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Relationship of *Chlamydia pneumoniae* Infection to Severity of Human Coronary Atherosclerosis

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Background—Infection with *Chlamydia pneumoniae* has been postulated to play a pathogenic role in atherosclerosis. We examined the role of infection with *C pneumoniae* in relation to the extent of coronary atherosclerosis.

Methods and Results—Coronary atherosclerosis was graded microscopically on a postmortem basis in a blinded fashion in 60 subjects as mild (n=18) or severe (n=42) atherosclerosis. Serum antibodies to *C pneumoniae* were measured by microimmunofluorescence test. Paraffin-embedded coronary artery specimens were examined for the presence of chlamydia by use of a genus-specific direct immunofluorescence monoclonal antibody. Frozen coronary artery specimens were examined by immunoperoxidase for the presence of *C pneumoniae* by use of a specific monoclonal antibody RR-402. Direct immunofluorescence was reactive in 86% of cases with severe atherosclerosis but in only 6% of cases with mild atherosclerosis ($P<0.01$), whereas immunoperoxidase staining was reactive in 80% and 38% of cases with severe and mild atherosclerosis, respectively ($P<0.01$). Elevated IgG and IgA levels against *C pneumoniae* were not different in cases with severe and mild atherosclerosis (61% and 30% for severe atherosclerosis and 67% and 42% for mild atherosclerosis, respectively).

Conclusions—This study supports the hypothesis that intracellular infection with *C pneumoniae* may relate to the severity of atherosclerosis in some subjects. Serum antibody titers against *C pneumoniae* do not differentiate between severe and mild atherosclerosis. (*Circulation*. 2000;101:2568-2571.)

Key Words: atherosclerosis ■ pathology ■ infection

There has been a resurgence of interest in the infectious basis of coronary atherosclerosis. Several recent reviews^{1,2} have suggested that coronary atherosclerosis may be an autoimmune process triggered by an infectious agent, most likely *Chlamydia pneumoniae*. This proposition is based on the observation of raised antibody titers to *C pneumoniae* in patients with coronary artery disease in some studies.^{3,4} Experimental studies have demonstrated that *C pneumoniae* can replicate and maintain infection in human macrophages, endothelial cells, and aortic smooth muscle cells.^{5,6} These cell types show particular susceptibility to infection with *C pneumoniae*.⁷ Recently, Muhlestein et al⁸ showed that intranasal inoculation of *C pneumoniae* accelerates the development of atherosclerosis in a rabbit model of cholesterol-induced atherosclerosis. They also showed that treatment with azithromycin prevented atherosclerosis in rabbits infected with *C pneumoniae*. In earlier clinical studies, Gupta et al⁹ and Gurfunkel et al¹⁰ found that antibiotic treatment of patients with high titers of antibody against *C pneumoniae* significantly reduced the number of cardiac events. However, other studies^{11,12} have failed to show similar seroepidemi-

logical association between infection with *C pneumoniae* and presence and extent of coronary atherosclerosis.

The aim of the present study was to correlate the severity of coronary atherosclerosis with intracellular infection with *C pneumoniae* and with seropositivity against *C pneumoniae*.

Methods

Autopsy Material

Between December 1995 and June 1996, tissues from 300 consecutive autopsy cases were collected at the Department of Forensic Medicine, University of Uppsala, Uppsala, Sweden. Tissues from 60 of these cases (45 men and 15 women) were examined in the present investigation. Thirty of these cases had a diagnosis of cardiac death. In the remaining 30 cases, the cause of death was noncardiac (suicide, 10; accident, 8; gastrointestinal bleeding, 4; hypothermia, 1; epilepsy, 1; diabetes, 1; aortic aneurysm, 1; bronchial asthma, 1; uremia, 1; cerebral bleeding, 1; and pulmonary embolism, 1). The mean (\pm SD) age of these cases was 61 ± 17 years. In these cases, serum samples were also available for serological testing (described below).

At autopsy, a 1×1 -cm section of heart muscle was frozen at -70°C . Coronary arteries were dissected, and a 2-cm-long segment of the left anterior descending coronary artery (LAD) proximal to the

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first diagonal branch was removed in each case and fixed in 4% buffered formalin. Pieces from the rest of the coronary arteries were frozen at -70°C . Postmortem blood was collected via a femoral vein. Sera were frozen at -70°C for measurement of lipoprotein(a) and other lipid fractions.

