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Relation Between Direct Detection of *Chlamydia pneumoniae* DNA in Human Coronary Arteries at Postmortem Examination and Histological Severity (Stary Grading) of Associated Atherosclerotic Plaque

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Background—Numerous studies have suggested a link between *Chlamydia pneumoniae* infection, atherosclerosis, and coronary artery disease. However, it is still unclear whether *C pneumoniae* plays a causal role in the pathogenesis of these conditions. Accordingly, we have performed a systematic dissection of the 3 coronary arteries on 33 postmortem subjects and studied the relationship in individual artery segments between the presence of *C pneumoniae* DNA and the severity of associated atherosclerosis.

Methods and Results—The prevalence of *C pneumoniae* DNA in arterial segments was determined by polymerase chain reaction (PCR) after controlling for the presence of PCR inhibitors. Atherosclerosis in each arterial segment was graded histologically with the Stary classification. *C pneumoniae* was detected by PCR in 78.8% of subjects, but there was no association between the presence of this DNA and cause of death or grade of atherosclerosis. When paired mild and severe atherosclerotic lesions within subjects were compared, mild lesions were as likely to be positive for *C pneumoniae* as severe lesions.

Conclusions—This study demonstrates that *C pneumoniae* can frequently be detected in atheromatous plaques in coronary arteries. However, its distribution did not correlate with severity or extent of disease. (*Circulation*. 1999;99:2733-2736.)

Key Words: *Chlamydia pneumoniae* ■ atherosclerosis ■ coronary disease ■ infection

Known risk factors account inadequately for the incidence of coronary artery disease (CAD),¹ suggesting that other factors, such as infection, may be important.² *Chlamydia pneumoniae* infection has been strongly implicated as a possible cause of atherosclerosis; most seroepidemiological and pathological studies have reported a positive association between prior or current infection with the organism and CAD.³ Furthermore, in 3 studies, viable *C pneumoniae* have been cultured from atherosclerotic tissue.⁴⁻⁶ However, there are problems with these data. First, many studies relied on the immunohistological detection of *C pneumoniae* antigen in atheroma. This method generally gives the highest prevalence of chlamydial infection but correlates poorly with detection of *C pneumoniae* DNA by polymerase chain reaction (PCR).^{4,5,7-9} Second, PCR-based studies have not been adequately controlled for the differential effect of PCR inhibitors. Third, the ubiquitous occurrence of atheroma from early age¹⁰ makes it difficult to select appropriately age-matched control vessels at similar risk of infection. Indeed, a number of studies either have not compared diseased with control

vessels or have compared material from subjects of widely different ages.^{4,11} We have overcome this problem by determining the relationship between *C pneumoniae* infection and the severity of associated CAD within individuals within whom there can be no variation in exposure to blood-borne infection. The effect of tissue inhibitors on PCR sensitivity has been determined by use of internal amplification controls.

Methods

Specimen Collection

All 3 coronary arteries plus segments of the lung and myocardium were collected from 33 consecutive adult subjects at autopsy. Twenty-one subjects (63.6%) had died of causes related to CAD, and 12 had died of noncardiac causes. Postmortem examinations were performed within 48 hours of death, and the heart was aseptically removed before removal of other organs. In a laminar flow cabinet, each coronary artery was inspected, and 1-cm segments of the most and least diseased regions were sampled. Half of each segment was frozen in liquid nitrogen before detection of *C pneumoniae* DNA by PCR; the remainder was processed for histology.

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TABLE 1. Characteristics of Patients With and Without *C pneumoniae* in Coronary Arteries

	Subjects With <i>C pneumoniae</i> in Coronary Arteries (n=26)	Subjects Without <i>C pneumoniae</i> in Coronary Arteries (n=7)
Male, n (%)	14 (53.8)	4 (57)
Age, y	72.4±9.7	78.3±7
Coronary deaths, n (%)	17 (65.4)	4 (57)
Noncoronary deaths, n (%)	9 (34.6)	3 (43)
Presence of plaque rupture, hemorrhage, or acute thrombosis, n (%)	12 (46.1)	2 (28.6)
<i>C pneumoniae</i> in lungs, n (%)	5 (19.2)	1 (14.3)
<i>C pneumoniae</i> in myocardium, n (%)	6 (23.1)	0

Polymerase Chain Reaction

DNA was extracted from coronary arteries by conventional proteinase K digestion and phenol-chloroform extraction; PCR was performed in triplicate as previously described.¹² To check for PCR inhibitors in the extracted DNA, samples were spiked with phage λ DNA and subjected to PCR with specific primers. If inhibition was present, samples were diluted 10-fold. A nested PCR was used to detect *C pneumoniae*,¹³ and all positive products were confirmed by Southern hybridization.¹² Mock extraction controls and PCR-negative controls were interspersed every 5 to 6 samples. A PCR-positive control of DNA equivalent to 1 to 10 elementary bodies was used for every PCR experiment.

