Confirmed Previous Infection With Chlamydia pneumoniae (TWAR) and Its Presence in Early Coronary Atherosclerosis

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Background—Chlamydia pneumoniae has been identified in coronary atheroma, but concomitant serum antibody titers have been inconsistently positive and unavailable before the detection of early or advanced atherosclerotic lesions.

Methods and Results—This retrospective investigation was performed on premortem serum specimens and autopsy tissue from 60 indigenous Alaska Natives at low risk for coronary heart disease, selected by the potential availability of their stored specimens. Serum specimens were drawn a mean of 8.8 years (range, 0.7 to 26.2 years) before death, which occurred at a mean age of 34.1 years (range, 15 to 57 years), primarily from noncardiovascular causes (97%). Coronary artery tissues were independently examined histologically and, for C pneumoniae organism and DNA, by immunocytochemistry (ICC) and polymerase chain reaction (PCR) with species-specific monoclonal antibody and primers. Microimmunofluorescence detected species-specific IgG, IgA, and IgM antibody in stored serum. C pneumoniae, frequently within macrophage foam cells, was identified in coronary fibrolipid atheroma (raised lesions, Stary types II through V) in 15 subjects (25%) and early flat lesions in 7 (11%) either by PCR (14, 23%) or ICC (20, 33%). The OR for C pneumoniae in raised atheroma after a level of IgG antibody $\geq 1:256 > 8$ years earlier was 6.1 (95% CI, 1.1 to 36.6) and for all coronary tissues after adjustment for multiple potential confounding variables, including tobacco exposure, was 9.4 (95% CI, 2.6 to 33.8).

Conclusions—Serological evidence for C pneumoniae infection frequently precedes both the earliest and more advanced lesions of coronary atherosclerosis that harbor this intracellular pathogen, suggesting a chronic infection and developmental role in coronary heart disease. (Circulation. 1998;98:628-633.)

Key Words: Chlamydia pneumoniae ■ coronary disease ■ atherosclerosis

In several populations worldwide, Chlamydia pneumoniae TWAR, a recently identified respiratory bacterial pathogen responsible for $\approx 10\%$ of community-acquired pneumonia in adults, has been reported in atherosclerotic lesions.1-3 Within macrophages of atheroma resected from coronary and carotid arteries, Chlamydia-specific structures and antigen have been identified by electron microscopy and immunocytochemistry (ICC), and species-specific nucleotide sequences have been identified by polymerase chain reaction (PCR). The organism is rarely found in vascular specimens from nonatherosclerotic patients and those with nonatherogenic arteriopathy.1-8 In addition, the agent has recently been directly isolated from human coronary7 and carotid9 artery atherosclerotic plaque. After rabbits received nasal inoculation of C pneumoniae, with their subsequent seroconversion for organism-specific IgG antibody, the organism was cultured from their early aortic atherosclerotic plaque.19

Published reports on the seroepidemiology of this agent and coronary heart disease in humans from 5 distinct populations have used various study designs and generally support an association.11-19 Prior infection has been defined by combinations of C pneumoniae-specific IgG or IgA antibody at various levels and circulating immune complexes of chlamydial lipopolysaccharide.11-14 An additional report from a multicenter US cohort study indicated an odds ratio (OR) of 2.0 between IgG and asymptomatic carotid atherosclerosis.18 Serum IgG antibody levels $\approx 1:64$ conferred a 2- to 7-fold risk for concurrent coronary artery disease, but neither the presence of serum antibody nor its dose response has been associated with a finding of C pneumoniae antigen or DNA in atherosclerotic lesions.1,3,7,20

At present, the evidence for an association of C pneumoniae and atherosclerosis does not constitute causation. Data regarding whether infection precedes disease (temporality) are circumstantial: the presence of IgG or IgA antibody and absence of IgM antibody simultaneously with the diagnosis of disease or the identification of the organism. Concern
persists that a finding of *C. pneumoniae* antigen or DNA in coronary atheroma or an immunologic response in patients with advanced disease may reflect only a relatively late-onset “passenger” role of the organism migrating within macrophages to the site of disease, rather than playing an early role in the endothelial injury hypothesized to initiate atherosclerosis. Only the Helsinki Heart Study provided serological data suggesting that infection does not represent a proclivity for *C. pneumoniae* to land in injured cardiac tissues or for myocardial damage to reactivate a latent infection.

