Detection of *Chlamydia pneumoniae* in Aortic Lesions of Atherosclerosis by Immunocytochemical Stain

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Recent evidence has shown the presence of *Chlamydia pneumoniae* antigens and nucleic acid in coronary artery atheromas from autopsy patients in South Africa. In this study, the immunocytochemical technique was used to demonstrate *C pneumoniae* antigens in atheromas of the aorta in autopsy patients from retrospective aortic atherosclerosis studies at the University of Washington. The patients were 34 to 58 years old. Immunoperoxidase staining using *Chlamydia*-specific monoclonal antibodies showed one of four fatty streaks and six of 17 fibrous plaques were positive for *C pneumoniae* antigens; four control aortic tissues were negative. Two of the positive plaques were from the same patient. Double-label immunocytochemical staining using *Chlamydia-* and tissue type-specific monoclonal antibodies demonstrated the antigens in the cytoplasm of macrophages and smooth muscle cells in the atheromatous lesion. This study suggested a wider involvement of *C pneumoniae* organisms in atherosclerotic lesions of the arterial system than has previously been documented. (Arterioscler Thromb. 1993;13:1501-1504.)

**KEY WORDS** • atherosclerosis of aorta • *Chlamydia pneumoniae* • immunocytochemistry

*Chlamydia pneumoniae*, also known as TWAR, is one of the three species of the genus *Chlamydia*. The name *TWAR* comes from the laboratory designation of the first two isolates, TW-183 and AR-39. It is primarily a human respiratory pathogen. Infection with *C pneumoniae* is very common; it is found in from 5% to 10% of community-acquired pneumonia, bronchitis, and sinusitis. Worldwide, more than 50% of adults have antibodies against *C pneumoniae*. Antibody is infrequent under the age of 5 years. The prevalence increases rapidly in teenagers, continues to increase throughout adulthood, and is highest in the elderly. Serological studies have shown an association of *C pneumoniae* antibody and acute myocardial infarction and coronary heart disease, angiographically diagnosed coronary artery stenosis, and ultrasonographically diagnosed carotid wall thickening. By electron microscopy, immunocytochemical stain, and polymerase chain reaction, we have demonstrated *C pneumoniae* organisms in atherosclerotic lesions of coronary arteries from autopsy patients in South Africa. In this report we describe the detection of *C pneumoniae* antigens by using immunocytochemical techniques in atheromas of the aorta in autopsy patients in the United States.

**Methods**

**Procurement of Tissue**

The specimens were a random sampling of the aortic tissues originally obtained for human atherosclerosis studies by Gown et al. 20 of the original 68 patients were represented. Segments of abdominal aorta were obtained from the Surgical Pathology and Autopsy Services of the University of Washington Medical Center (UWMC), Seattle. Autopsy material was obtained within 12 hours of death (range, 4 to 12 hours). Excised tissues were immersed in methanol-Carnoy's fixative (60% methanol, 30% chloroform, and 10% glacial acetic acid). After overnight fixation, tissues were embedded in paraffin. Atherosclerotic lesions selected were fatty streaks and fibrous plaques. Fatty streaks were identified grossly as characteristically flat or slightly raised, yellow streaks in the incised aorta. Fibrous plaques selected were small (0.2 to 2.0 cm in diameter), raised, gray lesions with or without evidence of an atheromatous base. Patients ranged from ages 34 to 58 years and were randomly selected from among this patient population to represent a wide spectrum of underlying diseases. Control nonatherosclerotic aortic tissues and one myocardial tissue were obtained from a different set of age-matched specimens from the Surgical Pathology and Autopsy Services at the UWMC. Nonatherosclerotic tissues were obtained from the same region of the aorta as the atherosclerotic tissues. All tissues were obtained with appropriate informed consent.

**Immunocytochemistry**

Paraffin blocks were sectioned for hematoxylin and eosin (Fig, A) and immunocytochemical stains. One lesion from each autopsy case was studied, except that in one case two fibrous plaques were examined. One section per tissue block was stained for each antibody dilution. For immunocytochemical staining for detection of chlamydial antigens, the avidin-biotin complex immunoperoxidase system with diaminobenzidine-
Immunolocalization of Chlamydia pneumoniae in sequential sections of a single representative human atherosclerotic lesion. A, Hematoxylin and eosin-stained section; note abundant foam cell population. B, Avidin-biotin immunoperoxidase procedure using negative control antibody (normal ascitic fluid) on same region of lesion (no immunostaining present; methyl green nuclear counterstain). C, Avidin-biotin immunoperoxidase procedure (black reaction product) using antibody to C pneumoniae (RR-402) on same region of lesion demonstrating diffuse foam cell localization of antigen (arrows). Some cells in the deeper part of the lesion manifest nuclear immunostaining pattern (arrowhead). D, Double-label immunostaining procedure showing simultaneous localization of C pneumoniae antigen by using Chlamydia genus-specific antibody CF-2 (black reaction product) to cells positive for anti-macrophage antibody HAM56 (red reaction product); see "Methods" for details of double-label procedure. E, Double-label immunostaining procedure showing simultaneous localization of C pneumoniae antigen (black reaction product) to cells positive for anti-smooth muscle antibody HHF35 (red reaction product) in media underlying atherosclerotic lesion (original magnification ×250 [A, B, C, and E], ×400 [D]).

