

CLINICAL STUDIES

ATHEROSCLEROSIS

## Increased Incidence of *Chlamydia* Species Within the Coronary Arteries of Patients With Symptomatic Atherosclerotic Versus Other Forms of Cardiovascular Disease

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**Objectives.** The objectives of this study were to test prospectively for an association between *Chlamydia* and atherosclerosis by comparing the incidence of the pathogen found within atherosclerotic plaques in patients undergoing directional coronary atherectomy with a variety of control specimens and comparing the clinical features between the groups.

**Background.** Previous work has suggested an association between *Chlamydia pneumoniae* infection and coronary atherosclerosis, based on the demonstration of increased serologic titers and the detection of bacteria within atherosclerotic tissue, but this association has not yet been regarded as established.

**Methods.** Coronary specimens from 90 symptomatic patients undergoing coronary atherectomy were tested for the presence of *Chlamydia* species using direct immunofluorescence. Control specimens from 24 subjects without atherosclerosis (12 normal coronary specimens and 12 coronary specimens from cardiac transplant recipients with subsequent transplant-induced coronary disease) were also examined.

Atherosclerotic cardiovascular disease is a major health problem causing nearly half of the deaths in the American population. Although significant efforts have been made to determine the etiology of this process, only epidemiologic associations have been found. An infectious cause for coronary artery disease has been proposed by some but is currently not accepted. However, the rise and fall of the incidence of coronary artery disease in the United States from the 1940s through the 1970s appears to emulate that of an infectious epidemic (1-3). Additionally, the recent discovery of *Helicobacter pylori* as the infectious etiology of another chronic

**Results.** Coronary atherectomy specimens were definitely positive in 66 (73%) and equivocally positive in 5 (6%), resulting in 79% of specimens showing evidence for the presence of *Chlamydia* species within the atherosclerotic tissue. In contrast, only 1 (4%) of 24 nonatherosclerotic coronary specimens showed any evidence of *Chlamydia*. The statistical significance of this difference is a  $p$  value  $<0.001$ . Transmission electron microscopy was used to confirm the presence of appropriate organisms in three of five positive specimens. No clinical factors except the presence of a primary nonstenotic lesion (odds ratio 3.0,  $p = 0.057$ ) predicted the presence of *Chlamydia*.

**Conclusions.** This high incidence of *Chlamydia* only in coronary arteries diseased by atherosclerosis suggests an etiologic role for *Chlamydia* infection in the development of coronary atherosclerosis that should be further studied.

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inflammatory/degenerative illness, peptic ulcer disease (4), suggests that an infectious etiology for atherosclerotic coronary heart disease should not be dismissed. Still, no one has yet discovered a clear association, let alone a causative relationship, for any particular infectious agent.

Recently, a small body of evidence has appeared, primarily from Europe, purportedly linking the respiratory bacterial pathogen *Chlamydia pneumoniae* (also labeled strain TWAR) with coronary artery disease (5-13). Evidence includes elevated serologic titers as well as the presence of *Chlamydia pneumoniae* within atherosclerotic lesions of both younger patients with more immature plaque and older patients with advanced disease as demonstrated by immunocytochemistry and polymerase chain reaction techniques (14-17). However, these are preliminary and uncontrolled findings that do not yet prove an etiologic link. Whether *Chlamydia pneumoniae* exists as an "innocent bystander" or has a direct causative role in the development of coronary artery disease remains to be seen.

Important preliminary information necessary to ascertain a causative relationship between *Chlamydia* and coronary ath-

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erosclerosis includes the true incidence of bacterial infection, a clinical correlation with the presence of infection, and the uniqueness of the association. If *Chlamydia* infection were the major cause of coronary atherosclerosis, one might expect a high incidence of bacteria detected within the plaque of a prospectively studied series of symptomatic patients. If *Chlamydia* infection were only one of a number of possible causes of coronary atherosclerosis, the incidence of bacterial detection in such a series might be lower, although a significant correlation might be found between the presence of *Chlamydia* and certain clinical characteristics. If the presence of *Chlamydia pneumoniae* within the atherosclerotic plaque were simply the manifestation of an "innocent bystander," one might also expect the bacteria to be found in other cardiovascular disease states as well as coronary atherosclerosis, especially those also associated with endothelial disruption.

