**Chlamydia pneumoniae** Infection Induces Inflammatory Changes in the Aortas of Rabbits

KIRSI LAITINEN,1,2 AINO LAURILA,2 LIISA PYHÄLÄ,1 MALIA LEINONEN,2 AND PEKKA SAIKKU2

National Public Health Institute, Helsinki1 and Oulu,2 Finland

Received 22 April 1997/Returned for modification 29 May 1997/Accepted 1 July 1997

**Chlamydia pneumoniae**, a common human respiratory pathogen, has been associated with atherosclerosis in several seroepidemiological studies. Moreover, its presence in lesions of vessels walls has been demonstrated by culture, immunohistochemistry, PCR, and electron microscopy. In this study, we infected intranasally with *C. pneumoniae* New Zealand White rabbits which had been fed a normal diet. Reinfecion was given 3 weeks later. Six of the nine reinfected animals showed inflammatory changes consisting of intimal thickening or fibroid plaques resembling atherosclerosis in 2 to 4 weeks after reinfection. One rabbit had calcified lesions. Immunohistochemistry for *C. pneumoniae* was strongly positive in the three older affected animals. No lesions were seen in the controls. The results suggest that *C. pneumoniae* infection is capable of inducing inflammatory atherosclerosis-like changes in the aortas of infected rabbits.

---

**Materials and Methods**

*C. pneumoniae* strain and inoculation of animals. *C. pneumoniae* Kajaani 7 (a mycoplasma-free Finnish epidemic strain) was used to inoculate the animals (5). The organisms propagated in an HL cell line (2) were purified by Renographin gradient ultracentrifugation. The inoculum preparations were stored in aliquots in sucrose-phosphate-glutamate (SPG) buffer at −70°C until used.

The animals used were *Bordetella bronchiseptica* and Pasteurella spp.-free male NZW (HsdPoc:NZW) rabbits (5 months old) purchased from Harlan BV (AD Zeist, The Netherlands). The rabbits were fed an Altromin 2113 breeding diet (Christian Pedersen A/S, Ringstedt, Denmark) ad libidum.

The lungs, heart, aorta, liver, and spleen were removed by using sterile instruments. The aorta was carefully dissected and examined for macroscopic changes. Small tissue pieces were placed in SPG for culture, and the rest were fixed in 10% buffered formalin immediately after removal. Transverse sections 2 to 3 mm thick were taken from the ascending and abdominal aorta, the heart, liver, spleen, and lungs. The tissues were fixed in 10% buffered formalin immediately after removal. Transverse sections 2 to 3 mm thick were taken from the ascending and abdominal aorta, the heart, liver, spleen, and lungs. The lungs were homogenized with a stomacher mechanical blender in SPG in sterile plastic bags. The tissues were centrifuged at 550 × g for 1 h. The inoculated cells were cultured in Dulbecco modified Eagle medium containing 5% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.5 μg/ml cycloheximide (0.5 μg/ml), gentamicin (20 μg/ml), and vancomycin (20 μg/ml) at 35°C with 5% CO₂. After 72 h, the HL cells were fixed and stained for inclusions with fluorescein isothiocyanate-conjugated *Chlamydia genus*-specific antibody (Pathfinder Chlamydia Confirmation System; Kallestad Diagnostic, Chaska, Minn.).

Serology. Antibodies to *C. pneumoniae* were measured by the microimmuno-fluorescence test using formalin-fixed whole elementary bodies of the Kajaani 7 strain as an antigen. Immunoglobulin G (IgG) antibodies in pre- and postinoculation sera were detected with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Sigma Chemical Co., St. Louis, Mo.).

Histology. The lungs, spleen, liver, heart, and thoracic and abdominal aorta were fixed in 10% buffered formalin immediately after removal. Transverse sections 2 to 3 mm thick were taken from the ascending and abdominal aorta, the aortic arch was embedded as a whole, and a 1- to 2-cm longitudinal section was taken from the thoracic aorta, choosing a macroscopically affected area. Samples were collected to an equal extent from the infected and control animals. Tissue slices were embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin; the aortic tissue also with Verhoeff's elastin stain.

