C-Reactive Protein Levels and Viable \textit{Chlamydia pneumoniae} in Carotid Artery Atherosclerosis

S. Claiborne Johnston, MD, PhD; Louis M. Messina, MD; Warren S. Browner, MD, MPH; Michael T. Lawton, MD; Caroline Morris, RN, CNS; Deborah Dean, MD, MPH;

\textbf{Background and Purpose}—An elevated serum level of C-reactive protein, an inflammatory marker, is an independent predictor of stroke and coronary artery disease. To determine whether chronic infection with \textit{Chlamydia pneumoniae}, which has been identified in atherosclerotic plaques, is responsible for systemic inflammation, we studied the association between serum C-reactive protein levels and infection of carotid artery atherosclerotic plaque with viable \textit{C pneumoniae}.

\textbf{Methods}—Serum C-reactive protein levels were obtained before endarterectomy for carotid artery stenosis. Plaques were tested for \textit{C pneumoniae} mRNA, an indicator of viability, and DNA by polymerase chain reaction of DNA and cDNA, respectively.

\textbf{Results}—Forty-eight samples were studied, of which 18 (38%; 95\% CI, 23 to 50) were infected with viable \textit{C pneumoniae} as evidenced by isolated chlamydial mRNA. All 18 of these samples, plus 1 additional sample, were positive for chlamydial DNA. Serum C-reactive protein levels were higher in those with viable \textit{C pneumoniae} compared with those without infection (median, 8 mg/L versus undetectable; \(P=0.045\) by Wilcoxon rank-sum test). In multivariable models, the only independent predictor of the presence of viable \textit{C pneumoniae} was a detectable C-reactive protein level (odds ratio, 4.2; 95\% CI, 1.1 to 17; \(P=0.04\)).

\textbf{Conclusions}—Viable \textit{C pneumoniae} are present in a substantial portion of carotid artery atherosclerotic plaques and are associated with increased serum C-reactive protein levels. These findings may explain the link between elevated C-reactive protein levels and the risk of cardiovascular disease and stroke but should be reproduced in a larger cohort. (\textit{Stroke}. 2001;32:2748-2752.)

\textbf{Key Words}: atherosclerosis ■ carotid arteries ■ \textit{Chlamydia pneumoniae} ■ C-reactive protein ■ inflammation

\textbf{C}-reactive protein is a sensitive indicator of acute and chronic inflammation.\textsuperscript{1} In apparently healthy men and women, an elevated serum level of C-reactive protein is a predictor of long-term risk of myocardial infarction and stroke.\textsuperscript{2, 3} It is also an independent predictor of the risk of cardiovascular events in patients with coronary artery disease.\textsuperscript{5} The association between serum C-reactive protein levels and subsequent risk of stroke, myocardial infarction, and death from cardiac causes supports the importance of inflammation in the pathogenesis of cerebrovascular and coronary artery disease.\textsuperscript{5, 6}

Focal inflammation is present in atherosclerotic plaques and has been associated with the progression of vascular stenosis and its complications.\textsuperscript{5−9} Chronic infection of arteries has been proposed as a cause of inflammation and a contributor to the initiation and progression of atherosclerosis. Polymerase chain reaction (PCR), immunocytochemical staining, and electron microscopy are among the techniques that have demonstrated evidence of \textit{Chlamydia pneumoniae} infection in coronary and carotid artery plaques.\textsuperscript{10−16} Detection of viable organisms, a clearer indicator of ongoing infection, has been more difficult. In 4 studies, viable \textit{C pneumoniae} has been cultured from a small proportion of atherosclerotic plaques.\textsuperscript{12, 14, 17, 18} In another study, evidence of viable \textit{C pneumoniae} was found in 10 of 30 patients by isolation of RNA and reverse-transcriptase (RT) PCR.\textsuperscript{19}

Chronic infection of blood vessels with \textit{C pneumoniae} could lead to elevation of C-reactive protein levels and contribute to the instability or progression of atherosclerotic plaques.\textsuperscript{20−23} Several studies have found an association between C-reactive protein and serological evidence of \textit{C pneumoniae} infection, whereas others have not.\textsuperscript{24−29} These variable results may be explained by poor correlation between positive chlamydial serologies and evidence of plaque

