

# Does *Chlamydia pneumoniae* Cause Atherosclerosis?

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**T**his presentation will be in 3 parts. First, there will be an introduction to the microbiology and epidemiology of *Chlamydia pneumoniae*. I will then present the data that establish an association between *C pneumoniae* and atherosclerosis, and finally, I will discuss some of the preliminary data concerning a causal role for *C pneumoniae* and atherosclerosis.

## C PNEUMONIAE, A NEWLY DESCRIBED SPECIES OF THE GENUS CHLAMYDIA

### History

We isolated the prototype *C pneumoniae* strain, TW-183, in 1965, but its role in respiratory infection was not demonstrated until the 1980s.<sup>1,2</sup> The laboratory designation of the first isolate from respiratory disease was AR-39 and led to the strain name TWAR (from the TW + AR of the first 2 isolates). In 1989, based on DNA homology and morphology of the elementary body, the TWAR organism was established as a new third species of the genus *Chlamydia* and named *C pneumoniae* after the most commonly recognized clinical syndrome associated with infection with the organism.<sup>3</sup>

### Microbiology

*Chlamydia* are unique intracellular bacteria. Their life cycle includes a metabolic inert infectious form, the elementary body, and a larger intracellular form, the reticulate body. The reticulate body divides by binary fission using the cells' energy. Only the reticulate body is susceptible to antibiotics, placing a premium on antibiotics with a high intracellular penetration and persistence. Unlike *Chlamydia trachomatis*, *C pneumoniae* does not cause sexually transmitted disease and is not transmitted sexually. Also, unlike the third species of

chlamydia, *Chlamydia psittaci*, the cause of psittacosis/ornithosis, *C pneumoniae* does not depend on an animal reservoir and is not transmitted from bird to man.<sup>4,5</sup>

### Epidemiology

*Chlamydia pneumoniae* causes about 10% of all community-acquired pneumonia in adults and perhaps 5% of bronchitis and sinusitis.<sup>6</sup> In most studies, *C pneumoniae* has been found to be the third most common cause of pneumonia. Most *C pneumoniae* infections are asymptomatic, especially in children. Transmission is person to person by the respiratory tract. The infection is periodic, with low rates in some years and much higher rates in others. Epidemics have been described and have played a major role in proving a causal relation of *C pneumoniae* and acute respiratory illness.<sup>6-8</sup>

Serologic studies have taught us that the most unusual aspect of *C pneumoniae* epidemiology is the high prevalence and universal nature of infection throughout the world. Most of us have our first *C pneumoniae* infection before age 20 years and reinfection is common. Despite evidence that antibody from a first infection is usually lost in 3 to 5 years, population prevalence antibody rates reach 50% by age 20 years and continue to rise in adults, reaching about 80% in men and 70% in women in old age.<sup>9</sup> The consistently higher prevalence of antibody in men after age 20 years has never been explained and possibly could reflect the higher incidence of atherosclerotic disease in men.

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The biological plausibility of chlamydia invading cardiovascular tissue is supported by human infections. All 3 *Chlamydia* species have been shown to cause myocarditis and endocarditis.<sup>10</sup>

### OBSERVATIONAL STUDIES PROVE THE ASSOCIATION OF *C PNEUMONIAE* AND ATHEROSCLEROSIS

The evidence for the association of *C pneumoniae* and atherosclerotic disease will be presented in 2 parts—seroepidemiological study and demonstration of the organism in atheroma.

#### Seroepidemiologic Study

The initial study indicating an association between *C pneumoniae* and coronary artery disease was seroepidemiological and was reported from Finland in 1988 by Saikku et al.<sup>11</sup> They showed that patients with coronary artery disease were significantly more likely to have serologic evidence of past infection with *C pneumoniae* than were population-matched controls. Saikku et al<sup>12</sup> extended their original finding in a larger nested case-control study involving patients enrolled in a study of lipid lowering drugs and by using immune complexes containing chlamydial proteins as another serologic marker.<sup>13</sup>

We found a similar association between Cpn IgG antibody and coronary artery disease in 2 studies in Seattle, Wash, involving patients with angiographically demonstrated disease. In the first, patients with at least 1 coronary artery with 50% or greater stenosis were more likely than patients with normal coronary angiograms to have IgG antibody.<sup>14</sup> In the second, patients with coronary artery disease were compared with patients from the same health maintenance organization who did not have a history of cardiac disease.<sup>15</sup> An association between IgG antibody and coronary disease was found. In this study, the increased risk was restricted to subjects who were cigarette smokers. **Table 1** gives a summary of these first 5 studies.

