplasmic space (Fig. 1a). The

inclusions were immunolabeled with uniform intensity. Some inclusions exceeded the size of the nucleus before bursting and destroying the cell. Smooth muscle cells also supported the growth of *C. pneumoniae*. Inclusions were round and stained uniformly with fluorescent antibodies (Fig. 1b). However, unlike inclusions in endothelial cells, inclusions in smooth muscle cells varied greatly in size. Cells containing more than one inclusion were frequently observed. No inclusions were detected in uninfected smooth muscle cells stained by the same procedure. *C. pneumoniae* inclusions in macrophages were smaller and less compact than those normally observed in HL cells, endothelial cells, and smooth muscle cells (Fig. 1c). The immunolabel varied in intensity over the area of the inclusion, creating a mottled appearance.

Susceptibilities of arterial cells. Endothelial cells, smooth muscle cells, and macrophages were all susceptible to infection with strain AR-39 (Tables 1 and 2). However, these artery cells were much less susceptible to AR-39 than HL cells. Macrophages were inoculated with AR-39 on 3, 4, 7, 8, or 14 days after culture. Monocytes from three donors showed different susceptibilities (Table 2).

Two commonly used methods for the enhancement of chlamydial infectivity in cell cultures, i.e., centrifugation of the inoculum onto cell monolayers and inhibition of host cell metabolism with cycloheximide, were evaluated. Endothelial cell culture inoculated with 5.7×10^3 IFUs produced an average of 572 inclusions per coverslip without any treatment. Cycloheximide treatment increased the average inclusion count to 2,817 per coverslip (fivefold). No results were obtained with smooth muscle cells treated with cycloheximide because of toxicity. Cell layers infected by centrifugation yielded an average of 12,054 inclusions per coverslip in endothelial cells when inocula containing 4.5×10^4 IFUs were used and 9,102 inclusions per coverslip in smooth muscle cells when an inoculum size of

TABLE 1. Susceptibilities of human arterial endothelial and smooth muscle cells to *C. pneumoniae* in comparison with susceptibilities of HL cells

Cell type	Susceptibility index ^a	Range	Burst size ^b
HL	1.0		73 ($n = 5$)
Endothelial	0.32 ($n = 8$)	0.07–0.55	25 ($n = 6$)
Smooth muscle ^c			
Primary	0.62 ($n = 2$)	0.57–0.67	10 ($n = 1$)
Restenotic	0.36 ($n = 2$)	0.06–0.66	11 ($n = 3$)

^a HL cells were assigned a susceptibility index of 1.0.

^b Number of IFUs per inclusion.

^c Smooth muscle cells were derived from tissue resected from primary and restenotic lesions of the aorta and femoral artery, respectively.

TABLE 2. Infection of macrophages derived from human peripheral blood monocytes with *C. pneumoniae*

Donor	Day of culture before inoculation	% Cells infected	Burst size ^a
A	7, 14	26.5 $\pm$ 0.9 ($n=3$)	0.35 ($n = 2$)
B	8	2.7 ($n = 1$)	0
C	3, 4, 7, 8	6.2 $\pm$ 4.4 ($n=5$)	0.07 ($n = 1$) ^b

^a Number of IFUs per inclusion.

^b Only day 7 macrophages resulted in productive infections.

2.4×10^5 IFUs was used. Thus, centrifugation enhanced infectivity 20-fold in endothelial cells and 3-fold in smooth muscle cells. However, these treatments are not necessary to infect these cells, as shown by an average of 615 inclusions per coverslip in endothelial cells and 2,952 inclusions per coverslip in smooth muscle cells without any treatment with inocula containing 4.5×10^4 IFUs and 2.4×10^5 IFUs for endothelial cells and smooth muscle cells, respectively. These enhancing factors were not examined in macrophages.

DISCUSSION

The in vitro experiments described here show that *C. pneumoniae* can infect, survive, and multiply in three cellular components of the human vascular wall, i.e., endothelial cells, smooth muscle cells, and monocyte-derived macrophages. Other investigators have reported that endothelial cells derived from human umbilical vein (2, 7, 19), aorta (2, 19), and pulmonary artery (19) and that an immortalized endothelial cell line, EA.hy926, which is derived from HUVEC (7), and a nonadherent human macrophage line, U937 (2), are also susceptible to infection with several strains of *C. pneumoniae* in vitro.