Formalin-fixed and paraffin-embedded coronary arteries were cut in 4- μm -thick serial sections for direct immunofluorescence examination and for May-Grünwald-Giemsa staining. Frozen 10- μm -thick tissue sections were also prepared for immunoperoxidase staining.

All LAD segments were examined under light microscope, and the degree of atherosclerosis was quantified by 2 independent investigators as follows: grade 0, no atherosclerosis; grade 1, arteries with intima plus media (I+M) thickness <0.25 mm; grade 2, arteries with I+M thickness 0.25 to 0.60 mm; grade 3, arteries with I+M thickness between 0.61 and 1.0 mm; grade 4, arteries with I+M thickness between 1.01 and 1.5 mm; and grade 5, arteries with I+M thickness >1.5 mm.

Atherosclerosis in the LAD was then divided into the following groups for the purpose of the study: no atherosclerosis, grade 0; mild atherosclerosis, grade 1 to 2; and severe atherosclerosis, grade 3 to 5.

Direct Immunofluorescence

The sections of the paraffin-embedded and formalin-fixed arteries were deparaffinized with xylene and alcohol. They were stained by use of a direct fluorescent *Chlamydia* genus-specific monoclonal antibody (Pathfinder, Kallestad Diagnostics), and the sections were examined under a UV microscope. The presence of apple-green fluorescent particles was recorded. Positive control was Imagen control slides (Dakopatts AB).

Immunoperoxidase Staining

Frozen sections of the coronary arteries were fixed in acetone and stained with *C pneumoniae*-specific monoclonal antibody RR-402 (Dakopatts AB) against the major outer membrane protein and then stained by the avidin-biotin complex immunoperoxidase method with the Dako-LSAB kit (Dakopatts AB). The sections were then counterstained by Mayer's hematoxylin. Coronary arteries positive for *C pneumoniae* by polymerase chain reaction served as positive controls. Adjacent sections stained without primary antibody were used as negative controls.

Serology

IgG and IgA antibodies to *C pneumoniae* were measured by a microimmunofluorescence test (Dakopatts AB). An IgG titer of $\geq 1/64$ and IgA titer of $\geq 1/16$ were considered positive.

Statistical Analysis

All data are expressed as mean \pm SD. Data in the 2 groups were corrected for multiple comparisons. Results of direct immunofluorescence and immunoperoxidase positivity were compared by χ^2 test. The relationship between IgG and IgA levels was examined by linear regression analysis. A *P* value of ≤ 0.05 was considered significant. Software from InStat 2.01 for McIntosh was used for these analyses.

Results

Degree of Atherosclerosis

On examination of multiple LAD sections, 18 cases were classified as mild atherosclerosis, and the remaining 42 cases were classified as severe atherosclerosis. The mean age of the 2 groups was 54 ± 18 and 64 ± 16 years, respectively (*P*=NS). All 60 cases had coronary atherosclerosis.

Data on immunofluorescence were available in all 60 cases, but other data were not available in some cases. The precise number of cases with the relevant study (and positivity, in percent) is expressed in the appropriate sections.

Direct Immunofluorescence

By direct immunofluorescence, 36 (86%) of 42 cases with severe atherosclerosis were reactive for chlamydia. In contrast, only 1 (6%) of 18 cases with mild atherosclerosis was reactive for chlamydia. The difference in the presence of *Chlamydia* genus-specific antibodies was highly significant between the 2 groups (*P* <0.01). An example of immunofluorescence reactivity to *C pneumoniae* in a case with severe atherosclerosis is shown in the Figure (left panel).

Immunoperoxidase Staining

Immunoperoxidase staining for the presence of *C pneumoniae* was reactive in 32 (80%) of 40 cases with severe atherosclerosis. In contrast, only 6 (38%) of 16 cases with mild atherosclerosis were reactive for *C pneumoniae*. The difference in immunopositivity was significant (*P* <0.01). An example of immunoperoxidase reactivity to *C pneumoniae* in a case with severe atherosclerosis is shown in the Figure (right panel).

Serology

Serological results were available in 59 of 60 subjects. Serum antibodies to *C pneumoniae* (IgG titer $\geq 1/64$) were present in 40 cases. Nine of these 40 were also positive for *Chlamydia trachomatis* and/or *Chlamydia psittaci*. These sera were excluded due to the possibility of cross-reactivity. Positive antibody levels to *C pneumoniae* were present in 23 (61%) of 38 subjects with severe atherosclerosis and 8 (67%) of 12 subjects with mild atherosclerosis.

