Presence of *Chlamydia pneumoniae* in Human Symptomatic and Asymptomatic Carotid Atherosclerotic Plaque

Ronald LaBiche, PhD; Deloris Koziol, PhD; Thomas C. Quinn, MD; Charlotte Gaydos, DrPH; Salman Azhar, MD; Gary Ketron, MD; Suman Sood, BS; Thomas J. DeGraba, MD

**Background**—*Chlamydia pneumoniae* has been identified in atherosclerotic plaques of patients with cerebrovascular and cardiovascular disease. However, the direct causative effect of *C pneumoniae* infection in the activation of atherosclerotic plaque to a prothrombotic state remains to be established. The aim of the present study is to examine the correlation between intraplaque presence of chlamydiae and symptomatic carotid disease in humans.

**Methods**—Plaques from 37 symptomatic and 57 asymptomatic consenting patients undergoing carotid endarterectomy were snap-frozen, and the tissue was prepared for polymerase chain reaction analysis for *Chlamydia pneumoniae* per Institutional Review Board–approved protocol. Blood was drawn from each patient at the time of surgery for serological analysis.

**Results**—The overall rate of plaques positive for *C pneumoniae* was 14.82%, with 5 of 37 (13.5%) plaques from symptomatic patients and 9 of 57 (15.8%) from asymptomatic patients, which revealed a definitive presence of the organism. No association existed between *C pneumoniae* presence and symptomatic disease (*P* = 1.0). Also, no association existed between presence of *C pneumoniae* and severity of stenosis. Finally, seropositivity for anti-chlamydial IgG, IgA, and IgM anti-chlamydial antibodies did not correlate with identification of *C pneumoniae* in the plaques. However, high-serum anti-chlamydial IgA levels (≥1:128) were associated with occurrence of symptomatic disease (*P* = 0.03; odds ratio, 2.86; 95% CI, 1.12 to 7.28).

**Conclusions**—Presence of *C pneumoniae* as a single factor does not appear to be sufficient to explain the occurrence of cerebrovascular symptoms. Low sensitivity of seropositivity for IgG, IgA, or IgM associated with PCR-identified *C pneumoniae* presence in the plaque makes it unlikely to be valuable as the single determining factor for actively infected plaque. Association of high-level anti-chlamydial IgA with symptomatic disease suggests that chronic or acute chlamydial infection anywhere in the body could play a role in atherosclerotic plaque activation and be used as a marker to target populations in future stroke prevention trials. (Stroke. 2001;32:855-860.)

**Key Words:** atherosclerosis ■ carotid arteries ■ chlamydia ■ immunoglobulin ■ symptoms

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The role of inflammation in the initiation, maturation, and activation of atherosclerotic plaque is emerging as a pivotal component that requires further investigation. Infectious agents have been postulated to be triggers to each of the major phases of atherosclerosis. Organisms such as the herpesvirus family, in particular cytomegalovirus, and *Chlamydia pneumoniae* have been the focus of research associating infection with atherosclerotic disease. This is in part due to the ability of these organisms to cause a chronic infectious state and to upregulate cytokines and adhesion molecules. *C pneumoniae* was chosen for the present study because it is an obligate intracellular parasite that commonly infects mononuclear phagocytes. Macrophages derived from monocytes characteristically are localized in human atherosclerotic plaque and provide a mechanism for entry of the organism into the vessel wall. Additionally, evidence exists for infection with *Chlamydia* species to create a persistent nonlethal infection, which can lead to a chronic inflammatory state by means of a variety of mechanisms. Endotoxin release and chlamydial heat-shock protein 60 have been associated with the increase of tumor necrosis factor-α and matrix metalloproteinase from macrophages. Chlamydial heat-shock protein 60 causes oxidation of LDL, which alters LDL to its highly atherogenic form. Finally, chlamydiae can activate CD4 and CD8+ T-lymphocytes through major histocompatibility complexes I and II. These factors demonstrate the
propensity and ability of *Chlamydia* to localize in atherosclerotic plaque and exacerbate, if not initiate, the atherosclerotic inflammatory process.