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One study compared C-reactive protein levels in patients with and without PCR evidence of \textit{C pneumoniae} in atherosclerotic plaques and failed to find a statistically significant difference.\textsuperscript{15} Thus, the connection between \textit{C pneumoniae} plaque infection, systemic inflammation, and symptomatic vascular disease remains uncertain. To determine whether viable \textit{C pneumoniae} organisms are present in atherosclerotic plaques and whether these organisms are associated with inflammation and the risk of stroke, we evaluated vascular risk factors, signs and symptoms of infection, preoperative serum C-reactive protein levels, and presence of viable \textit{C pneumoniae} in carotid tissue in a prospective study of patients undergoing elective carotid endarterectomy.

**Subjects and Methods**

**Subjects**

Between March 1998 and August 2000, all patients scheduled for elective carotid endarterectomy with preoperative evaluations at the University of California, San Francisco, were approached for participation in the study, which was approved by the local institutional review board for human research. All those providing consent from whom carotid plaque was available were enrolled. A clinician completed a structured interview to obtain information on risk factors for atherosclerosis (including elevated cholesterol, hypertension, diabetes, obesity, coronary artery disease, and smoking history), recent use of antibiotics, and signs and symptoms that might be considered compatible with \textit{C pneumoniae} infection (questionnaire available at www.neurology.ucsf.edu), although these infections may be asymptomatic in many cases. Medical records were reviewed to obtain information about medical history and medications during the prior year and at presentation.

Symptomatic carotid arteries were defined as those associated with stroke or transient ischemic attack. An operation on a previously treated artery was defined as a repeated endarterectomy. Antibiotics considered effective in treating \textit{C pneumoniae} included tetracycline, doxycycline, erythromycin, clarithromycin, azithromycin, and ofloxacin. Symptoms and diagnoses of infection and antibiotic usage were recorded if they occurred during the year before surgery. Cough, sore throat, nasal congestion, and sinus congestion were defined as symptoms of upper respiratory infection. Respiratory infections included diagnoses of common cold, flu, pneumonia, bronchitis, and sinusitis.

**C-Reactive Protein Assay**

All blood samples were drawn before surgery. C-reactive protein levels were determined with fixed-rate nephelometry (Nichols Institute, Quest Diagnostics), the most sensitive test available at study initiation; the technique is sensitive to levels below the detection threshold (4 mg/L).\textsuperscript{31} Levels below the detection threshold (<4 mg/L) were defined as not detectable.

**Carotid Samples**

Atherosclerotic tissue was removed by the operating physician using standard surgical techniques. The tissue was sectioned immediately through the center of the widest portion of arterial wall. One section was placed in a sterile Eppendorf tube and another in a tube containing 500 µL RNAzol B (Tel-Test, Inc). Both vials were stored at \(-80^\circ\text{C}\) until processed. DNA was extracted and purified as described above. In addition, primers specific for HPRT, a housekeeping gene, were used in PCR for each cDNA sample to determine the adequacy of the sample; all samples were amplified by these primers. Quantification of bacterial load was measured in all samples positive for \textit{C pneumoniae} by use of competitive PCR with known amounts of a polycompeitor cDNA containing \textit{C pneumoniae}. The polycompeitor contains an insert that increases its molecular weight compared with the wild type.\textsuperscript{37} Briefly, the polycompeitor was used in concentratons of 1, 10, 50, and 100 copies and run with each batch of patient samples for the PCR. A master mix was created that included all the ingredients for PCR except for the patient and polycompeitor samples. The master mix was divided equally among these samples; the respective sample cDNA or polycompeitor sample was added to the respective tube; and all tubes were subjected to the same PCR thermocycling parameters at the same time. Ten microliters of each patient and polycompeitor sample was run on a 1.5% agarose gel stained with ethidium bromide. The quantity of cDNA in the sample was determined by comparison with the results for the 4 concentrations of polycompeitor with the use of a Bio-Rad gel documentation system according to the manufacturer’s instructions. Samples that contained \(\geq 1\) copy of mRNA in the quantitative assay were considered to have viable organisms.

