Failure to Detect Chlamydia pneumoniae in Coronary Atheromas of Patients Undergoing Atherectomy

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To further investigate a proposed relationship between Chlamydia pneumoniae and coronary heart disease, coronary atheromas were collected from patients undergoing percutaneous atherectomy. Fifty-eight atheroma specimens were examined by culture and polymerase chain reaction (PCR) and 22 by electron microscopy. All were negative for C. pneumoniae, except a single specimen that was PCR-positive. These results differ from studies in other populations, in which this organism was identified by nonculture methods within coronary atheromas obtained at autopsy. Anti-C. pneumoniae antibody titers from 65 of the patients were compared with those of 28 asymptomatic controls. IgG titers were higher in controls than in patients. There is no evidence that C. pneumoniae exists within atheromas in this study population, nor does seroprevalence correlate with the presence of coronary disease in these patients.

Chlamydia pneumoniae, a common cause of both upper and lower respiratory tract infection [1, 2], has been investigated as a possible cause of coronary heart disease (CHD) and atherosclerosis. Collaborating investigators have demonstrated a statistical correlation between serologic evidence of C. pneumoniae infection and the presence of atherosclerotic vascular disease in populations in Finland and Seattle [3–6]. Subsequent reports have described the detection of this organism by several nonculture methods in coronary atheromas obtained from autopsies [7, 8] or atherectomy specimens [9]. These methods included polymerase chain reaction (PCR), immunohistochemistry, and transmission electron microscopy [7–9]. It has been hypothesized that C. pneumoniae is transported from the lungs into the circulation by pulmonary macrophages, where it then infects vascular tissue [4]. This infection, through mechanisms that remain to be elucidated, may then lead to the development of atherosclerosis.

Isolation of C. pneumoniae in culture from coronary atheromas was attempted in an autopsy study by Kuo et al. [8] but not in an atherectomy study by Campbell et al. [9]. Kuo et al. [8] were unable to isolate C. pneumoniae using an HL cell tissue culture system, even from specimens in which nonculture tests were positive. This may have been because the autopsy specimens were collected in South Africa but cultured in Seattle. Delays between death and specimen collection, and difficulty in maintaining ideal transport conditions, may have rendered any organisms nonviable.

To further investigate the possible presence of C. pneumoniae within coronary tissue, we attempted to isolate this organism from atheromas obtained from patients with clinically significant CHD undergoing percutaneous coronary atherectomy. Through collaborations at Johns Hopkins University and the University of Washington, cultured specimens were also examined by PCR, and a separate group was examined by transmission electron microscopy.

Methods

Patients. Atherectomy specimens were obtained from patients undergoing clinically indicated coronary atherectomy in the Cardiac Catheterization Laboratory of the Maimonides Medical Center. Only patients undergoing primary atherectomy on native vessels were enrolled. On completion of the procedure, atherectomy tissue (~10–20 mg) was placed either into Chlamydia transport medium and frozen at −70°C or into Karnovsky’s glutaraldehyde–paraformaldehyde fixative and refrigerated.
Cultures. Specimens were periodically removed from the freezer and hand-carried the 4 km to our laboratory in an insulated container filled with dry ice. On arrival in the laboratory, the specimens were again stored at -70°C until processing. Specimens were then thawed and divided under sterile conditions in a laboratory separate from the Chlamydia laboratory. One portion was placed into PCR buffer and refrozen. The other portion was homogenized in a ground glass tissue grinder and then sonicated. Monolayers of cycloheximide-treated HEp-2 cells grown on multiwell microtiter plates were then inoculated and incubated as described previously [10]. Each plate included a positive and a negative laboratory control, and two passes were done on each sample. The monolayers were stained with a fluorescein-conjugated genus-specific anti-Chlamydia antibody for detection of inclusions.

PCR. Frozen specimens in PCR buffer were shipped from Brooklyn to Baltimore on dry ice. After thawing, they were ground using disposable grinders. Gloves were changed between each specimen. As previously described, the specimens were treated with 300 µL of lysis buffer, consisting of PCR buffer containing proteinase K and NP-40/Tween 40 at concentrations of 100 µg/mL and 0.5%, respectively, at 60°C for 1 h [11-13]. After heating for 5 min at 100°C, 50 µL was subjected to PCR for a 463-bp sequence of the 16S rRNA gene of C. pneumoniae. Detection of PCR products was by hybridization with a 270-bp nested, biotin-labeled RNA probe and subsequent EIA, which used anti-biotin-coated microtiter plates and an anti–DNA–RNA monoclonal antibody conjugated to alkaline phosphatase. After addition of a substrate of methylumbelliferyl phosphate, the end product, methylumbelliferone, was detected in a fluorometer. Positive values were calculated at 5 SD above the mean of negative controls. Positive laboratory controls were included in each batch in serial dilutions ranging from 200 to 0.1 IFU/reaction. Negative laboratory controls were processed along with patient specimens. PCR was done, using aerosol barrier pipette tips, in a room separate from the laboratory where the EIA was done. Rigorous attention was paid to decontamination procedures with 1 N HCl between runs [11-13].