Our previous studies showed that human peripheral blood monocytes cultured for 7 days or less were efficiently microbicidal against the human biovars of *Chlamydia trachomatis* (21). Although the lymphogranuloma venereum biovar survives in mononuclear phagocytes infected after 8 days or more in culture, the trachoma biovar continued to be killed by such cells. The present study shows that *C. pneumoniae* has the same resistance against human mononuclear phagocytes as the lymphogranuloma venereum biovar of *C. trachomatis*.

These data support the immunocytochemical findings of Kuo et al. (9) which showed the presence of *C. pneumoniae* in foam cells of macrophage and smooth muscle cell origin in atherosclerotic lesions of the aorta as well as those studies in which chlamydial DNA was detected in plaque specimens (11). We have demonstrated by in vitro techniques that cultures whose histological characteristics showed them to be smooth muscle cells or macrophages can be infected with and support the growth of *C. pneumoniae*. These methods also allowed evaluation of endothelial cells, which are more difficult to assess in vivo because they make up a very small fraction of the cells in the artery wall. Our experiments were designed to detect as efficiently as possible the entire number of mature infectious particles that could be produced in one cycle of infection of healthy cells. Our harvests were passed into the most susceptible cell line known in order to allow the maximum number of infectious progeny to form inclusions. The burst size reported is the closest approximation to the number of infectious particles produced by each inclusion in a particular cell type.

The in vivo susceptibilities of arterial wall cells to *C. pneumoniae* cannot be assessed from these experiments. However, the present in vitro study showed that arterial wall cells were less susceptible than the commonly used HL epithelial cell line in this system. It is possible that the isolate used for testing in the present study had adapted to HL cells by continuous passage in that cell line. However, adaptation alone may not explain the magnitude of the difference in susceptibility between arterial wall cells and HL cells. The greater susceptibilities of HL cells to *C. pneumoniae* is consistent with *C. pneumoniae* being primarily a respiratory pathogen whose initial target tissue is the epithelium of the respiratory tract. The comparative susceptibilities of arterial wall cells and nonrespiratory epithelial cells to *C. pneumoniae* were not evaluated in

the present study. The low-grade infectivity demonstrated in our experiments is also compatible with the histological picture of subacute or chronic inflammatory reactions observed in atherosclerotic and restenotic lesions (13) and can be compared with chronic or persistent infection of the conjunctiva (trachoma) and the fallopian tube (salpingitis) with *C. trachomatis* (8).

The outer membrane of chlamydia contains LPS. LPS can combine with immunoglobulins to form immune complexes. Circulating immune complexes containing chlamydial LPS and *C. pneumoniae* proteins have been associated with coronary artery disease (12). Both LPS and immune complexes cause endothelial cell injury (5, 6). Many changes could result from the interaction between artery wall cells and *C. pneumoniae*. Increased surface expression of adhesion molecules such as vascular cell adhesion molecule by *C. pneumoniae*-infected endothelial cells may facilitate the recruitment of inflammatory cells to that locus and may be an early event in lesion formation. LPS is also a potent immune stimulator. Prescott et al. (14) demonstrated the migration of leukocytes to and the development of atherosclerotic lesions specifically in regions that had been directly exposed to LPS, even without perturbation of the endothelium and normal lipid levels. Infection of human monocytes with *C. trachomatis* has been shown to induce the production of interleukin 1 (17). Interleukin 1 elaborated by infected macrophages could cause the proliferation of nearby smooth muscle cells, which are also capable of secreting growth factors, thus perpetuating a paracrine system of growth control and inflammation (16).

In summary, endothelial cells, smooth muscle cells, and macrophages play important roles in the formation of arterial lesions and vessel wall inflammation. *C. pneumoniae* is capable of infecting all three cell types. Although the pathogenic role of *C. pneumoniae* in atherosclerosis is yet to be determined, experiments can now be carried out in vitro to determine, at a molecular level, the effect of *C. pneumoniae* on the expression of key molecules that are known to contribute to atherogenesis.

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