**Statistical Analysis**

Dichotomous variables were compared with Pearson’s \( \chi^2 \) test or Fisher’s exact test when any cell of a \( 2 \times 2 \) table was <5. Exact 95% CIs for prevalent \textit{C pneumoniae} were calculated by use of exact binomial probabilities. C-reactive protein levels, age, and pack-years of smoking were compared with the Wilcoxon rank-sum test, because these variables were not normally distributed. Multivariable logistic regression analysis was performed, including all variables associated with the presence of viable \textit{C pneumoniae} (at \( P<0.20 \)) and removing those no longer contributing (at \( P>0.10 \)) in a stepwise manner. All statistical analyses were performed with the STATA statistical package (version 6.0).

**Results**

Seventy-four patients were scheduled for elective carotid endarterectomy during the study period. Scheduling prevented enrollment of 26 patients (most commonly because the carotid plaque was removed when laboratory staff was unavailable); 1 patient refused consent; and plaque could not be removed intact in 2 others.

A total of 48 endarterectomy samples were obtained from 46 patients (mean \( \pm \text{SD} \) age, 72 \pm 8 years), 14 (30%) of whom were female. In 15 patients (31%), the treated carotid artery was associated with symptoms caused by stroke or transient ischemic attack; the remaining patients were asymptomatic. The procedure was a reoperation in 4 patients (8%). Vascular risk factors were common, including smoking (\( n=37 \)), hypercholesterolemia (\( n=33 \)), hypertension (\( n=31 \)), and diabetes mellitus (\( n=10 \)). Of the 44 patients who provided information on signs and symptoms of infection, 35 (80%) identified a diagnosis or symptoms of infection in the year before endarterectomy, most frequently consistent with respiratory infection (\( n=32, 73\% \)).

Serum C-reactive protein levels before endarterectomy ranged from undetectable to 86 mg/L, with a median below the detection threshold; 21 patients had detectable levels. Detectable C-reactive protein levels were associated with a
history of vomiting, diarrhea, or the taking of antibiotics in the prior year (the Table).

PCR revealed evidence of *C pneumoniae* in 19 carotid plaques (40%). Eighteen of these showed evidence of viable *C pneumoniae* (38%; 95% CI, 23 to 50) on the basis of the presence of chlamydial RNA. Two patients had bilateral endarterectomies; 1 had evidence of viable *C pneumoniae* in both plaques, whereas neither plaque from the other patient was infected. Patients with viable *C pneumoniae* were somewhat more likely to report signs and symptoms of infections in the prior year (the Table).

Serum C-reactive protein levels were greater in patients with viable *C pneumoniae* than in those without evidence of viable chlamydial organisms (median, 8 mg/L versus not detectable; *P* = 0.045; the Figure). Viable *C pneumoniae* were twice as common in those with detectable serum C-reactive protein levels than in other patients [52% (11 of 21) versus 26% (7 of 27); *P* = 0.06]. In multivariable models, the only independent predictor of the presence of viable *C pneumoniae* was a detectable C-reactive protein level (odds ratio, 4.2; 95% CI, 1.1 to 17; *P* = 0.04); the presence of signs or symptoms of infection in the prior year was retained in the model but was not a significant predictor (odds ratio, 6.6; 95% CI, 0.7 to 64; *P* = 0.10). No other demographic characteristics or vascular risk factors were predictors. The presence of viable *C pneumoniae* was not associated with neurological symptoms at presentation.