The purpose of this study was, therefore, 1) to determine the incidence of the *Chlamydia* pathogen within atherosclerotic plaques in a prospectively studied consecutive series of symptomatic American patients requiring coronary revascularization using directional coronary atherectomy, 2) to compare the clinical features of those demonstrating the pathogen to those not, and 3) to compare the incidence of *Chlamydia pneumoniae* in patients with symptomatic atherosclerotic coronary disease to that of patients with other forms of cardiovascular disease, including cardiac transplant rejection arteriopathy and idiopathic cardiomyopathy.

## Methods

**Atherosclerotic cohort.** In order to obtain atherosclerotic plaque specimens from a consecutive cohort of patients with symptomatic coronary artery disease, all patients undergoing directional coronary atherectomy at LDS Hospital between January 1993 and July 1994 who gave informed consent were included in the study. A total of 90 patients were enrolled.

All patients were pretreated with aspirin 325 mg daily for at least 24 h before atherectomy. Concomitant medical therapy such as beta-adrenergic blocking agents, calcium channel blockers, nitrates and dipyridamole were continued at the discretion of the attending physician. Atherectomy was performed via the femoral approach using 10F arterial sheaths and guide catheters. Heparin (10,000 units) was administered at the beginning of each procedure after vascular access had been established, and further heparin was administered as necessary during the procedure to maintain an activated clotting time at >350 s. A directional coronary atherectomy catheter (Simpson Coronary AtheroCath, Devices for Vascular Intervention, Inc.) suitably sized to produce an approximate device-to-artery ratio of 1.1:1 was prepared and advanced across the lesion over a 0.014-inch (0.36-mm) exchangeable guide wire. Using the lowest possible inflation pressure, as many atherectomy cuts were made as were required to obtain a satisfactory result as determined by the operator. After the procedure, all atherectomy specimens were sent for pathologic

examination. All specimens underwent direct immunofluorescence testing.

**Nonatherosclerotic cohort.** In order to provide a variety of control specimens from nonatherosclerotic cardiovascular disease processes, pathologic specimens were obtained from a number of sources, including 1) normal coronary artery tissue from 12 patients (3 with idiopathic cardiomyopathy, 1 infant with congenital heart disease and 8 with traumatic death) suffering death for reasons other than coronary artery disease, and 2) diffusely diseased coronary artery tissue from 12 patients with chronic transplant rejection-induced ischemic coronary heart disease obtained either at autopsy or repeat heart transplantation. All specimens were sent for direct immunofluorescence testing.