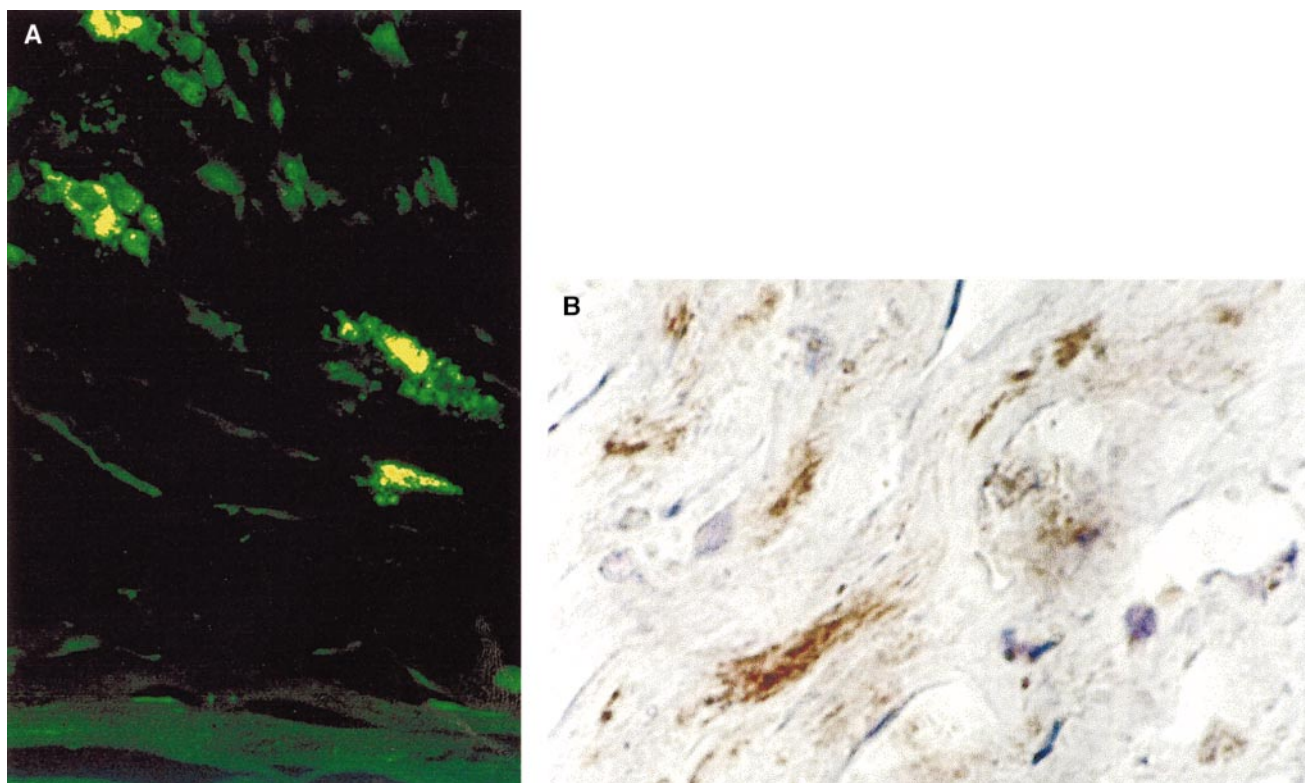
Serum antibodies to *C pneumoniae* (IgA titers $\geq 1/16$) were present in 20 cases. Three cases were also positive for *C trachomatis* and/or *C psittaci*. These sera were excluded due to the possibility of cross-reactivity. Positive antibody levels to *C pneumoniae* were present in 12 (30%) of 39 subjects with severe atherosclerosis and 5 (42%) of 12 subjects with mild atherosclerosis. The difference in seropositivity was not significant between the 2 groups.

IgG levels correlated with IgA levels with a high degree of correlation coefficient (*P* <0.001).

Discussion

In the present carefully conducted study in cases of autopsy-proven coronary atherosclerosis, several key points associated with *C pneumoniae* became evident.