This study was designed to determine whether infection with *C. pneumoniae*, diagnosed by the host *C. pneumoniae*–specific antibody response, preceded any direct evidence of this organism in coronary artery tissue from low-risk subjects with early disease. We studied indigenous Alaska Natives with a lower mortality rate from coronary heart disease compared with whites both in Alaska and the rest of the United States. A recent forensic autopsy study, including 66% of subjects with a violent cause of death, demonstrated a lower prevalence of raised atherosclerotic lesions in Alaska Natives than in non-Natives. The basis of the present report is the analysis of coronary artery tissue specimens from Alaska Natives in that study and their stored serum specimens obtained earlier for other reasons.

### Methods

#### Cases

Sixty subjects were selected by matching all 103 Alaska Natives previously autopsied and reported elsewhere to a population-based serum bank of >300,000 Alaska Native specimens maintained by the Centers for Disease Control and Prevention in Anchorage, Alaska. Subjects selected were 75% male and included 47 Eskimos, 5 Aleuts, and 8 Indians residing in 40 communities statewide. They died between February 1989 and December 1992 at a mean age of 34.1 years (range, 15 to 57 years). Of these deaths, 97% were due to other causes.

#### Specimens

After forensic autopsy, the right coronary artery was dissected from the heart, fixed in 10% buffered formalin, stained with Sudan IV stain, and evaluated for lesions of the intimal surface as described elsewhere. Slides were read by a cardiovascular histopathologist, masked to any other laboratory results, who used light microscopy and the classification of Herbert Stary to grade atherosclerosis, measure coronary intimal thickness, and assess foam cells, lipid, and calcium within atheroma.

Fifty-six subjects each had 1 accessible premortem serum specimen, a mean of 8.8 years (range, 0.7 to 26.2 years) before death, and 4 subjects had inadequate amounts of serum available. For 22 subjects, a second serum specimen was available that was drawn a mean of 8.2 years (range, 0.7 to 26.2 years) before death, and all stored serum specimens were collected for noncardiovascular health screening programs, including hepatitits, and none were obtained for acute respiratory disease investigations. Blood specimens at autopsy, obtained by the prosector from either the vena cava, heart, or aorta, were centrifuged, frozen, and available from only 45 subjects for serum thiocyanate levels.

#### Analysis of Specimens

**Immunocytochemistry**

Tissue sections sequential to those examined for histology were stained by ICC with the genus-specific monoclonal antibody, CF-2, that recognizes the lipopolysaccharide antigen of *Chlamydia* species. Positive-staining tissues were subsequently tested with the *C. pneumoniae*–specific monoclonal antibody, RR-402, with appropriate controls of normal mouse ascitic fluids and HL cell monolayers infected with *C. pneumoniae* or *C. trachomatis*. Slides were read independently of other study results.

**Polymerase Chain Reaction**

PCR was performed on DNA obtained from fixed tissue sections 16 mm thick with *C. pneumoniae*–specific HL-1, HR-1 primer sets that amplified a 437-bp *C. pneumoniae*–specific DNA sequence. DNA was purified by boiling the sections in a 5% suspension of Chelex 100 chelating resin (Sigma Chemical Co) in sterile water. The resulting supernatants were extracted with phenol/chloroform by standard methods and precipitated with ethanol, and the DNA pellets were resuspended in 50 μL Tris-ethanolamine buffer at pH 8. Mock extractions of buffer were done and amplified to ensure that no contamination occurred. A control consisting of PCR reagents without any specimen and various dilutions of purified *C. pneumoniae* DNA were done in each PCR run as the negative and positive controls, respectively. Presumptive positives and detection of products below the sensitivity of agarose gels were confirmed by immunocchiochemiluminescence as previously described.