These seroepidemiologic findings have now been confirmed by several investigators from various countries. Thirty-eight studies with similar findings have been reported. Four additional studies have failed to find the association. It should be pointed out that with the high prevalence of antibody in middle-aged and older adults, and therefore in control populations, there is a reduced opportunity to show a difference in the frequency of antibody in cases vs controls.

More precision from seroepidemiologic studies is limited by the serologic test. There is little evidence that it can separate those with prior infection from those with chronic infection. The most important result of the seroepidemiological studies is to raise the question of a possible causative relation of *C pneumoniae* to atherosclerosis.

#### Direct Demonstration of *C pneumoniae* in Atherosclerotic Lesions

Morphologic and microbiologic evidence of the presence of *C pneumoniae* in atheromatous plaques of coronary ar-

**Table 1. Summary of Results From the First 5 Seroepidemiologic Studies Reporting an Association Between *Chlamydia pneumoniae* Antibody and Coronary Artery Disease**

Location	No. of Cases/ Controls	Antibody Titer Cutoff, g/L	Odds Ratio (95% Confidence Interval)
Finland <sup>11</sup>	70/41	IgG ≥ 32	3.8 (1.4-10.7)
Seattle <sup>14</sup>	461/95	IgG ≥ 16	1.6 (1.0-2.7)
Finland <sup>12</sup>	102/102	IgA ≥ 64	2.3 (0.9-6.2)
Seattle <sup>15</sup>	171/120	IgG ≥ 8	2.6 (1.4-4.8)
Finland <sup>13</sup>	46/46	Immune complex*	3.9 (1.3-12.0)

\*Presence of absence of immune complexes used as assay.

teries and other large vessels that develop atherosclerotic disease has been reported by multiple investigators in various countries. While the organism has been demonstrated primarily by immunocytochemistry (ICC) (using species-specific monoclonal antibodies) and the polymerase chain reaction (PCR), it has also been shown by electron microscopy and isolation of the organism.

To our knowledge, the most extensive series of studies with atherosclerotic tissues and *C pneumoniae* come from our laboratories (**Table 2**).<sup>3</sup> The first indication that the organism existed in atherosclerotic lesions was made by electron microscopy. The pear-shaped elementary bodies of *C pneumoniae* are unique among *Chlamydia* species and provided the method for the original presumptive identification of the organism in atheromata of coronary arteries. Alan Shor, MD, a pathologist in South Africa, made the electron microscopy observation. Using ICC and PCR with his material, we showed that the structures were *C pneumoniae*.<sup>16</sup>

The tissues of the 362 persons we studied came from autopsies, surgeries, and atherectomies and included atheromatous lesions of coronary, carotid, and femoral/popliteal arteries and the aorta. The populations studied came from South Africa and throughout the United States, including Alaska. Persons studied included whites, blacks, Asians, and Alaskan natives.<sup>16-24</sup>

*Chlamydia pneumoniae* was present in atheromata of all these populations in both sexes. The organism was found in lesions in young persons as well as in extensive disease in older persons. Overall, 55% of the developed lesions were positive for *C pneumoniae*. In these studies, the organism was not found in normal artery specimens. However, true matched controls without atherosclerosis were available only in the study of young persons (aged 15-34 years) from a US multicenter study where 31 persons without evidence of atherosclerosis in the studied coronary artery failed to show *C pneumoniae*.<sup>19</sup>

The positive specimens in Table 2 could be positive as determined by ICC or PCR. Almost all specimens were tested by both methods. We have detected *C pneumoniae* less frequently by PCR than by ICC in tissue specimens. The reason for the lower detection rate is not completely understood, but is thought to be due to inhibitors that most often cannot be removed by standard methods.

The first isolation of *C pneumoniae* from an atheroma was made in 3 separate laboratories culturing an

**Table 2. Results of 8 University of Washington Studies of Atherosclerotic Tissue for the Presence of *Chlamydia pneumoniae***

Source (No.) of Specimens	Artery	Type of Specimen	Normal Tissue*	Atherosclerotic Tissue	
				Type of Lesion	No. (%)
South Africa (16)	Coronary	Autopsy	0/8†	Early	7/16† (44)
University of Washington, Seattle (18)	Coronary	Atherectomy	None	Advanced	13/20 (65)
				Primary	10/23 (43)
PDAY study‡ (19)	Coronary	Autopsy	0/31	Restenotic	10/15 (67)
				Early	2/11 (18)
Alaskan Natives (24)	Coronary	Autopsy	0/3	Advanced	6/7 (86)
				Early	6/19 (32)
University of Washington (23)	Coronary	Autopsy	0/4	Advanced	17/40 (43)
				Early	6/18 (33)
University of Washington (17)	Aorta	Autopsy	0/4	Advanced	6/16 (38)
				Early	1/4 (25)
California and University of Washington (20)	Carotid	Endarterectomy	0/6§	Advanced	6/17 (35)
University of Washington (22)	Carotid	Endarterectomy	None	Advanced	37/61 (61)
California (21)	Femoral/popliteal	Vascular bypass	None	Advanced	12/16 (75)
				Advanced	11/23 (48)

\*Without histological evidence of atherosclerosis.