**Pathologic examination. Immunofluorescence.** Specimens for immunofluorescence were frozen in OCT, cut at 4  $\mu$ m onto glass slides, and air dried. After 30 min, they were layered with the test antibody, obtained prediluted from Baxter Scientific. The antibody is a mouse monoclonal antibody directed at a 3,000-Da lipoprotein common to all known types of *Chlamydia* species, *trachomatis*, *psittaci* and *pneumoniae*. The antibody has been directly conjugated with fluorescein isothiocyanate. Slides were washed in phosphate-buffered saline (PBS), incubated with the antibody for 30 min in a moist chamber at room temperature, and washed three times in PBS before coverslipping in Aquamount. Slides were reviewed in an epifluorescent microscope equipped with filter combination to detect fluorescein isothiocyanate. Positive and negative slides were photographed using ASA 640 film with manual exposure times of 15 s. Positive and negative controls were run with each batch of test slides. These consisted of the antigen controls received with the antibody, which were monkey kidney cells infected and uninfected with *Chlamydia*. Elementary bodies of *Chlamydia* fluoresce apple green and measure 350 to 450 nm. Controls for nonspecific binding of immunoglobulin consisted of air-dried sections of test samples (atherectomy, coronary artery or myocardial samples) incubated with antibodies against mouse immunoglobulin (Ig), human IgG, human IgM, human C3c, human fibrin and human HLA-DR. Slides were scored as positive only if the *Chlamydia*-specific antibody stained organisms morphologically consistent with *Chlamydia* and no similar staining was observed with all control antibodies. Other positive staining was usually limited to staining of entrapped histiocytes with HLA-DR within the atherectomy specimen. Organisms that stained positively were usually located superficially in the atherectomy specimen. Rarely were they associated with atheromatous debris (three cases). Usually such debris was independent of the stained structures. The amount of staining was graded on the basis of the number of organisms as being markedly positive (too numerous to count), positive (10 to 100) or rare (<10/plaque). The latter cases were considered equivocal. All cases were reviewed by an experienced pathologist who was uninformed of the clinical circumstances at the time of review. Slides of the preparation were reviewed by the other authors. Figure 1 shows a typical positive control stain; Figure 2 shows a positive atherectomy specimen



Figure 1. Positive control specimen of monkey kidney cells infected with *Chlamydia* species reacted with antibody against *Chlamydia* antigen that is fluorescein isothiocyanate labeled. The preparation is simultaneously treated with 0.016% Evans blue powder, which stains nuclei of cells red. The solution also contains methylene blue as a counterstain. The organisms appear as apple green dots. The picture was exposed for 15 s.  $\times 250$ , reduced by 32%.

with organisms found extensively within the atherosclerotic plaque, and Figure 3 shows a plaque without organisms.

**Transmission electron microscopy.** Transmission electron microscopy was used to confirm the presence of appropriate *Chlamydia* organisms in five of the specimens staining positive by immunofluorescence. Tissues were fixed with Karnovsky's glutaraldehyde-paraformaldehyde followed by fixation in 1% osmium tetroxide. Specimens were embedded in Epon (E1nest F. Fullman), thin-sectioned, stained with uranyl acetate and

Figure 2. Frozen section of coronary atherectomy plaque reacted with antibody against fluorescein isothiocyanate-labeled *Chlamydia* antigen. A large number of organisms are seen, some of which are in aggregates. No autofluorescent atheromatous debris was present in this region. Sections stained with other antibodies were negative. The section is interpreted as markedly positive. Exposure time 15 s.  $\times 250$ , reduced by 34%.

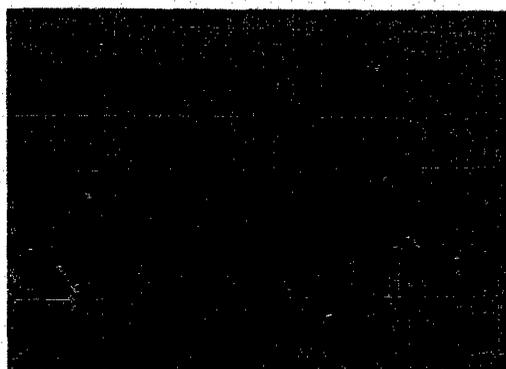


Figure 3. Section of atherosclerotic plaque in which no organisms are found. This was stained and exposed exactly as Figures 1 and 2. Because no organism was found, the specimen was interpreted as negative. Exposure time 15 s.  $\times 100$ , reduced by 34%.

lead citrate and examined in a JEOL 100S transmission electron microscope.

**Data collection and analysis.** For each atherectomy and control transplant patient, routine demographic and clinical data were prospectively entered into a data bank. All data were verified by retrospective review of patient records. Data are presented as proportions or means and SD for continuous variables. For atherectomy patients, univariate analysis was performed on all clinical variables to determine possible correlations between patient clinical and tissue characteristics. Statistical comparisons were performed using chi-square statistic or, when appropriate, the Fisher exact test, for categorical and Student *t* test for continuous variables. A *p* value  $< 0.05$  was considered significant.