#### Serology

Serum was examined for IgG, IgM, and IgA antibody to *C. pneumoniae* with the microimmunofluorescence test developed by the coauthors (S.-P.W., J.T.G.). Formalin-fixed whole elementary bodies of *C. pneumoniae* strain AR-39, a pharyngeal isolate obtained in Seattle, were used as antigen to determine the presence of species-specific antibodies in stored serum specimens. This assay is not cross-reactive and is reproducible within a 2-fold variation. Serum specimens were tested independently of all other results.

**Serum Thiocyanate**

Serum thiocyanate levels were measured as in previous autopsy studies. A smoker was defined as having a serum thiocyanate level ≥90 μmol/L, a threshold previously established in living smokers and nonsmokers.

#### Statistical Analysis

Data were analyzed with univariate and multivariate programs (SAS Institute). Geometric means of IgG antibody were adjusted with multiple potentially confounding variables were entered into a multivariate logistic model as independent variables, with the response variable as *C. pneumoniae* organism demonstrated in coronary artery by either ICC or PCR. Univariate analyses used appropriate Student’s *t* test and χ2 test.

### Results

#### Detection of *C. pneumoniae*

*C. pneumoniae* organism was identified with PCR or ICC staining in coronary arteries of 37% of all subjects (22/60). Data in Table 1 indicate that ICC staining was more often positive than PCR (20 versus 14 specimens, respectively), and both techniques identified the organism in 55% (12/22) of all positive subjects. Positive specimens included 35% (14/40) of those with a raised atheroma and 39% (7/18) of those with early flat lesions, including adaptive intimal thickening. Among cases positive or negative for *C. pneumoniae*...
of the organism, there was a similar distribution of the established causes of death, sex, and similar median ages.

**Histopathology**

Raised fibrolipid plaques (Stary type III through V) were found in coronary arteries in 40 individuals. Lower-grade lesions were present in 18 subjects, including adaptive intimal thickening in 13 (Table 1). Two specimens could not be graded. Macrophage foam cells were identified in 77% of specimens that were positive for *C pneumoniae*, compared with 8% with no organism (P, 0.0001). Foam cells were identified in 54% (7/13) of specimens with only adaptive intimal thickening, including 3 of 5 with *C pneumoniae* demonstrated. In specimens with *C pneumoniae*, the mean thickness of the intima was 16% greater and a lipid core within these atheroma was more common than in specimens without the organism, although neither difference was statistically significant (Table 1).

**Serological Testing**

Premortem serum specimens were available for 95% of all subjects who were positive for *C pneumoniae* organism and for 92% of those who were negative, with similar time intervals between collection of serum and death (Table 1). Among 56 subjects, the proportions with a 1:16 level of *C pneumoniae*-specific IgG, IgA, and IgM antibody in serum specimens obtained 1 to 24 years before death were 84%, 57%, and 5%, respectively, and for a level of 1:128 they were 63%, 13%, and 0%, respectively. Although IgA antibody was detectable more frequently at higher levels of IgG (82% at $\geq 1:256$ IgG compared with 68% for $\geq 1:16$ IgG, P, 0.05), IgA was not associated with *C pneumoniae* in tissue. There was no sex-specific difference in seropositivity; however, the power of the study to detect this was only 55%.

As presented in Table 2, the unadjusted serum geometric mean titer (GMT) of IgG antibody for subjects with *C pneumoniae*-positive coronary arteries, all ages combined,
was 94.9, versus 54.9 \((P=0.114)\) for those with organism-negative specimens. Most of this difference occurred in subjects >35 years of age who had an almost 5-fold higher GMT preceding a finding of organism in tissue \((P=0.024)\). Although no difference was noted in younger subjects, interaction between age and the presence or absence of \(C\) pneumoniae did not achieve statistical significance. After adjustment for age, smoking, and the interval between dates of serum acquisition and death, the almost 2-fold difference in the GMTs of IgG antibody still did not reach significance.