### Characteristics of Patients by Presence of Viable *C pneumoniae* and Detectable Serum C-Reactive Protein

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Viable <em>C pneumoniae</em></th>
<th>C-Reactive Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Present (n=18)</td>
<td>Absent (n=30)</td>
</tr>
<tr>
<td>Age (mean±SD) y</td>
<td>71±11</td>
<td>73±8</td>
</tr>
<tr>
<td>Female sex, n (%)</td>
<td>5 (28)</td>
<td>10 (33)</td>
</tr>
<tr>
<td>Non-Hispanic, white</td>
<td>18 (100)</td>
<td>25 (83)</td>
</tr>
<tr>
<td>Symptomatic carotid, n (%)</td>
<td>6 (33)</td>
<td>9 (30)</td>
</tr>
<tr>
<td>Repeated endarterectomy, n (%)</td>
<td>1 (6)</td>
<td>3 (10)</td>
</tr>
<tr>
<td>Ever smoker, n (%)</td>
<td>15 (88)</td>
<td>22 (79)</td>
</tr>
<tr>
<td>Current smoker, n (%)</td>
<td>5 (29)</td>
<td>4 (14)</td>
</tr>
<tr>
<td>Smoking (mean±SD), pack-y</td>
<td>47±37</td>
<td>40±41</td>
</tr>
<tr>
<td>Hypercholesterolemia, n (%)</td>
<td>10 (56)</td>
<td>23 (77)</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>11 (61)</td>
<td>20 (67)</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>5 (28)</td>
<td>5 (17)</td>
</tr>
<tr>
<td>Coronary artery disease, n (%)</td>
<td>10 (56)</td>
<td>14 (47)</td>
</tr>
<tr>
<td>Overweight, n (%)</td>
<td>7 (41)</td>
<td>8 (29)</td>
</tr>
<tr>
<td>Antibiotics, symptoms, and diagnosed infections in the prior year, n (%)</td>
<td>95x654</td>
<td>95x654</td>
</tr>
<tr>
<td>Prescribed antibiotics</td>
<td>10 (59)</td>
<td>11 (39)</td>
</tr>
<tr>
<td>Prescribed antibiotics covering chlamydia</td>
<td>3 (18)</td>
<td>3 (10)</td>
</tr>
<tr>
<td>Fever</td>
<td>4 (25)</td>
<td>5 (18)</td>
</tr>
<tr>
<td>Cough</td>
<td>4 (25)</td>
<td>8 (29)</td>
</tr>
<tr>
<td>Nasal congestion</td>
<td>7 (44)</td>
<td>9 (32)</td>
</tr>
<tr>
<td>Sinus congestion</td>
<td>6 (38)</td>
<td>7 (25)</td>
</tr>
<tr>
<td>Sore throat</td>
<td>6 (38)</td>
<td>5 (18)</td>
</tr>
<tr>
<td>Vomiting</td>
<td>4 (25)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>4 (25)</td>
<td>3 (11)</td>
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<tr>
<td>Bladder discomfort</td>
<td>2 (13)</td>
<td>1 (4)</td>
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<tr>
<td>Any symptom of respiratory infection</td>
<td>9 (56)</td>
<td>16 (57)</td>
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<tr>
<td>Any symptom of infection</td>
<td>12 (75)</td>
<td>16 (57)</td>
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<tr>
<td>Common cold</td>
<td>10 (63)</td>
<td>15 (54)</td>
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<tr>
<td>Flu</td>
<td>2 (13)</td>
<td>2 (7)</td>
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<tr>
<td>Pneumonia</td>
<td>0 (0)</td>
<td>2 (7)</td>
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<tr>
<td>Bronchitis</td>
<td>3 (19)</td>
<td>3 (11)</td>
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<tr>
<td>Sinusitis</td>
<td>1 (6)</td>
<td>4 (14)</td>
</tr>
<tr>
<td>Respiratory infection</td>
<td>11 (69)</td>
<td>15 (54)</td>
</tr>
<tr>
<td>Any diagnosed infection</td>
<td>13 (81)</td>
<td>16 (57)</td>
</tr>
<tr>
<td>Any symptom or diagnosis of infection</td>
<td>15 (94)</td>
<td>20 (71)</td>
</tr>
</tbody>
</table>

*Derived from the Wilcoxon rank-sum test for age and pack-years; for dichotomous outcomes, Fisher's exact test was used when any cell value in a 2×2 table was <5, and Pearson’s χ² test was used for others.
C-reactive protein level by presence of viable \textit{C pneumoniae} as detected by PCR of cDNA generated by RT–PCR of mRNA isolated from carotid artery atherosclerotic plaques. Median serum C-reactive protein levels (solid lines) were greater for those with viable \textit{C pneumoniae} (\(P=0.045\)). Dashed line represents the detection limit of the test.