Immunohistochemistry. A species-specific monoclonal antibody, RR 401 (Washington Research Foundation), was used to demonstrate *C. pneumoniae* antigen in aortic tissue. Immunostaining was performed by the avidin-biotin-
peroxidase method of Hsu et al. (13), using the Vectastain ABC kit (Vector Laboratories, Burlingame, Calif.). Diaminobenzidine was used as a chromogen, and hematoxylin was used as a counterstain. 

**RESULTS**

Growth of the experimental animals was clearly retarded after the reinfection. The average weight gain was 800 g per week in the control animals and after the first infection but only 50 g per week after the reinfection. However, no other clinical signs of disease (disturbances in food intake, hydration, and defecation) were observed except in two animals after the reinfection. One animal developed severe lung edema 24 h after the reinfection and died. The other rabbit had diarrhea, ocular discharge, and weight loss 2 days after the reinfection and was euthanized. Histopathologic findings showed that this animal had massive pneumonia.

All sample homogenates (lung, liver, and spleen) remained culture negative during the course of the infection. The samples were passaged once in HL cells and still remained culture negative at every time point, all animals remained culture negative.

The lungs of the infected animals (except the two with symptoms) were macroscopically normal, and only mild perivascular and peribronchial inflammatory infiltrate consisting mainly of lymphocytes and eosinophilic granulocytes was seen in histology. The control animals showed no macroscopic or histologic changes. The spleens of both the infected and the control animals remained macroscopically and histologically normal during the experiment. In the liver specimens, mild portal inflammation was seen in one animal after the primary infection and in three animals after the reinfection. The livers of the control animals were normal.

The amount of pericardial fat was macroscopically increased in the infected animals 2 weeks after the reinfection. In histology, only slight myofibrosis of the cardiac muscle was seen in three animals. Macroscopic changes in the aorta were seen in two animals 2 weeks postreinfection and in three animals 4 weeks postreinfeciton (Table 1). The changes consisted mainly of pale flat streaks or spot-like thickenings of the vessel wall and were located in the aortic arch and thoracic aorta. In histology, proliferative lesions with focal intimal thickening and disruption of elastin fibers were seen (Fig. 1a). No foam cells or lipid accumulation was found in any of these lesions. In one of these animals, more advanced, calcified atherosclerotic plaques were found in the aortic arch 4 weeks postreinfeciton. Histologically, a fibrous plaque with calcification, smooth muscle cell proliferation, and inflammatory cell infiltration was seen (Fig. 1b).

Immunostaining with anti-*C. pneumoniae* antibodies showed an equivocal positive reaction in aortic endothelial cells in three animals 2 weeks after the reinfection (Table 1). Immunostaining was clearly positive in three animals at 4 weeks (Fig. 1c). All these animals also had macroscopic and/or microscopic changes. The controls remained negative.

**DISCUSSION**

We showed here that infection with *C. pneumoniae*, a respiratory pathogen, led to the formation of *C. pneumoniae* antigen-positive atherosclerosis-like changes in the aorta in healthy rabbits given a normal diet in 5 to 7 weeks. The finding indicates that *C. pneumoniae* is capable of infecting the vessel wall, which may lead to the development of lesions resembling atherosclerosis.

The rabbit model has been widely used for studies on atherosclerosis since the early 20th century (1). The disease is usually induced in normal NZW rabbits with unnatural diets containing lipids or proteins or by aggravating the arteries with mechanical denuding of vascular endothelium (1, 4, 6, 22, 31). In our rabbit model, atherosclerosis-like changes in the aortas of rabbits fed a normal diet were induced simply by giving them infectious chlamydial intranasally. After the infection, *C. pneumoniae* antigen could be demonstrated in the vessel walls, and its presence was associated with the rapidly developing proliferative lesions. This observation points to the possibility that these lesions in the infected rabbits were caused by the *C. pneumoniae* infection and that the pathogen had not been merely deposited in preformed lesions due to other reasons. The model is comparable to the human situation, where most people become infected by *C. pneumoniae* at least once during
their lifetimes (21), and C. pneumoniae can be found in atherosclerotic lesions at a young age (20).