nickel chloride color modification as previously described was employed.\textsuperscript{13} Methyl green was used as counterstain (Fig, B). Murine monoclonal antibodies (ascitic fluid) used were Chlamydia genus specific (CF-2, 1:1000 dilution), C pneumoniae species specific (RR-402, 1:200 dilution), and Chlamydia trachomatis species specific (KK-12, 1:2000 dilution). Dilutions of these antibodies were determined by titrations. The specificity of these monoclonal antibodies has been described.\textsuperscript{14,15} Ascitic fluid, prepared from mice injected with NS-0 myeloma cells, was used as a control. Positive and negative antigen controls included HeLa cell monolayers infected with C pneumoniae and C trachomatis. All tissue sections were first screened with the Chla-
mydia genus–specific antibody. Positive tissues were sectioned and then reacted with C pneumoniae and C trachomatis antibodies. For identification of cell types that contained chlamydial antigens, a double-label immunostaining technique was applied according to the previously described method. In this procedure, tissue sections were immunostained sequentially with antibodies to chlamydial antigens using CF-2 in the avidin–biotin immunoperoxidase system with dianinobenzidine–nickel chloride color modification (Vector Laboratories, Burlingame, Calif) to yield a black reaction product. This was followed by immunostaining for cell type–specific antibodies by using either murine monoclonal antibody HAM56 for macrophages or HHF35 for smooth muscle cells in the avidin–streptavidin alkaline phosphatase–Vector Red system to yield a red reaction product. Controls for staining the procedure included a stain containing all components except one of the two primary antibodies; i.e., antibody against either Chlamydia, macrophages, or smooth muscle cells was omitted. The specificities of monoclonal antibodies HAM56 and HHF35 have been described.

Results

C pneumoniae antigens were detected in one of four fatty streaks and six of 17 fibrous plaques (two positive plaques were from the same patient). All positive tissues were positive by both the Chlamydia genus– and C pneumoniae species–specific monoclonal antibodies and were negative by the C trachomatis species–specific monoclonal antibody. Four control aortic tissues were negative; all tissues were negative by normal ascitic fluid. Cell monolayers infected with C pneumoniae and C trachomatis reacted in the appropriate manner. Cross-reaction of these antibodies with the myocardium was not observed.

C pneumoniae organisms were localized to the cytoplasm of cells within atheromatous lesions in a patchy fashion predominantly in the intima (Fig, C). Both the Chlamydia genus– and C pneumoniae species–specific monoclonal antibodies produced a similar immunostaining pattern.

The double-label immunostaining experiments with a positive tissue sample revealed both macrophages and smooth muscle cells to contain chlamydial antigens (Fig, D and E, respectively). Chlamydial antigen–positive macrophages were observed in the whole thickness of atheroma; however, they were more predominant in the subendothelial regions. Chlamydid antigen–positive smooth muscle cells were located mainly in the deeper regions of the intima and were fewer in number than chlamydial antigen–positive macrophages.

Discussion

We previously demonstrated the presence of C pneumoniae in the atheromas of coronary arteries in autopsy patients from South Africa. This study shows that this phenomenon also occurs in North Americans and that the atherosclerotic lesions in the aorta are involved in addition to the coronary arteries.

The role of C pneumoniae in atherosclerosis is unknown. However, unlike herpesviruses (herpes simplex and cytomegalovirus), which are found in both lesions and uninvoluted portions of arteries, C pneumoniae has been found only in the lesions and not in the normal arterial tissues. Furthermore, C pneumoniae was found in early (fatty streaks) and late (fibrous plaques) lesions and in macrophages as well as smooth muscle cells. Finding C pneumoniae in smooth muscle cells in addition to macrophages suggests that C pneumoniae is not merely a passenger in macrophages but can contribute to the disease process because of its ability to colonize cells of the vessel wall. Our recent in vitro experiments showed that C pneumoniae survives in both mouse peritoneal macrophages and in human peripheral blood monocytes (C-c. Kuo, MD, PhD, et al, unpublished data), suggesting that macrophages may be a source of infection. The inflammatory response to infected smooth muscle cells may contribute to the atherosclerotic process.

The demonstration of C pneumoniae in atherosclerotic lesions does not in itself prove a causal relation between the organism and atherosclerosis. It is possible that other underlying disease processes may be, in part, account for our findings. However, the findings of this study do provide justification for further studies of the pathogenic role of C pneumoniae in atherosclerosis.

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References


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