Animal studies have demonstrated an association between inoculation of *C. pneumoniae* and rapid development of atherosclerotic disease in New Zealand White rabbits. Also, the atherosclerotic process has been demonstrated to be accelerated in apolipoprotein E-deficient mice with injection of *C. pneumoniae*. Numerous studies have been reported to show an association between chlamydial infection by immunohistochemical staining, polymerase chain reaction (PCR), and serological positive studies and coronary artery or cerebrovascular disease. Despite the association, no definitive proof exists that presence of *Chlamydia* species or other infectious agents causes either initiation of atherosclerosis or progression of atherosclerotic plaque to a symptomatic state in humans. Preliminary studies with antibiotics for secondary prevention of coronary disease have been performed, with mixed results. However, criteria for similar studies in cerebrovascular disease are not well defined. The aim of the present study was to examine the association between presence of *C. pneumoniae* in human carotid atherosclerotic plaque and occurrence of ischemic symptoms. We also studied the predictive value of anti-chlamydial antibody seropositivity for plaque infection and its association with symptomatic disease.

Given the potential for enhancing proinflammatory mediators that could lead to plaque rupture or luminal thrombosis, we hypothesized that *C. pneumoniae* would be present in a significantly larger percentage of symptomatic versus asymptomatic carotid atherosclerotic plaque. We further hypothesize that elevated anti-chlamydial antibodies would be associated with presence of *C. pneumoniae* in the plaque and symptomatic disease. To test these hypotheses, carotid plaque specimens from symptomatic and asymptomatic patients were examined by PCR for chlamydial DNA. Serological analysis also was performed to determine whether circulating anti-chlamydial antibodies are markers for symptomatic disease and *C. pneumoniae* presence in the plaque.

**Subjects and Methods**

**Subjects**

Ninety-four patients undergoing carotid endarterectomy at the National Naval Medical Center consented to participate in this Institutional Review Board–approved protocol to study presence of *C. pneumoniae* in symptomatic versus asymptomatic patients. Patients were identified as candidates for carotid endarterectomy according to standard medical practices of the vascular surgery service at the participating hospital, following the North American Symptomatic Carotid Endarterectomy Trial (NASCET) and Asymptomatic Carotid Endarterectomy Study (ACAS) criteria. Patients were invited to participate in the protocol after the decision was made for the patient to undergo endarterectomy. Full history of prior medical conditions was taken, and neurological examination was performed and recorded by a neurologist from the Stroke Branch/National Institute of Neurological Disorders and Stroke (NINDS). Patients were classified as symptomatic (prior transient ischemic attack or stroke within 6 months) or asymptomatic (no prior history of cerebral ischemic event). Computed tomographic scans were obtained on all patients before surgery, and imaging studies were used to rule out silent infarcts in the “asymptomatic” population.

Stroke risk factors for hypertension (blood pressure >140/90 mm Hg for ≥1 year), past history of smoking (≥5 pack-years), diabetes (oral agent or insulin dependent for >1 year), and hypercholesterolemia (LDL cholesterol >160 mg/dL untreated, fasting triglycerides >200 mg/dL, or on cholesterol-lowering medications for >1 year), were recorded in all patients. Patients with atrial fibrillation or other conditions highly suspected to be cardiac sources of emboli were excluded from the symptomatic group to avoid possible confusion between a cardiac versus a carotid source of ischemic events. A screening for fasting blood glucose and a lipid profile were performed on all patients who had no known history of diabetes or hypercholesterolemia.

Plaques were obtained at the time of surgery in a sterile fashion, and blood was drawn for serological testing. Carotid stenosis was measured on angiogram by use of NASCET criteria.