Transmission electron microscopy. Atherectomy specimens in Karnovsky’s fixative were processed and viewed as described by Kuo et al. [8].

Controls. Patients undergoing predication screening for elective surgical procedures or visiting the Family Practice Clinic were recruited as controls. Those aged ≥40 with neither a history nor symptoms of CHD or atherosclerosis were enrolled.

Nasopharyngeal cultures and serology. Nasopharyngeal specimens for C. pneumoniae culture were obtained from patients and controls using Dacron-tipped, aluminum-shafted swabs. Cultures were done on HEp-2 cells as described [10]. Blood was collected from patients and controls for C. pneumoniae serum. Serum was assayed for anti-TW183 IgG and IgM using the microimmuno-fluorescence method [2].

Statistical analysis. Differences in clinical characteristics between patient and control groups were analyzed by χ² (discrete values) or t test for independent samples (means of continuous variables). Differences in antibody titers between groups were analyzed by logistic regression, adjusted for baseline clinical characteristics. The above analyses were done on SPSS software (SPSS, Chicago). Results of nasopharyngeal cultures were analyzed by Fisher’s exact test, and our results were compared with those of Kuo et al. [8] and Campbell et al. [9] by the two-tailed binomial test (StatXact-Turbo and StaTable, respectively; Cytel Software, Cambridge, MA).

Results

Patients and controls. Seventy-two patients and 28 controls were enrolled. The predominant indications for atherectomy included angina pectoris (45 patients, 63%) and acute myocardial infarction (25 patients, 35%). Eight (18%) of the patients with angina had had a prior myocardial infarction. Fifteen controls were undergoing presurgical evaluation and 13 were attending the Family Practice Clinic.

Clinical characteristics are presented in table 1. Controls were younger and more likely to be female and nonwhite, to have a smoking history, and to have used antibiotics in the 2 months before enrollment.

Atherectomy cultures and PCR-EIA. Fifty-eight atheroma specimens were collected from 50 patients for C. pneumoniae culture. All were negative. Fifty-six specimens from these 50 patients were studied by PCR. One specimen was positive twice; a duplicate specimen from the same lesion was negative.

Electron microscopy. Twenty-two specimens from 22 other patients were examined by transmission electron microscopy. These specimens were not cultured or tested by PCR. Structures resembling C. pneumoniae were not found. However, each specimen did contain typical foam cells, smooth muscle cells, and cholesterol clefts (figure 1).

Nasopharyngeal cultures and serology. Nasopharyngeal cultures for C. pneumoniae were obtained from 50 patients and all 28 controls. A single culture in each group was positive (2% of patients, 3.6% of controls).

Serologic results were available for 65 patients and all 28 controls (table 2). When IgG titers ranging from ≥1:8 through ≥1:64 were defined as positive, controls were more likely to be positive than patients at all titers. This difference was statistically significant for IgG titers of ≥1:32 and ≥1:64 (P = .041 and .027, respectively, by logistic regression). When adjusted for baseline clinical characteristics, this difference was significant only for an IgG titer of ≥1:64. Five patients (8%) and 5 controls (18%), all with IgG titers of ≥1:64, had IgM titers ranging from 1:8 to 1:64. This difference was not significant (P = .23 and .58 by unadjusted and adjusted regression, respectively). The patient with a positive nasopharyngeal culture had an IgG titer of 1:256 and an IgM titer of 0, and the control who was culture-positive had IgG and IgM titers of 0.

Discussion

We were unable to isolate C. pneumoniae from 58 atherectomy specimens collected from 50 patients. Similarly, Kuo et al. [8] did not isolate this organism from 36 autopsy specimens,
In contrast, our specimens were collected from living patients and quickly frozen at −70°C in Chlamydia transport medium. We process nasopharyngeal cultures for C. pneumoniae in a similar manner and have been successful in isolating the organism under these conditions. Thus, it is likely that our results are in fact true-negative results, although the possibility that our atheromas contained C. pneumoniae in numbers too few to infect HEp-2 cells cannot be excluded. In addition, we were unable to detect C. pneumoniae in all but 1 of our specimens with the use of PCR. A specimen from 1 patient was PCR-positive, but a second specimen from the same lesion was negative. Even if this single result represents a true-positive result, the prevalence of 2% (1 of 50 patients) is significantly lower than the 43% found by Kuo et al. [8] and the 32% found by Campbell et al. [9] (P < .001 by the two-tailed binomial test). Although our primers differ from those used by Kuo et al. and Campbell et al., they have been used successfully for the diagnosis of respiratory tract C. pneumoniae infection in both immunocompetent and immunocompromised patients [12, 13]. Results of C. pneumoniae cultures in HEp-2 cells and PCR using our primers correlate well in the diagnosis of respiratory infection [14].