As presented in Table 3, the unadjusted OR for \(C\) pneumoniae–specific IgG antibody and a subsequent finding of this organism in the coronary artery is significant for an antibody level of \(\geq1:256\). The serum specimens with this level of IgG antibody preceding this finding were obtained a mean of 8.3 years (median, 6.6 years) before death. Serum specimens with this level of antibody were followed by the absence of organism at autopsy by a mean of 14.4 years \((P=0.04\) for the difference) and median of 16.1 years \((P=0.03)\). For lower thresholds of IgG antibody, the presence of \(C\) pneumoniae in coronary artery was not statistically significant.

Other stratified univariate analyses included examining the association of this threshold of IgG antibody, \(\geq1:256\), and \(C\) pneumoniae organism in coronary artery by grade of atheromatous lesion. This OR among subjects with raised atheroma (Stary types III through V) was 6.08 (95% CI, 1.11 to 36.6; \(P=0.03)\), but the OR of 3.11 for those with flat lesions (Stary types I or II), including adaptive intimal thickening, did not achieve statistical significance (95% CI, 0.28 to 40.64; \(P=0.35)\). In addition, smoking status, defined by a postmortem serum thiocyanate of \(\geq90\) \(\mu\)mol/L, was unrelated to either the presence of \(C\) pneumoniae organism in coronary arterial tissue (OR, 0.64; 95% CI, 0.15 to 2.77) or to premortem serum IgG antibody of \(\geq1:256\) (OR, 0.22; 95% CI, 0.03 to 1.36). Smoking status was more commonly determined in individuals with any atherosclerotic lesion compared with those with only adaptive intimal thickening (OR, 7.78; 95% CI, 0.82 to 182), but this difference was of marginal statistical significance \((P=0.06)\).

Multivariate analysis permitted an adjustment for the potential confounders of age, raised histological lesion, interval from serum to death, and smoking. Those produced statistically significant ORs of 3.65 and 9.40 for IgG antibody titers of \(\geq1:128\) and \(\geq1:256\), respectively. After the backward elimination of statistically insignificant variables from this full model, the only remaining covariate directly associated with the presence of \(C\) pneumoniae in coronary arteries at autopsy was an antemortem level of \(C\) pneumoniae–specific IgG antibody of \(\geq1:256\) (OR, 8.01; 95% CI, 2.46 to 25.99). The time interval from this earliest identified seropositivity to death showed a modest inverse relationship (OR, 0.86; 95% CI, 0.76 to 0.99).

Persistence of seropositivity for \(C\) pneumoniae was examined in 22 subjects with a second subsequent serum specimen available before their death, but persistence was difficult to correlate with infection at autopsy because only 5 subjects in this subgroup had demonstrable organism. A total of 19 subjects had IgG antibody \((\geq1:8)\) in their first specimen, and 20 were positive in their second serum. There was little trend over time for either IgG or IgA antibody \((r=0.255, P=0.265)\) compared with the initial antibody levels, which were used in all primary analyses. Persistent IgG and IgA antibody titers were 82% (18) and 27% (6), respectively, including lower but present second values of IgG in 56% (10) and of IgA in 50% (3) of those subjects with both serum specimens positive. More than one fourth of subjects with declining serum antibody levels, whether IgG (3/11) or IgA (2/7), had a finding of \(C\) pneumoniae organism at autopsy. Of those 9 subjects with multiple serum specimens and initial IgG antibody levels of \(\geq1:256\), 78% (7/9) remained positive at the same level, higher, or only 1 dilution less than the initial value. However, the organism was not identified in tissue from 86% (6/7) of these subjects with persistently high antibody levels.