\textbf{Discussion}

Our study of atherosclerotic carotid arteries suggests that chronic infection with viable \textit{C pneumoniae} is common: \(\approx40\%\) of plaques removed at endarterectomy had evidence of viable organisms. Infection appeared to be unrelated to vascular risk factors, signs and symptoms suggestive of infection, or presentation with stroke or transient ischemic attack. Similar to results of prior studies,\textsuperscript{12,15,20} we found that vascular infection with \textit{C pneumoniae} appears to be asymptomatic.

Other groups have demonstrated viable \textit{C pneumoniae} from cultures of atherosclerotic plaques in the coronary,\textsuperscript{12,18} femoral,\textsuperscript{17} and carotid arteries,\textsuperscript{14,17} albeit in only 4\% to 16\% of specimens. A single study used RT-PCR in carotid endarterectomy tissue and found evidence of viable \textit{C pneumoniae} in 10 of 30 patients,\textsuperscript{19} a detection rate much greater than those seen in studies that used culture. We found a similar rate of infection, which was corroborated by PCR of independently extracted DNA. Furthermore, we showed that plaques with viable infections contained a large number of organisms, suggesting that these infections could be clinically relevant. Characteristics of the referral population we studied—largely non-Hispanic white and referred for elective treatment—may have influenced the prevalence of \textit{C pneumoniae} we found.

This is the first study to demonstrate higher serum C-reactive protein levels in patients with viable carotid \textit{C pneumoniae}. This association may explain why the C-reactive protein level is a risk factor for stroke and cardiovascular events: Higher levels may indicate chronic arterial wall infection with \textit{C pneumoniae}, which in turn may contribute to plaque progression.\textsuperscript{20–23} Recent in vitro studies have shown that \textit{C pneumoniae} infection of human smooth muscle cells results in production of interleukin-6,\textsuperscript{18} which is the major regulator of C-reactive protein production,\textsuperscript{39} providing a plausible pathophysiological link.

Our findings could also be explained by an unrecognized factor that increases C-reactive protein levels and increases the risk of \textit{C pneumoniae} infection. For example, an underlying susceptibility to infection could be associated with both elevated C-reactive protein levels and \textit{C pneumoniae} infection without a causal link between the 2 factors. We did not identify such a factor in this study, but our sample was small.

Others have studied C-reactive protein in \textit{C pneumoniae} infection, but they have not attempted to detect viable organisms. One study that compared C-reactive protein levels in those with and without PCR evidence of \textit{C pneumoniae} DNA\textsuperscript{16} reported that mean levels were higher (but not significantly so) in those who were PCR positive. Several groups have studied the association between C-reactive protein and positive serologies for \textit{C pneumoniae}, with conflicting results.\textsuperscript{24–29} Recent studies have shown, however, that serology is a poor marker for the presence of \textit{C pneumoniae} DNA or RNA.\textsuperscript{12,16,18,30}

If chronic vascular infection with \textit{C pneumoniae} is responsible for C-reactive protein elevation, treatment should be associated with reduced C-reactive protein levels. Three small trials have tested whether antibiotics directed at \textit{C pneumoniae} reduce vascular events and inflammatory markers in high-risk patients. All 3 studies demonstrated reductions in C-reactive protein levels in those treated with antibiotics,\textsuperscript{40–42} and 1 study demonstrated a reduction in vascular events.\textsuperscript{40} The reduction in C-reactive protein after treatment could be due to eradication of \textit{C pneumoniae} or, alternatively, of other infectious agents treated by these antibiotics.

The association that we found between the presence of viable \textit{C pneumoniae} and detectable levels of C-reactive protein should be reproduced in a larger group of patients. More sensitive tests for C-reactive protein are now available and could provide additional information, particularly because cohort studies have shown that differences in levels below the detection limit of our assay are associated with the risk of cardiovascular events.\textsuperscript{3,4} The fact that we detected a difference with a less sensitive, first-generation test suggests that the association between levels of C-reactive protein and the presence of viable \textit{C pneumoniae} is strong. However, because the positive predictive value of a detectable C-reactive protein level is low with the less sensitive test, it should not be used as a screening test to identify those with viable \textit{C pneumoniae}.

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\textbf{References}

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