The results of transmission electron microscopy in our study are also in contrast to those of Kuo et al., who found “typical” forms in 6 and “atypical” forms in 11 of 21 autopsy specimens [8]. Our 22 electron microscopy specimens were processed and examined in Seattle by the same investigators (D.L.P., P.C.), so differences in technique and expertise cannot explain our discordant findings.

One possible explanation for our negative results is that our specimens were smaller than those examined by Kuo et al. and that by sampling variation they lacked what appears to be a patchy infection within atheromas [8]. Since each specimen was divided into separate portions for culture and PCR, the pieces of tissue studied by each method were indeed quite small. However, we find this explanation for our negative results unlikely. All of our specimens studied by electron microscopy and, by analogy, all of our other specimens contained foam cells, smooth muscle cells, and cholesterol clefs, the areas in which Kuo et al. [8] identified structures resembling C. pneumoniae. Even if the smaller size of our specimens reduced the likelihood of capturing the portion of atheroma containing the organism, our volume of specimens was such that >1 specimen would likely have been positive were C. pneumoniae in fact present. When analyzed by the binomial test, our combined positive results (1 of 80 specimens) remain significantly different from those of Kuo et al. (20 of 36 specimens), even if each of our specimens had one-eighth the pretest probability of infection within atheromas [8]. Since each specimen was divided into separate portions for culture and PCR, the pieces of tissue studied by each method were indeed quite small. However, we find this explanation for our negative results unlikely. All of our specimens studied by electron microscopy and, by analogy, all of our other specimens contained foam cells, smooth muscle cells, and cholesterol clefs, the areas in which Kuo et al. [8] identified structures resembling C. pneumoniae. Even if the smaller size of our specimens reduced the likelihood of capturing the portion of atheroma containing the organism, our volume of specimens was such that >1 specimen would likely have been positive were C. pneumoniae in fact present. When analyzed by the binomial test, our combined positive results (1 of 80 specimens) remain significantly different from those of Kuo et al. (20 of 36 specimens), even if each of our specimens had one-eighth the pretest probability of containing the organism. Furthermore, Campbell et al. [9] detected C. pneumoniae in 32% of 38 atherectomy specimens by PCR and in 45% by immunohistochemistry, suggesting that their smaller size does not affect the test results significantly.

Finally, our IgG and IgM results do not support an association between seropositivity for C. pneumoniae and CHD in our population. Our patient and control groups were not well-matched in several characteristics, and our data should therefore be interpreted with caution. However, it has been shown that seroprevalence increases slightly with age and that men are more likely than women to be seropositive [1]. Thus, any bias due to age or sex differences in our study would have favored an association between C. pneumoniae and CHD. Seroprevalence for C. pneumoniae has not been shown to vary according to race. In a series of 98 asymptomatic adults, 46 of whom were nonwhite, seroprevalence was 81% in whites and 83% in nonwhites [15] (C. Hyman, personal communication).

Curiously, while investigators in Seattle and Finland have reported a correlation between seroprevalence of C. pneumoniae and CHD [3–6], and one study found an increasing correlation with increasing IgG titers [3], positive specimens in the study of Kuo et al. [8] were from patients with IgG

20 (56%) of which were positive by one or more nonculture methods. In their study, electron microscopy demonstrated "typical" Chlamydia-like structures with the infectious elementary body form in 6 specimens (17%), suggesting that isolation of this organism would have been possible. The unavoidable delay between their subjects' deaths and specimen collection and suboptimal transport conditions may explain their negative culture results [8]. In contrast, our specimens were collected from living patients and quickly frozen at −70°C in Chlamydia transport medium. We process nasopharyngeal cultures for C. pneumoniae in a similar manner and have been successful in isolating the organism under these conditions. Thus, it is likely that our results are in fact true-negative results, although the possibility that our atheromas contained C. pneumoniae in numbers too few to infect HEp-2 cells cannot be excluded. In addition, we were unable to detect C. pneumoniae in all but 1 of our specimens with the use of PCR. A specimen from 1 patient was PCR-positive, but a second specimen from the same lesion was negative. Even if this single result represents a true-positive result, the prevalence of 2% (1 of 50 patients) is significantly lower than the 43% found by Kuo et al. [8] and the 32% found by Campbell et al. [9] (P < .001 by the two-tailed binomial test). Although our primers differ from those used by Kuo et al. and Campbell et al., they have been used successfully for the diagnosis of respiratory tract C. pneumoniae infection in both immunocompetent and immunocompromised patients [12, 13]. Results of C. pneumoniae cultures in HEp-2 cells and PCR using our primers correlate well in the diagnosis of respiratory infection [14].