**DNA Isolation**

Carotid atherosclerotic plaques were obtained from symptomatic and asymptomatic patients undergoing carotid endarterectomy at the National Naval Medical Center, Bethesda, Md. DNA was isolated from approximately 5 to 10 serial sections of about 16-μm thickness from snap-frozen excised tissues by use of the QiAamp tissue kit (Qiagen Inc) according to the manufacturer’s instructions, except that DNA was eluted in 2 steps of 50 μL each.

Care was taken to maintain aseptic handling of tissue samples. Patient samples and primary PCR reaction assembly were kept isolated in a separate laboratory to eliminate contamination of DNA samples by primary or secondary PCR products. The laboratory used to prepare the primary product was supplied separately from the main laboratory. Each sample was analyzed in duplicate or triplicate to insure reliability.

**PCR Amplification**

To detect *C. pneumoniae* DNA at the required level of sensitivity, a 2-step nested PCR protocol was implemented. The sequence of the 473-bp PstI fragment was used as the basis for this PCR, because it previously had been shown to have little sequence similarity to other members of this genus by dot-blot hybridization. Outer primers for the primary PCR step were designated CP1-L (5'-TTATTCACCCTCTCTACAGCAGAAA-3') and CP2-R (5'-GGGGTTTCAGGGATCATTTGT-3') and produced a 404-bp sequence that corresponds to a chlamydial polymerase. Inner primers (nested) contained within the 404-bp sequence were designated CPN1-L (5'-TTACGAAACGGCATTACAAACGCTAGAAATCATTTGT-3') and CPN1-R (5'-TTATGGCATATCCGCTTCGGGAAATCTAC-3') and were a 214-bp product.

After an initial 9-minute reaction at 95°C to activate the PCR enzyme, the outer PCR reaction was performed. This reaction consisted of 40 cycles of 30 s at 95°C to melt, 50 s at 60°C to anneal, and 30 at 72°C to extend the primers; was performed in 25 μL of PCR buffer, 50 nmol/L of each chlamydial primer (5'-ATCG-3', 5'-ATCG-3'), and 0.25 U of AmpliTaq Gold (Perkin Elmer Inc); and was templated with 1 μL of the DNA isolated from the patient samples. The inner, nested PCR reaction was performed with between 25 and 30 cycles of 15 s at 94°C to melt, 1 minute at 60°C to anneal, and 15 s at 72°C to extend the primers, in the same PCR reaction buffer as above. The nested reaction was templated with 1 μL of the primary PCR product hot-started at 50°C. PCR products were visualized under 300 nm of UV transillumination (Fotodyne Inc) after electrophoresis in 2% ultrapure agarose, 1:10 000 SYBR Green II (Molecular Probes Inc). Specific nested PCR product was identified by use of 5 to 15 ng of dH174 DNA restricted with HaeIII as a size marker. All PCR reactions were performed in a Perkin-Elmer model 9700 gene amplification PCR system (PE Applied Biosystems).
TABLE 1. Relationship Between C pneumoniae PCR Status of Carotid Plaque and Potential Risk Factors to Cerebrovascular Symptoms