We found no lesions in the control rabbits, nor did Dailey et al. (4) or Richardson et al. (31), who followed up rabbits fed a normal diet until 6 months. Xu et al., however, found a few mild lesions in 2 (18%) of 11 control rabbits aged 26 to 27 weeks and in 3 (25%) of 12 controls aged 42 to 43 weeks (40). Our animals were 20 to 21 weeks old at the onset of the study, and it is hence possible that some of the lesions were spontaneous. However, spontaneous calcified lesions are very rare.

C. pneumoniae is an obligatory intracellular bacterium capable of multiplying in endothelial and smooth muscle cells and macrophages (8, 9, 14, 15), and it has been shown recently that macrophages can disseminate C. pneumoniae (41). It induces the production of cytokines (15, 25) and adhesion molecules (16, 25), and it possesses an endotoxin, a lipopolysaccharide of a gram-negative organism (27) capable of inducing profound responses in the host organism. Furthermore, it can cause a persistent infection (12). Because of these properties, C. pneumoniae seems to be a highly suitable candidate for triggering the chronic inflammation found in atherosclerosis (32). However, the presence of C. pneumoniae in atherosclerotic lesions has also led to suggestions that it is deposited from blood circulation only in areas rich in oxidized fat as an innocent bystander. This theory is not supported by the fact that C. pneumoniae is a nonmotile pathogenic bacterium incapable of multiplying outside living cells (21). The finding of C. pneumoniae present in deep-site cells of arterial walls further speaks against passive sedimentation from blood circulation (10, 19, 20, 26, 28, 30, 35, 38).

Fong et al. (7) have recently shown that NZW rabbits infected once with C. pneumoniae develop pneumonia, and they also found fatty streaks and grade III atherosclerotic lesions in two of six animals 1 to 2 weeks after infection. In both rabbit models, therefore, atherosclerotic changes developed after infection with C. pneumoniae. However, there are some differences. In our animals, no microscopic lipid accumulation or foam cells were found, whereas Fong et al. (7) demonstrated accumulation of foamy macrophages in the aortic arch 1 week after infection. We inspected our rabbits 2 weeks after the primary and secondary infections, and it is thus possible that we have missed some early changes. Furthermore, we did not see any atherosclerotic changes in the rabbits infected only once. Even though the doses used for the infection of the animals were practically the same in both studies, Fong et al. (7) used a catheter to inoculate the bacteria deep in the trachea, whereas we used a syringe into the nostrils. Thus, the number of chlamydiae invading the circulation in our model would be quite low and possibly mimic the situation in humans more closely than the study of Fong et al. (7). The inflammatory reaction of the lungs was also milder in our study than in the study of Fong et al. (7). Interestingly, both pulmonary and arterial lesions, which were severe and lethal in some cases, were only seen in the animals after the reinfection. In humans, the most severe pneumonias caused by C. pneumoniae are similarly reinfections (17). Whether reinfections promote or are essential for the development of atherosclerosis in humans remains to be studied.

Our results provide further evidence on the putative role of C. pneumoniae in the development of atherosclerosis. It has been demonstrated (10, 18–20, 26, 28, 30, 35, 38) and even isolated in human atherosclerotic lesions (30); it has been cultivated in pure culture; and in the study reported here, when inoculated into rabbits, it induced arterial inflammatory lesions resembling atherosclerosis in experimental animals, and its presence in the produced lesions was demonstrated. The mod-
el that we developed for this study provides possibilities to study the infection-induced atherosclerotic changes, the properties of the infecting chlamydiae, and the effect of antichlamydial therapy. We believe that these results will serve as an impetus to intervention studies with antibiotics effective against chlamydiae. There is a possibility that antibiotics are effective against chronic C. pneumoniae infection and, further, against morbidity and mortality from cardiovascular diseases.

ACKNOWLEDGMENTS

We are grateful for Leena Liesiörova, Mika Paldauni, and Tuula Hietalahdi, for skilled technical assistance. We thank the Juselius Foundation for financial support.

REFERENCES