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Table 1. Characteristics of patients and controls.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patients (n = 72)</th>
<th>Controls (n = 28)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>49 (68)</td>
<td>10 (36)</td>
<td>.005</td>
</tr>
<tr>
<td>Mean age (range)</td>
<td>65 (43–86)</td>
<td>58 (40–82)</td>
<td>.007</td>
</tr>
<tr>
<td>White race</td>
<td>6/72 (93)</td>
<td>7/28 (25)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Hypertension</td>
<td>35/71 (49)</td>
<td>13/28 (46)</td>
<td>.80</td>
</tr>
<tr>
<td>Diabetes</td>
<td>19/70 (27)</td>
<td>6/28 (21)</td>
<td>.56</td>
</tr>
<tr>
<td>Smoking</td>
<td>26/70 (37)</td>
<td>17/28 (61)</td>
<td>.03</td>
</tr>
<tr>
<td>Family history of CHD</td>
<td>32/69 (46)</td>
<td>9/28 (32)</td>
<td>.20</td>
</tr>
<tr>
<td>Elevated cholesterol</td>
<td>34/67 (51)</td>
<td>10/23 (43)</td>
<td>.55</td>
</tr>
<tr>
<td>URI within 2 months</td>
<td>9/38 (24)</td>
<td>3/28 (11)</td>
<td>.18</td>
</tr>
<tr>
<td>Antibiotic use within 2 months</td>
<td>5/56 (9)</td>
<td>7/27 (26)</td>
<td>.04</td>
</tr>
</tbody>
</table>

NOTE. Unless indicated, data are no./no. with information available (%). All comparisons were by χ² test for independent samples. CHD, coronary heart disease; URI, upper respiratory infection.
Figure 1. Transmission electron micrographs of atherectomy specimens. Top specimen contains normal-appearing smooth muscle (SM) cells. Collagen fibrils are interspersed between cells. Bottom specimen contains cholesterol clefts (CC) and numerous foam cells (FC). No C. pneumoniae particles are seen (original magnification, ×3300; bar = 1 μm).
titers ranging from 0 to 1:32. Patients with titers above 1:32 were entirely negative. This led to the speculation that high IgG titers might be protective against infection of vascular tissue. However, positive specimens in the atherectomy series by Campbell et al. [9] included those from patients with IgG titers as high as 1:256. Indeed, it would have been paradoxical to have found organisms predominantly in seronegative or low-titer positive patients when the original hypothesis that C. pneumoniae and CHD were related was based on increased seroprevalence in patients with CHD compared with controls.

C. pneumoniae has been shown to disseminate systemically in mice after respiratory inoculation [16]. That this occurs in humans is a plausible but unproven explanation by which C. pneumoniae may have infected coronary arteries in the patients studied by Kuo et al. [8] and Campbell et al. [9]. If so, why this phenomenon would occur in South Africa and Seattle but not Brooklyn is not easily explained. Local epidemiology may be involved, since C. pneumoniae, although found worldwide, exhibits different patterns of infection in different populations [1]. If patterns of infection do indeed differ between South Africa and Seattle and Brooklyn, this may partially explain our discordant findings, although the mechanism by which this would affect dissemination is not immediately obvious. Variation in virulence and invasiveness among geographically distinct strains of C. pneumoniae may be involved but has not been demonstrated.

The significance of identifying C. pneumoniae in atheromas remains to be determined, since the organism’s presence does not necessarily prove that it plays an etiologic role in CHD and atherosclerosis. Mechanisms by which C. pneumoniae infection may lead to atheroma formation have been proposed but are largely speculative. At present, it appears that if this organism can cause CHD, its role varies in different populations. The possible relationship between C. pneumoniae and CHD appears to be complex and will require further population-based studies as well as studies designed to determine the mechanisms of disease.

### Table 2. Seropositivity in patients and controls.

<table>
<thead>
<tr>
<th>IgG titer</th>
<th>Patients (n = 65)</th>
<th>Controls (n = 28)</th>
<th>P by logistic regression</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥1:8</td>
<td>51 (78)</td>
<td>26 (93)</td>
<td>.109</td>
</tr>
<tr>
<td>≥1:16</td>
<td>49 (75)</td>
<td>26 (93)</td>
<td>.067</td>
</tr>
<tr>
<td>≥1:32</td>
<td>47 (72)</td>
<td>26 (93)</td>
<td>.041</td>
</tr>
<tr>
<td>≥1:64</td>
<td>40 (62)</td>
<td>24 (86)</td>
<td>.027</td>
</tr>
</tbody>
</table>

**NOTE:** Data are no. (%). 5 patients had IgM titers of 1:8–1:64; 5 controls had IgM titers of 1:8–1:64.

### Acknowledgments

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### References