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Chlamydial PCR+ (n=5)</th>
<th>Chlamydial PCR- (n=32)</th>
<th>P</th>
<th>Chlamydial PCR Association With Risk Factor</th>
<th>Effect of Chlamydial PCR on Symptoms, Controlling for Risk Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertension, n (%)</td>
<td>Symptomatic (n=4)</td>
<td>Asymptomatic (n=8)</td>
<td>4 (80)</td>
<td>8 (89)</td>
<td>27 (84)</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>Symptomatic (n=0)</td>
<td>Asymptomatic (n=0)</td>
<td>0 (0)</td>
<td>8 (17)</td>
<td>11 (34)</td>
</tr>
<tr>
<td>Smoking, n (%)</td>
<td>Symptomatic (n=2)</td>
<td>Asymptomatic (n=4)</td>
<td>2 (40)</td>
<td>8 (89)</td>
<td>24 (75)</td>
</tr>
<tr>
<td>Hypercholesterolemia, n (%)</td>
<td>Symptomatic (n=1)</td>
<td>Asymptomatic (n=9)</td>
<td>1 (20)</td>
<td>9 (100)</td>
<td>23 (72)</td>
</tr>
<tr>
<td>Coronary artery disease, n (%)</td>
<td>Symptomatic (n=2)</td>
<td>Asymptomatic (n=6)</td>
<td>2 (40)</td>
<td>6 (67)</td>
<td>16 (50)</td>
</tr>
<tr>
<td>Men, n (%)</td>
<td>Symptomatic (n=5)</td>
<td>Asymptomatic (n=8)</td>
<td>5 (100)</td>
<td>8 (89)</td>
<td>26 (81)</td>
</tr>
<tr>
<td>Age±SD, y</td>
<td>74.6±7.1</td>
<td>70.1±8.4</td>
<td>67.2±8.8</td>
<td>68.9±9.0</td>
<td>77.9±15</td>
</tr>
<tr>
<td>Stenosis±SD, %</td>
<td>77.7±18.6</td>
<td>73.6±11.3</td>
<td>77.9±15</td>
<td>72.1±13</td>
<td>77.9±15</td>
</tr>
</tbody>
</table>

*Fisher’s Exact Test, †Student’s t test, ‡Mantel-Haenszel χ² analyzing the effect of C pneumoniae PCR status on occurrence of symptoms, stratified by each risk factor; §Logistic regression modeling symptoms as dependent variable with chlamydial PCR status and continuous variable (age or stenosis) as independent covariates.

Serological Testing

Microimmunofluorescence was performed on serum samples from 91 of 94 patients for anti-IgG, anti-IgA, and anti-IgM for C pneumoniae by use of formalin-treated whole elementary bodies of organisms (Washington Research Foundation). This test is considered to be the reference standard for determination of anti-chlamydial IgG, IgM, and IgA antibody levels. Purified elementary bodies from high-titer C pneumoniae preparations were mixed with purified yolk sac and applied to glass slides (The Washington Research Center) Only 1 serovar of C pneumoniae existed, and purified antigen was made from strain AR39. Antigen dots for C trachomatis also were included in the series of antigen dots, so that specificity of the anti-C pneumoniae antibody in human serum could be confirmed. The highest-dilution serum that demonstrated good, even fluorescence of the elementary bodies was recorded as the titer for each group. The laboratory used for the present study participates with others in quality assurance studies for microimmunofluorescence. Adequate serum sample for serological testing was available for 91 of 94 [97%] of the patients. Dilutions were initiated at ratios of 1:8 through 1:1024. A value of ≥1:16 was considered positive.

Data Analysis

For comparisons of categorical variables, Fisher’s Exact Test (2-sided P value) was used. Continuous variables were compared by 2-sample t tests. For exploration of possible confounding risk factors, Mantel-Haenszel χ² was used for stratification analysis of dichotomous variables, whereas logistic regression was used in the analogous situation to control for continuous variables. For a final multivariate analysis, logistic regression was used, which modeled symptomatic state as the dependent variable and all other possible risk factors as covariates. For the logistic regression, Wald χ² was used to determine significance, and odds ratios and 95% confidence intervals were calculated. For all tests, a value of P≤0.05 was considered significant.

Results

Major risk factors, age, and degree of stenosis were similar in both Chlamydia-positive and -negative groups (Table 1). Only diabetes was nearly significant (Fisher’s Exact Test, P=0.07).

PCR Detection of C pneumoniae

DNA isolated from the carotid atherosclerotic plaques of 94 endarterectomy patients were tested in a 2-stage nested PCR protocol. Plaques from 37 symptomatic and 57 asymptomatic patients were studied. Overall rate of plaques positive for C pneumoniae was 15%; 5 of 37 (13.5%) plaques from symptomatic patients and 9 of 57 (15.8%) from asymptomatic patients revealed a definitive presence of the organism. No association was observed between presence of C pneumoniae and symptomatic disease (Fisher’s Exact Test, P=1.00).