### Discussion

This study provides direct evidence of infection with \(C\) pneumoniae in coronary arteries obtained at autopsy and a serological diagnosis of infection in the same individuals 5 to 14 years earlier, consistent with \(C\) pneumoniae playing a role in the pathogenesis of atherosclerosis. This significant relationship with organism-specific DNA or antigen appears only with the highest levels of preexisting \(C\) pneumoniae–specific IgG antibody established for the serological diagnosis of respiratory infections. \((P=0.024)\) This correlation of prior infection and subsequent molecular and immunologic evidence of the organism in atheromatous tissue has not been reported previously and strongly suggests a persistent or chronic infection. In South African subjects, \(^3\) high antibody titers were not correlated with the finding of \(C\) pneumoniae organism in atheroma at autopsy, nor was there any difference in the detection of \(C\) pneumoniae in coronary atherectomy or carotid endarterectomy specimens from US patients with undetectable, low, or high IgG antibody titers. \(^3\) Another study of explanted hearts indicated seropositivity in patients both with and without demonstrable organism and coronary atherosclerosis. \(^7\) In a recent trial, however, persistent seropositivity \(\geq1:64\) during a 3-month interval was related to secondary cardiovascular events. \(^3\) After the administration of antibiotic treatment directed at \(C\) pneumoniae, this level declined, along with the cardiovascular event rate.

A higher seroprevalence rate may be the driving force behind the high levels of IgG antibody defining \(C\) pneu-
moniae cardiovascular infection in the Alaska Native pop-
ulation we studied. Prevalence rates of 77% and 49% for levels of ≥1:16 and ≥1:128, respectively, were noted in subjects in whom no C pneumoniae was demonstrated in coronary tissue. In other populations studied, lower antibody levels (1:8 to 1:64) defining infection in patients with coronary atherosclerosis have been accompanied by lower seroprevalence rates in healthy control subjects, 42% to 59%, for a titer of ≥1:16. It is noteworthy that in Alaska Natives in 1994, pneumonia, usually nonbacteremic, persisted as the third most common reason for hospitalization, consistent with an undiagnosed burden of C pneumoniae respiratory infection.

The finding in this study of C pneumoniae organism in coronary arterial lesions described as adaptive intimal thickening is consistent with previous reports that the organism is never or rarely found in normal coronary artery. Arterial locations of intimal thickening correspond to regions of altered mechanical stress and increased turnover of endothelial cells and smooth muscle cells and increased concentrations of LDLs. These areas have been referred to as atherosclerosis-prone areas, and eccentric intimal thickening indicates a region of increased susceptibility to plaque formation. At the University of Washington, C pneumoniae was previously demonstrated in 18% (2/11) and 44% (7/16) of adaptive intimal thickening and fatty streaks, respectively, in young adults. In the present study, macrophage foam cells were present in more than half of the specimens with only adaptive intimal thickening, with and without C pneumoniae. A marked increase in the prevalence of these progenitor atheromatous cells characterized our subjects’ specimens with C pneumoniae and suggests the coexistence of this organism and early pathogenesis. The recently reported replication of C pneumoniae within human macrophages, endothelial cells, and vascular smooth muscle cells gives biological plausibility to the concept of a chronic intravascular infection that produces rather than follows an immune response. Our finding of an associated serological response to the presence of the organism in raised coronary lesions supports this sequence of events.