## Results

Demographic data for the atherosclerotic patients undergoing coronary atherectomy as well as the heart transplant patients with nonatherosclerotic coronary disease are listed in Table 1. The only significant difference noted in the baseline clinical characteristics between the two populations was the incidence of a positive family history of coronary artery disease which was present in 57% of atherectomy patients compared with only 25% in transplant recipients, although the numbers of transplant recipients were small.

Table 2 shows the results of immunofluorescence testing for *Chlamydia* species in atherosclerotic and control tissue specimens. A total of 90 coronary atherectomy and 24 control specimens were tested. Coronary atherectomy specimens were definitely positive (Fig. 2) in 66 (73%) and equivocally positive in 5 (6%), resulting in 79% of specimens showing evidence for the presence of *Chlamydia* species within the atherosclerotic tissue. In contrast, only 1 (4%) of 24 nonatherosclerotic

**Table 1. Demographic Data of Patients Undergoing Atherectomy and Control Transplant Recipients**

	Atherectomy (n = 90)	Transplant (n = 12)	p Value
Age (yr) (median, 25th-75th percentiles)	57, 50-65	58, 50-66	0.48
Male	87%	83%	0.44
<b>Atherosclerotic risk factors</b>			
Systemic hypertension	51%	17%	0.07
Diabetes mellitus	14%	0%	0.28
Hypercholesterolemia	49%	42%	0.59
Family history of CAD	57%	25%	0.03
Cigarette smoking	46%	42%	0.65
<b>Indication for procedure</b>			
Stable angina	18%	NA	
Unstable angina	44%	NA	
Post-myocardial infarction	38%	NA	
Restenotic lesion	17%	NA	
Multivessel disease	37%	NA	
<b>Vessel involved</b>			
LAD	51%	NA	
LCx	10%	NA	
RCA	32%	NA	
LMCA	1%	NA	
Saphenous vein graft	6%	NA	
<b>Position in vessel</b>			
Proximal	64%	NA	
Middle	29%	NA	
Distal	7%	NA	

CAD = coronary artery disease; LAD = left anterior descending coronary artery; LCx = left circumflex coronary artery; LMCA = left main coronary artery; NA = not applicable; RCA = right coronary artery.

coronary specimens showed any evidence of *Chlamydia*. The statistical significance of this difference is a p value <0.001.

Five specimens staining positive for *Chlamydia* by immunofluorescence were examined by transmission electron microscopy (see Fig. 4). In three cases structures similar in morphology and size to *Chlamydia* were found within the atherectomy material. Both inclusion-like structures containing round and pear-shaped structures similar to *Chlamydia* reticulate bodies and elementary bodies, respectively, and free pear-shaped particles were found. The size of the elementary bodies was measured at close to 0.22  $\mu\text{m}$ , which is similar in size to those seen in cell culture (18). Organisms were found attached to debris but not within entrapped macrophages within the plaques. The lack of organisms in two of the positive samples relates to the small size of the samples used for electron

microscopy. These samples usually represented 10% to 25% of the sample used for immunofluorescence.