Importantly, this study did not identify an association between seropositivity to *C pneumoniae* and the degree of atherosclerosis. In the past, the causative relationship between *C pneumoniae* and atherosclerosis was speculated on the basis of seropositivity in previous studies.^{2,3} Clinical trials to examine the causative role of *C pneumoniae* in cardiac events were designed exclusively on the basis of seropositivity. Two recent clinical trials have shown reduction in acute cardiac events in a small number of patients with coronary artery disease treated with macrolide antibiotics.^{9,10} However, a recent trial of azithromycin¹³ in a relatively large number of patients did not show any benefit (in terms of reduction of acute cardiac events), although the therapy decreased markers of infection and inflammation. The results of the latter large clinical trial



Left, Immunofluorescence staining of atherosclerotic plaques from human coronary artery. Direct fluorescent *Chlamydia* genus-specific antibody (Kallestad) (positive stain). (Original magnification $\times 400$). Right, Immunohistochemical staining of atherosclerotic plaques from human coronary artery. *C pneumoniae*-specific antibody RR 402, 1:5 dilution (positive stain). (Original magnification $\times 400$.)

and the present study indicate that seropositivity to a ubiquitous organism, such as *C pneumoniae*, is not an index of severity of atherosclerosis in coronary arteries. Results of several other large studies have also yielded data that do not show a direct correlation between seropositivity and extent of coronary atherosclerosis and its complications.^{11,12,14-16}

Infection with *C pneumoniae* is common, especially in the Scandinavian countries, where populations live in a closed environment for a large part of the year. This may well explain the high incidence of seropositivity (62% for IgG and 33% for IgA) in our patients with coronary atherosclerosis.

The absence of correlation between atherosclerosis and serum markers of prior infection with *C pneumoniae*, however, does not exclude a causative role of *C pneumoniae* in coronary atherosclerosis. Experimental studies have indicated that nasal inoculation with this organism causes localization of this bacteria in arteries and accelerates the process of atherosclerosis in coronary arteries.¹⁷ *C pneumoniae* has also been identified in human atherosclerotic specimens.¹⁸⁻²³ A variety of techniques, including immunocytochemistry, polymerase chain reaction, electron microscopy, and bacterial culture, have been used to confirm the presence of *C pneumoniae*. All these techniques have limitations in terms of identification of the presence of *C pneumoniae*. The choice of method used to identify *C pneumoniae* may explain why some investigators have failed to identify *C pneumoniae* in coronary atherectomy specimen,¹² whereas others have found positivity rates as high as 80% in similar tissues.²² We used

2 different methods to define the presence of *C pneumoniae*: direct immunofluorescence using a genus-specific probe and immunoperoxidase stain with a highly specific monoclonal antibody. Both methodologies revealed a high rate of reactivity (86% and 80%) in severe atherosclerosis and a much lower rate (6% and 38%) in mild atherosclerosis. These observations clearly suggest that *C pneumoniae* exists and grows in the atherosclerotic regions and that its growth correlates with the degree of atherosclerosis. Fryer et al²⁴ showed that *C pneumoniae* can infect human vascular endothelial cells and stimulate a 4-fold increase in the expression of tissue factor and platelet adhesion. *C pneumoniae* can also accumulate, replicate, and maintain infection in human macrophages and smooth muscle cells^{5,6}; these cells show particular susceptibility to infection with *C pneumoniae*.⁷ It may be speculated that bacteria precipitate local thrombosis and lipid peroxidation in vascular tissues,²⁵⁻²⁷ and the bacterial load determines the extent of atherosclerosis.

There is much emphasis on genetic predisposition to development of coronary atherosclerosis. In related unpublished data, we found a greater prevalence of immunoperoxidase staining for *C pneumoniae* and certain HLA-DR genotypes (13, 15, and 17) in subjects with severe atherosclerosis than in those with mild atherosclerosis. These observations obviously raise the possibility of genetic predisposition to accumulation of bacteria in arterial tissues and development of atherosclerosis.

These data suggest a link between localization of common bacteria, such as *C pneumoniae*, in the arterial tissues and

degree of atherosclerosis. Once the vessel wall is infected, the process of atherosclerosis is accentuated by activation of clotting system and inflammatory mediators. However, the central role of infection in initiating atherosclerosis in most patients with atherosclerosis remains far from clear. The activation of inflammation and an immune response locally in the coronary arteries may be a response to infection in some patients. Nonetheless, the degree of immune response in circulation (antibody titers) is not a predictor of the degree of infection or the extent of atherosclerosis.

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