Histology

Sections of coronary arteries adjacent to those studied by PCR were stained with hematoxylin and eosin. Most sections were also stained with Schmorl's stain for lipofuscin and with alizarin for calcium. The severity of atherosclerosis was graded with the Stary classification.¹⁴ This classification ranges from grade 0 (normal) to grade 6 (plaque rupture, fissure, or hemorrhage); grade 4 represents the earliest lesions visible macroscopically.

Results

Characteristics of the 33 recruited subjects are shown in Table 1. Three coronary arteries were obtained from 30 subjects and 2 coronary arteries were from 3 subjects, for a total of 96 arteries. Two segments were available from all but 5 of these arteries, resulting in 187 segments. Segments with atherosclerosis of Stary grade 3 or less were considered to have mild disease; those of grade 4 or greater were considered to have moderate or severe disease. To assess the reproducibility of Stary grading, 48 segments were recoded and then regraded by the same specialist cardiac pathologist. Thirty-four segments were graded identically on both occasions, with the remaining segments differing by only 1 grade (κ test, $P=0.78$). On classifying into mild or severe groups, only 3 of 48 segments changed groups on repeated grading.

Only 1 of 187 segments was Stary grade 0. Thirty-one subjects had ≥ 1 atherosclerotic lesion in each artery of Stary grade 4 or more, and 26 of these subjects were positive for *C pneumoniae* DNA in their coronary arteries. The remaining 2 subjects had mild atherosclerosis of Stary grade 3 or less, and neither had evidence of *C pneumoniae* DNA. There was no statistical difference in age, sex, or cause of death in subjects with or without *C pneumoniae* DNA in their coronary arteries.

Distribution of *C pneumoniae* in Coronary Arteries

Subjects were as likely to have *C pneumoniae* in 1 (8 of 26) as in 2 (9 of 26) or 3 (9 of 26) coronary arteries. Considering

the individual arteries, 41 of 96 had both mild and moderate or severely diseased segments. In these arteries, the mild segments were just as likely to be positive for *C pneumoniae* as the severe segments (Table 2; McNemar test, $P=1.0$). Similarly, arteries that had mild disease only were just as likely to have *C pneumoniae* as arteries that had moderate or severe disease only (9 of 14 versus 20 of 36; χ^2 test, $P=0.77$). When all arterial segments were considered as a group, no correlation was observed between Stary classification and the presence of *C pneumoniae* DNA (Table 3; χ^2 test, $P=0.57$). Thus, in 1 subject with severe atherosclerosis throughout, *C pneumoniae* was found at only 1 site, whereas in another subject who had only mild disease, every arterial segment was positive for *C pneumoniae*.

Effect of PCR Inhibitors

As anticipated, DNA samples extracted from coronary artery segments with moderate or severe disease were more likely to contain PCR inhibitors than samples extracted from mildly diseased segments (40 of 114 versus 15 of 73; odds ratio [OR], 2.1; 95% CI, 1.1 to 4.1). Inhibition was associated with the presence of lipid (44 of 128 versus 10 of 54; OR, 2.3; CI, 1.1 to 5.0) and especially calcium (42 of 103 versus 12 of 79; OR, 3.8; CI, 1.9 to 8.0) and was eliminated in all but 1 case by 10-fold dilution. Sixteen of 55 inhibited samples (29.1%) were positive for *C pneumoniae* compared with 52 of 132 uninhibited samples (39.4%; χ^2 test, $P=0.17$). We tested each coronary artery segment on 3 separate occasions, but repeated PCR did not always give a consistent result. Nine inhibited samples were positive 1 time; 7 samples, 2 times; and 0 samples, 3 times. For uninhibited samples, 34 were positive 1 time; 8 sample, 2 times; and 10 samples, 3 times.