**Clinical correlations.** The clinical and angiographic characteristics and related univariate predictors of a positive tissue test for *Chlamydia* species in atherectomy specimens are listed in Table 3. Clinical factors of age, male gender, smoking, hyperlipidemia, hypertension, diabetes, positive family medical history, unstable angina and post-myocardial infarction status did not significantly affect the incidence of a positive tissue test for *Chlamydia*. Angiographic factors of multivessel disease and treatment of a saphenous vein graft likewise were not predictive of the presence of *Chlamydia*. The only nearly significant predictor of the presence of *Chlamydia* within the tissue specimen was whether the sample originated from a primary or restenotic coronary lesion. Restenotic vessels accounted for

**Table 2. Results of Direct Immunofluorescence for *Chlamydia* Species on Atherosclerotic and Nonatherosclerotic Specimens**

Specimen Type	Level of Specimen Staining for <i>Chlamydia</i> Species			
	Markedly Positive	Positive	Equivocal	Negative
<b>Atherosclerotic tissue</b>				
Coronary atherectomy specimens (n = 90)	7 (8%)	59 (65%)	5 (6%)	19 (21%)
<b>Nonatherosclerotic tissue</b>				
Diseased transplant coronary tissue (n = 12)	—	—	—	12 (100%)
Normal coronary tissue (n = 12)	—	—	1 (8%)	11 (92%)



**Figure 4.** Transmission electron microscope image of a chlamydial organism found within a positively staining atherectomy specimen. It is seen directly beneath the dark arrowhead. The small dark central "elementary body" can be seen surrounded by a membranous structure. Other organism-like structures are seen nearby. The organism is not within a cell, although fragments of cells (C) can be seen. Collagen fibrils and elastin surround the cell fragments and organism.  $\times 37,500$ , reduced by 35%.

32% of negative but only 13% of positive specimens, resulting in an odds ratio of 0.33 ( $p = 0.06$ ). Neither the specific native coronary artery involved nor the position of the lesion within the vessel was predictive of the presence of *Chlamydia*.

### Discussion

To our knowledge, this is the first report of the differential incidence of *Chlamydia* species within the coronary artery wall of patients with atherosclerosis versus those with other forms of cardiovascular disease. The extremely high rate of possible infection in patients with symptomatic atherosclerotic disease (79%) compared to the very low rate in patients with normal coronary arteries or coronary artery disease from chronic transplant rejection (4%) provides new evidence for a direct link between the atherosclerotic process and *Chlamydia* infection. If *Chlamydia* were to be present only as an "innocent

bystander," finding fertile ground to grow within the diseased atherosclerotic arterial wall, it should also be found within the walls of arteries diseased by processes other than atherosclerosis. The process of accelerated coronary artery disease associated with chronic cardiac transplant rejection is very similar histologically to atherosclerosis (19) but is believed to result from an immunologic mechanism different from that of atherosclerosis (20). This absence of *Chlamydia* infection within diseased coronary segments of transplanted hearts appears to increase the likelihood that *Chlamydia* plays an active role in the pathogenesis of natural atherosclerosis. The absence of *Chlamydia* within native coronary arteries in 21% of patients suggests that other factors may be involved in some cases, that *Chlamydia* infection (once present) has resolved, or that, as postulated by Kuo et al. (14), because of the small quantity of tissue (on average 18.5 mg [21]) in samples tested, the study is limited by the sensitivity of the technique. It is therefore possible that, given an appropriately sensitive test, chlamydial infection may be present in an even higher percentage of patients with symptomatic coronary atherosclerotic disease. However, because a history of chlamydial infection is so prevalent in the population (50% of subjects are exposed by middle age [22]), the issue of causality remains.

How might the colonization of the arterial wall with *Chlamydia* species contribute to the development of atherosclerosis and ischemic coronary heart disease? On a physiologic and pathologic level, abnormal interactions among endothelial cells, platelets, macrophages and lymphocytes may lead to a cascade of events resulting in acute endothelial damage, thrombosis and repair, chronically leading to the development of atheroma in blood vessels (23,24). It is known that tissue factor is a major inducer of thrombus formation. In blood vessels that are injured, stimulated endothelial cells exhibit increased tissue factor procoagulant activity that promotes thrombosis and platelet adhesion at the site of injury, which in turn may promote the development of atherosclerosis (25). Infection by *Chlamydia pneumoniae* has been shown to produce a similar effect. In studies performed by Fryer et al. (26), *Chlamydia pneumoniae* was made to infect human umbilical