†Number positive as determined by immunocytochemistry and/or polymerase chain reaction testing.

‡PDAY indicates Pathobiological Determinants of Atherosclerosis in Youth study (US multicenter study).

§Autopsy specimens, not age matched.

||*Chlamydia pneumoniae* isolated from 1 patient.

atherosclerotic coronary artery.<sup>25</sup> We have isolated *C pneumoniae* from a carotid artery plaque removed at surgery.<sup>22</sup> When these artery isolates were tested by electron microscopy, by chromosomal fingerprinting, and by sequencing the variable area of the major outer membrane protein, they were found to be identical with the prototype *C pneumoniae* respiratory isolates.<sup>22</sup> Recently, 18 *C pneumoniae* isolates were obtained from 102 coronary artery or bypass vessel specimens obtained at open heart surgery.<sup>26,27</sup> Isolation of the organism provides additional evidence for the presence of viable organisms in atheroma.

There are now 50 reports on studies of *C pneumoniae* in atherosclerotic tissue. All but 4 succeeded in demonstrating the organism in atheroma. Those that failed used PCR only. Other studies using PCR only had lower frequency of positive results than studies using ICC alone or ICC and PCR.

These studies clearly establish that *C pneumoniae* is frequently present in atherosclerotic lesions and only rarely in normal arteries. They do not prove that the organism plays a causal role in the disease.

Further studies either of seroepidemiology or of the frequency of the organism in different tissue specimens will not help us answer the most important question: Does *C pneumoniae* play a causative role in the pathogenesis of atherosclerosis or its disease complications?

## ETIOLOGIC STUDIES

I will discuss 3 types of studies that will eventually determine if *C pneumoniae* plays a causal role in atherosclerosis.

### Animal Models

Two groups, 1 in Finland and 1 in Toronto, Ontario, have found aortic lesions after intranasal inoculation of *C pneu-*

*moniae* in New Zealand white rabbits.<sup>28,29</sup> There was a higher rate of aortic lesions after 2 inoculations compared with 1. The rabbits were killed from 2 weeks to 3 months after the final inoculation. Sham-inoculated control rabbits showed no aortic lesions, as would be expected in New Zealand white rabbits, which do not develop atherosclerosis while being fed a regular diet.

The Toronto studies of animals killed at age 3 months presented histological findings with some similarities to pathological findings of human atherosclerosis. Such lesions were not found in extensive controls, including rabbits infected with another pulmonary pathogen, *Mycoplasma pneumoniae*.<sup>30</sup> While it may be unclear how typical of atherosclerosis the early aortic lesions are, these experiments offer evidence that *C pneumoniae* pulmonary infection is capable of causing aortic lesions where they do not otherwise occur. If these rabbit experiments can be repeated with the development of disease observed over a longer period, they will be even more valuable.

Mühlestein et al<sup>31</sup> have used a different approach to the New Zealand white rabbit model. They enhanced the diet of their rabbits with a small amount (0.25%) of cholesterol and observed some aortic lesions in the control rabbits. However, when the rabbits were killed 3 months after receiving 3 intranasal inoculations, the aortic lesions in rabbits infected with *C pneumoniae* were greater by several different measurements. They also showed that infected rabbits treated with azithromycin for 7 weeks after the last intranasal challenge had aortas that resembled those of control animals and differed significantly from the infected untreated rabbits.

We have worked with a mouse model of atherosclerosis, using the Apo E-deficient mouse. This knockout mouse spontaneously develops atherosclerosis that has many similarities to the human disease. After intranasal inoculation, *C pneumoniae* persists in the aorta for at least 20 weeks and the organisms can be demonstrated in aortic atheroma. Using precise computer morphometric

analysis, it was possible to show that infected mice had a lesion area significantly larger than sham-inoculated mice. The area was more than 100% larger at age 16 weeks.<sup>32</sup>

A group from the University of Manitoba, Winnipeg, working with mice with low-density lipoprotein receptor deficiency and a cholesterol-enriched diet found that infection with *C pneumoniae* but not a mouse *Chlamydia trachomatis* strain significantly exacerbated the hypercholesterolemia-induced atherosclerosis.<sup>33</sup>

These preliminary experiments in 2 animal species suggest that *C pneumoniae* may be capable of initiating atherosclerosis and causing acceleration of the atherosclerotic process.