The potential limitations of this study merit discussion. We are aware of the criticism that “shopping” for optimal cut points in continuous data of prognostic factors to obtain statistical significance or a minimum P value for a threshold can elevate the global error rate with false-positives. However, our data were ordinal, and our choice of thresholds was guided by the biological basis of our reasoning that severe, persistent, or recurrent infections more likely to generate a high level of antibody are most likely to be associated with cardiovascular disease. The earliest date of seropositivity was determined only by the availability of the serum specimen and may still not have preceded the biological onset of atherosclerosis in these subjects. Because IgM was not present, seropositivity most likely reflected an earlier seroconversion. Moreover, a risk factor for this disease need not be primordial. Early evidence of coronary atherosclerosis in adolescence precedes exposure to many of the acknowledged risk factors that occur only in adulthood. Because small amounts of tissue were examined by ICC staining, misclassification of tissues read as negative was possible. However, the marked difference in accompanying macrophage foam cells is consistent with our results. Our opportunity to correlate persistence of antibody with confirmed infection was restricted by the limited availability of paired serum specimens from individuals with demonstrable C pneumoniae at autopsy. Although this study did not include control subjects, our results permit comparisons between subjects with and without demonstrated C pneumoniae and raised atheroma. Although we did not use parallel assays in collaborating laboratories, as others have recently done to confirm their findings of an association of C pneumoniae and coronary atherosclerosis, all of our tissue specimens were uniformly examined independently by all assay methods used. And finally, our conclusions offer no insights regarding which of the several biological effects of chronic C pneumoniae infections are the most likely pathogenic mechanisms postulated for this bacterium in coronary atherosclerosis.

In this study of Alaska Natives, the evidence for infection preceding or accompanying early asymptomatic lesions in young, low-risk adults is consistent with the expectation that exposure to risk factors for coronary atherosclerosis should

<table>
<thead>
<tr>
<th>Premortem Serum IgG Antibody to C pneumoniae</th>
<th>No. (%) of C pneumoniae Present in Coronary Artery (n=21)</th>
<th>No. (%) of Subjects With C pneumoniae Absent in Coronary Artery (n=35)</th>
<th>Univariate OR (95% CI)*</th>
<th>Adjusted OR (95% CI)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3 (14)</td>
<td>4 (11)</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>≥1:8</td>
<td>18 (86)</td>
<td>31 (86)</td>
<td>0.77 (0.12, 5.02)</td>
<td>0.83 (0.70, 3.42)</td>
</tr>
<tr>
<td>≥1:16</td>
<td>18 (86)</td>
<td>27 (77)</td>
<td>1.78 (0.35, 9.88)</td>
<td>2.18 (0.57, 8.29)</td>
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<tr>
<td>≥1:32</td>
<td>17 (81)</td>
<td>24 (69)</td>
<td>1.95 (0.46, 8.81)</td>
<td>2.37 (0.72, 7.82)</td>
</tr>
<tr>
<td>≥1:64</td>
<td>16 (76)</td>
<td>23 (66)</td>
<td>1.67 (0.43, 6.79)</td>
<td>1.91 (0.63, 5.82)</td>
</tr>
<tr>
<td>≥1:128</td>
<td>15 (71)</td>
<td>17 (49)</td>
<td>2.65 (0.73, 9.93)</td>
<td>3.65 (1.25, 10.65)</td>
</tr>
<tr>
<td>≥1:256</td>
<td>12 (57)</td>
<td>8 (23)</td>
<td>4.50 (1.21, 17.37)</td>
<td>9.40 (2.61, 33.84)</td>
</tr>
</tbody>
</table>

*Referent category is all subjects with lower and/or absent antibody levels.
†OR obtained by multiple logistic regression analysis including the variables of continuous age, raised histological lesion, interval from serum to death, and smoking defined by a serum thiocyanate level of 90 μmol/L.
occur before or during the earliest stages of disease development. This study also suggests some additional evidence for the dose-response criterion of causality both with respect to the grade of atherosclerotic lesion and the level of Chlamydia pneumoniae–specific antibody. Stored serum specimens antedating the direct demonstration of the organism in atheroma or confirmed coronary atherosclerosis should be used in current cardiovascular cohort studies with this resource and well-documented clinical end points with access to coronary artery tissue. Correlation of these data in individuals may indicate where in the natural history of Chlamydia pneumoniae infection the primary prevention of cardiovascular disease might be effected with antibiotics or vaccination, thereby demonstrating the ultimate criterion of causality, the cessation of the exposure followed by a reduction of disease.

Acknowledgments

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References

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