**Table 3.** Correlations With the Presence of *Chlamydia* Species

	<i>Chlamydia</i> (+) Group (n = 66)	<i>Chlamydia</i> (-) Group (n = 19)	Odds Ratio	Univariate p Value
Smoking history	47%	38%	1.44	0.56
Positive family history	58%	50%	1.38	0.62
Hypertension	52%	46%	1.29	0.68
Post-myocardial infarction	38%	37%	1.04	0.95
Multivessel disease	37%	37%	1.00	1.00
Unstable angina	53%	56%	0.90	0.85
Hypercholesterolemia	46%	62%	0.52	0.30
Diabetes	12%	23%	0.45	0.29
Saphenous vein graft	4%	11%	0.39	0.30
Restenotic lesion	13%	32%	0.33	0.06
Male	84%	95%	0.29	0.22
Median age (yr)	57	57	—	0.99

arterial endothelial cells and produced an increase in tissue factor procoagulant activity of 4 to 13 times over the control level. Additionally, *Chlamydia pneumoniae*-infected cells were shown to enhance platelet adhesion compared with noninfected control cells. It is therefore feasible that these or other as yet undetermined physiologic effects of *Chlamydia pneumoniae* infection of arterial tissues may play a significant role in the initiation and progression of coronary atherosclerosis.

As in any possible infection, in order to establish a direct etiologic link between the disease process and the infecting agent, a series of tests must be performed to satisfy the criteria that have come to be known as "Koch's postulates" (27). Under these rules, the microorganism, to be pathologic, must be present in all or nearly all cases of the disease, inoculations of its pure cultures must produce disease (for example, when injected into susceptible animals), and from these diseased organisms, it must again be able to be obtained and be propagated in pure cultures. Of these, the first postulate appears to be true with *Chlamydia* species and atherosclerotic coronary heart disease.

**Limitations.** This prospective study provides a convincing association between *Chlamydia* infection and coronary atherosclerosis. The presence of a series of negative controls of coronary arteries with diseased intima, but from mechanisms other than atherosclerosis, further strengthens that association. However, several limitations do exist in this study. First, specimens were so small that sampling error may be present in the specimens negative for the presence of bacteria. Second, because these specimens came from a somewhat select population of patients, namely those with coronary lesion characteristics suitable for referral for coronary atherectomy, they may not be totally representative of the overall population of patients with coronary atherosclerosis. Third, this study still does not define a definite etiologic role for *Chlamydia* infection in the development of coronary atherosclerosis, and this will require further studies. It is still possible that atherosclerotic plaque is merely a more fertile ground for *Chlamydia* to be deposited and grow. If this were the case, the presence of the pathogens would be a result rather than a cause. Viability of the bacterial elements also needs to be confirmed through successful cultures, although this has proven to be difficult. Animal models need to be developed that will allow for the careful study of the pathophysiology of coronary arterial infections. Fourth, the precise speciation of *Chlamydia* will require further study. However, our preliminary identification of *Chlamydia pneumoniae* strain TWAR by polymerase chain reaction in two specimens is consistent with other reports that associate this strain with atherosclerosis (17). Finally, large randomized clinical trials of antibiotic treatment or prophylaxis will be required to determine any effect on patient outcome.

**Conclusions.** Positive or equivocal evidence for the presence of *Chlamydia* was present in 79% of atherosclerotic specimens tested. In contrast, evidence of *Chlamydia* was found in only 4% of the control, normal coronary specimens or specimens associated with nonatherosclerotic forms of coro-

nary artery disease. This high incidence of *Chlamydia* only in coronary arteries diseased by atherosclerosis as compared to other forms of coronary disease provides evidence for an etiologic role of *Chlamydia* infection in the development of coronary atherosclerosis. Further studies are required to confirm all of Koch's postulates concerning this hypothesis.

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