Effect of Chlamydia-positive or -negative plaque on symptomatic state was explored further, and the study was controlled for each of the possible confounding risk factors listed in Table 1. These bivariate analyses did not reveal any association between presence of C pneumoniae and symptomatic plaques (all P>0.58).

Serological Detection of C pneumoniae

Serological studies of anti-chlamydial IgG, IgA, and IgM revealed no association between seropositive samples and presence of C pneumoniae within the plaque as measured by PCR. (Table 2). Only high anti-chlamydial IgA titers (≥1:128) were associated with symptomatic versus asymptomatic disease (Fisher’s Exact Test, P=0.03; Table 3). However, high titers of anti-chlamydial IgA, similar to the other immunoglobulin levels, were not associated with presence of intraplaque C pneumoniae. Four of 14 (28.6%) patients with PCR-positive plaque had high titers of IgA compared with 22 of 77 (28.6%) of patients who were PCR negative (Fisher’s Exact Test, P=1.00; Table 2). No serological level of any antibody class was associated with PCR-positive symptomatic plaque versus patients who were PCR positive and asymptomatic (data not shown; all P>0.58, Fisher’s Exact Test).

Multivariate Analyses

To explore definitively whether any factor was associated with symptoms, a logistic regression was modeled with symptom presence or absence as the dependent variable and all other possible risk factors (demographic, medical conditions, chlamydial PCR status, and chlamydial serologies) as
Discussion

Infection with *Chlamydia* represents a plausible and intriguing factor leading to atherosclerotic plaque progression and activation. As an obligate parasite, it can persist in a chronic state, increasing inflammatory mediator release and resulting in oxidation of LDL, among other mechanisms. The possibility that chlamydial infection increases the risk of stroke is so compelling that preliminary trials with antibiotics for primary prevention have been initiated. However, in the present study, no clear preponderance of occurrence of *C pneumoniae* presence in carotid atherosclerotic plaque could be associated with symptomatic patients. In fact, frequency of occurrence of *Chlamydia* was nearly identical between symptomatic and asymptomatic patients. No risk factor for stroke that is positively identified as a covariate in symptomatic disease appears to exist. At face value, these data would suggest that *C pneumoniae* does not play a major role in converting atherosclerotic plaque to the symptomatic state. If *C pneumoniae* could cause symptomaticity solely based on its presence, one could assume that a majority if not most of the plaques with *C pneumoniae* would fall in the symptomatic category. However, the presence of contributory factors may increase susceptibility of plaque to respond to infectious agents in an enhanced proinflammatory fashion in certain patient populations. For example, a potential scenario of plaque activation by foreign antigen is the proliferation of T cells, which are known components of atherosclerotic plaques. Plaques that possess resident T cells that have been previously sensitized to *C pneumoniae* would have a greater propensity for inflammatory activation and symptomaticity if reexposed. Symptomaticity could be mediated through increased risk of plaque rupture or increased luminal thrombosis.

Elevated anti-chlamydial IgA titers were significantly associated with symptomatic versus asymptomatic disease. Elevated anti-chlamydial IgA levels are believed to occur with reinfection of *C pneumoniae*. These titers will begin to decline within weeks to several months after reinfection. Persistently elevated levels are believed to be associated with a chronic infection state and have been noted to be associated with both chronic and acute coronary disease.