Discussion

A feature of this study was the careful control of PCR inhibition. DNA extracts of coronary arteries caused signifi-

TABLE 2. Prevalence of *C pneumoniae* DNA in Paired Mild and Severe Segments From 41 Coronary Arteries

	Severe Lesions	
	<i>C pneumoniae</i> Positive	<i>C pneumoniae</i> Negative
Mild lesions		
<i>C pneumoniae</i> positive	7	11
<i>C pneumoniae</i> negative	10	13

TABLE 3. Prevalence of *C pneumoniae* in Atherosclerotic Coronary Artery Segments According to Severity of Disease (Stary Classification)

	Stary Classification				
	Mild Lesions		Severe Lesions		
	≤2	3	4	5	6
Coronary artery segments positive for <i>C pneumoniae</i> , n (%)	18/41 (43.9)	9/32 (28.1)	17/45 (37.8)	18/56 (32.1)	6/13 (46.2)

cant levels of PCR inhibition, associated particularly with severe atherosclerosis. Lipid and particularly calcium were the main sources of inhibition, illustrating the importance of using PCR inhibition controls when studying atheroma lesions of differing severity. A similar use of λ DNA for this purpose was recently reported by another group.¹⁵ Inhibition was eliminated in all but 1 case by 10-fold dilution, but this was associated with a reduction in chlamydial detection from 39% to 29%. As reported by others,^{4,16} repeated testing of samples by PCR did not always produce consistent results. We attribute this to the low amounts of *C pneumoniae* DNA present in coronary arteries, to the sampling errors arising from use of small sample volumes, and to PCR inhibition. Our strategy of testing all samples in triplicate should have reduced any resulting underestimation of the prevalence of *C pneumoniae*.

Unlike some other studies, we did not use immunocytochemistry (ICC) to detect *C pneumoniae*. In general, ICC gives a higher prevalence of *C pneumoniae* in coronary tissue than PCR.^{4,5,7-9} Unfortunately, it is not known whether this is due to better sensitivity or worse specificity. Careful control experiments in a specialist immunohistochemical laboratory with a range of *C pneumoniae*-specific monoclonal antibodies failed to convince us of the specificity of ICC for *C pneumoniae* in atheromatous plaque. Nonspecific binding of immunoglobulin in atheromatous plaque can be a problem, and other workers have commented on difficulties in interpreting ICC for *C pneumoniae* in atheromatous plaque.¹⁶ For *C trachomatis* genital infections, nucleic acid amplification-based methods are clearly established as the most sensitive method for detecting the organism. Furthermore, PCR enables positive results to be confirmed by sequencing or, as here, by hybridization.

Our main finding was that *C pneumoniae* DNA was common in coronary arteries but that its distribution did not match the extent or severity of atherosclerosis. Only 1 previous study has looked for *C pneumoniae* at multiple sites from the coronary tree.⁴ Although severity of atherosclerosis was not graded, that study also found that the distribution of *C pneumoniae* was patchy. *C pneumoniae* has been detected in vessels not usually associated with atherosclerosis, such as the internal mammary artery and saphenous vein.^{12,17} Furthermore, in a recent study of 60 Alaskan natives dying mainly of noncardiac causes (mean age, 34.1 years), there was no difference in the severity of atherosclerosis in subjects with or without *C pneumoniae* infection, although a high *C pneumoniae* IgG titer of ≥ 256 an average of 8 years before death was associated with the presence of *C pneumoniae* in coronary arteries.⁹ Other studies, however, have found that

C pneumoniae was more prevalent in severely diseased than mildly diseased arterial segments. In 1 study, 15% of carotid endarterectomy samples were found to be positive for *C pneumoniae* by PCR, whereas the organism could not be detected in macroscopically normal segments adjacent to the diseased areas.¹⁸ Similarly, in an autopsy study of young persons in which samples were age and sex matched,⁷ *C pneumoniae* could not be found in normal segments but was found in 2 of 11 segments with intimal thickening and in 6 of 7 samples with atheroma.

In this study, the finding that *C pneumoniae* vascular infection is focal and not associated with the extent or severity of atherosclerosis does not disprove a role for the organism in CAD. After all, if *C pneumoniae* causes atherosclerosis, its presence would be expected to precede that of disease. Results from rabbit models indicate that intranasal inoculation with *C pneumoniae* either may result in aortic changes consistent with early atherosclerosis^{19,20} or may accelerate its development.²¹ However, if *C pneumoniae* merely colonizes diseased tissue and has no pathological role in CAD, its distribution would also be consistent with that observed here. The ability of *C pneumoniae* to induce or exacerbate atheroma in a population probably depends on more complex interactions with other factors than is generally appreciated. It is likely that the question of whether *C pneumoniae* causes or exacerbates CAD can be answered only by well-controlled animal studies and by large-scale antibiotic intervention trials.

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