### Mechanisms

There have been several recent reports of studies directed at potential mechanisms by which *C pneumoniae* could influence atherosclerosis development. Kalayoglu and Byrne<sup>34</sup> showed that human macrophages in cell culture with added lipid in the media ingested some of the lipid. However, if the cells were infected with *C pneumoniae*, greater amounts of cholesterol esters could be measured in the cells. Many of the infected cells took on the characteristic appearance of foam cells found in atheroma. Investigators at Harvard<sup>35</sup> have shown that *Chlamydia* heat shock protein 60 localizes in human atheroma and may regulate macrophage tumor necrosis factor  $\alpha$  and matrix metalloproteinase expression, functions considered relevant to atherosclerosis and its complications. Investigators in Kentucky<sup>36</sup> reported that infection of human endothelial cells with *C pneumoniae* stimulated transendothelial migration of neutrophils and monocytes. Another group from Toronto<sup>37</sup> found sequence homology between a murine heart muscle peptide and an outer membrane protein of *Chlamydia*. Both peptides caused severe heart damage in the mouse.

### Antibiotic Treatment Trials

**Retrospective Studies.** There have been 2 reports of retrospective antibiotic treatment studies in which the outcome was first myocardial infarction. These studies used large patient populations on which computerized pharmacy records of drug use over 3 to 5 years were available. One study found that prior use of certain antibiotics known to be effective against *C pneumoniae* reduced the incidence of coronary artery disease (CAD) events<sup>38</sup> and the other failed to find this effect.<sup>39</sup> I believe that it is unlikely that the short-term antibiotic therapy given for most illnesses would be long enough to affect chronic chlamydia infection.

**Prospective Studies.** Because of the enormous importance of atherosclerosis and especially CAD to human health, an argument can be made for studying the effect of antibiotic treatment directed at *C pneumoniae* on CAD events before it has been proven that the organism plays a causative role in the disease. This is particularly true if there is little danger to the subjects of a trial.

The causes of the process leading to myocardial infarction and other complications of atherosclerosis may

be different from the cause(s) of atherosclerosis. Because there are no animal models of the important complications of atherosclerosis, the antibiotic treatment trials will offer one way to get evidence about the effect of *C pneumoniae* on complications of atherosclerosis.

Three small, underpowered pilot trials have been reported. Two found a reduction in coronary artery events after antibiotic therapy<sup>40,41</sup> and the third did not.<sup>42</sup> One of the studies used hospitalized patients with coronary syndromes as subjects and treated them for 1 month. The significant reduction in additional events seen at the end of the first month of observation decreased over 6 months of observation, suggesting that 1 month of treatment might be insufficient.<sup>41</sup> Because of the small number of subjects, the results of all 3 of these studies are not reliable.

There is no justification for treatment of patients with CAD with antibiotics. The introduction of yet another unproven treatment into practice should be discouraged. The use of antibiotics for unproven indications can add to the increasing problem of antibiotic-resistant organisms.

At least 2 antibiotic treatment trials are now underway that are adequately powered to answer the question of whether antibiotic treatment can reduce the incidence of coronary artery events. The WIZARD trial is sponsored by Pfizer Inc, New York, NY, and the ACES trial by the National Heart Lung and Blood Institute, Bethesda, Md. The trials use 3500 to 4000 subjects with proven CAD and observation periods of up to 4 years. Azithromycin is the antibiotic chosen for both trials because of its proven effectiveness against *Chlamydia* and its long intracellular half-life allowing a once per week dosage. In the WIZARD trial the subjects received 600 mg of azithromycin or placebo once per week for 3 months. The ACES trial differs in that the subjects will receive the treatment for 1 year.<sup>43</sup> The long treatment is in recognition of the difficulty of successfully treating chronic *Chlamydia* infection. The endpoint events are coronary artery death, myocardial infarction, revascularization procedures, and hospitalization for unstable angina. Results of these studies will be available in 2 to 4 years and will provide indirect evidence of whether *C pneumoniae* plays a causative role in the complicating disease events of coronary atherosclerosis.

### CONCLUSIONS

An association of *C pneumoniae* and atherosclerosis has been clearly established. Studies with *C pneumoniae* in animal models, of possible basic mechanisms, and human secondary prevention antibiotic trials will eventually provide the information to determine the role, if any, of *C pneumoniae* in the cause of atherosclerosis. The answer to the question posed in the title of this lecture is that it is still not known if *C pneumoniae* causes atherosclerosis.

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