However, no association was seen between presence of *C pneumoniae* in atherosclerotic plaques and immunoglobulin titers. More than 70% of patients with plaque positive for *C pneumoniae* by PCR did not have high anti-chlamydial IgA titers. This lack of association makes anti-chlamydial IgA titer levels a poor indicator for demonstrable intraplaque presence of *C pneumoniae*. Because elevated anti-chlamydial IgA levels are associated with symptomatic disease, as previously reported in other studies, but not with intraplaque *C pneumoniae* presence, elevation of anti-chlamydial IgA may represent a more general chronic infection state, which might result in a greater likelihood of activation of generalized atherosclerotic plaque by circulating activated leukocytes. Another possible explanation for association of IgA levels to *Chlamydia* and symptomatic disease is the potential for a generalized increased immunoglobulin response to antigens not specific for *Chlamydia*. Persons who have been previously infected with *Chlamydia* could display an elevated anti-chlamydial IgA after exposure to a variety of antigens. This supports the hypothesis that generalized inflammatory response can activate atherosclerotic plaque, with
anti-chlamydial IgA acting only as a marker and not as an indicator of the specific antigen exposure. Further studies that focus on intraplaque differences between symptomatic and asymptomatic patients in presence of C pneumoniae need to be performed to identify any potential causative effect of infectious agents in thromboembolic atherosclerotic disease.

Several other findings and techniques necessitate brief discussion. First, the observed overall occurrence of intraplaque C pneumoniae by PCR in our population is lower than previously reported. The probes used in the present study were highly reproducible. Potential reasons are lack of sensitivity of the technique, although dilutional studies would indicate that this was not the case. Great care was taken to avoid contamination both from external sources and during initial amplification of the chlamydial DNA. The use of nested PCR was chosen to heighten the sensitivity of identifying Chlamydia within the atherosclerotic plaques. To avoid false-positives, primary PCR was performed in a separate laboratory with separate equipment. The technique of nested PCR is beneficial for reducing the likelihood that inhibitors within the tissue used would prevent identification of Chlamydia within the plaque.

Additionally, other studies used immunohistochemical staining, PCR, and serological testing as indicators of C pneumoniae presence. Another potential influencing factor of chlamydial presence in the plaque is that the population studied, which is characterized by military retirees and their dependents, has had supportive research. In conclusion, presence of C pneumoniae in carotid atherosclerotic plaque is not associated with the symptomatic state and therefore is unlikely to be sufficient as a single factor to cause thromboembolic stroke. C pneumoniae serological studies reveal that elevated anti-chlamydial IgA microimmuno-fluorescence was found to be a predictor of symptomatic disease but not a marker for presence of C pneumoniae in plaques as identified by PCR. Chlamydia still could be a modifier of atherosclerosis. However, future studies need to be performed to identify mechanisms by which C pneumoniae presence may be associated with the symptomatic state. Even if it is found that C pneumoniae, under some circumstances, enhances a proinflammatory and prothrombotic state in atherosclerotic plaques, our data suggest that it affects 5% to 15% of patients in our population. Future trials along this vein will need either to identify a surrogate marker that is consistent with intraplaque infection or to hypothesize one, given that our data suggest that reducing chronic infection with Chlamydia at any site in the body may be beneficial for reducing activation of atherosclerotic plaque.

Conclusions

In conclusion, presence of C pneumoniae in carotid atherosclerotic plaque may be associated with the symptomatic state and is therefore unlikely to be sufficient as a single factor to cause thromboembolic stroke. C pneumoniae serological studies reveal that elevated anti-chlamydial IgA microimmunofluorescence was found to be a predictor of symptomatic disease but not a marker for presence of C pneumoniae in plaques as identified by PCR. Chlamydia still could be a modifier of atherosclerosis. However, future studies need to be performed to identify mechanisms by which C pneumoniae presence may be associated with the symptomatic state. Even if it is found that C pneumoniae, under some circumstances, enhances a proinflammatory and prothrombotic state in atherosclerotic plaques, our data suggest that it affects 5% to 15% of patients in our population. Future trials along this vein will need either to identify a surrogate marker that is consistent with intraplaque infection or to hypothesize one, given that our data suggest that reducing chronic infection with Chlamydia at any site in the body may be beneficial for reducing activation of atherosclerotic plaque.

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