**Chlamydia pneumoniae** Burden in Carotid Arteries Is Associated With Upregulation of Plaque Interleukin-6 and Elevated C-Reactive Protein in Serum

S. Claiborne Johnston, Hui Zhang, Louis M. Messina, Michael T. Lawton, Deborah Dean

**Objective**—*Chlamydia pneumoniae* (Cpn) infection of vascular smooth muscle cells increases interleukin-6 (IL-6) secretion in vitro. In vivo, IL-6 stimulates liver C-reactive protein (CRP) production. Because serum levels of IL-6 and CRP are independent risk factors for stroke and myocardial infarction (MI), we investigated whether Cpn burden in carotid plaques might provide a link between plaque IL-6 expression and elevated serum levels of IL-6 and CRP.

**Methods and Results**—Consecutive patients undergoing elective carotid endarterectomy were studied. Serum levels of CRP and IL-6 were measured before surgery. Immunohistochemistry and real-time quantitative (k)RT-PCR were used to detect Cpn and the expression of IL-6 within carotid plaques. Cpn mRNA was present in 19 (37%) of 51 patients, suggesting viable infections. These patients had evidence for infection by PCR and immunohistochemistry. The Cpn burden, measured by real-time quantitative (k)-PCR using the number of organisms normalized against the number of eukaryotic cells in the tissue, was associated with plaque expression of IL-6 (Spearman R = 0.55; P < 0.0001), which was associated with serum levels of IL-6 (R = 0.56; P < 0.0001) and CRP (R = 0.80; P < 0.0001).

**Conclusions**—IL-6 secretion in atherosclerotic plaques infected with Cpn could explain elevated serum inflammatory markers in individuals at risk for stroke and MI. (Arterioscler Thromb Vasc Biol. 2005;25:2648-2653.)

**Key Words:** inflammatory markers ■ carotid atherosclerosis ■ IL-6 ■ *Chlamydia pneumoniae* ■ risk factors

Inflammation is an important component of atherosclerosis and likely contributes to atherogenesis and plaque rupture in coronary, cerebral, and peripheral vascular disease. Systemic markers of inflammation, such as the acute-phase reactant CRP, are associated with risk of myocardial infarction (MI) and stroke, and with poor outcomes in patients with symptomatic atherosclerosis. Elevated serum levels of CRP could indicate the degree of localized inflammation in plaques or a more systemic inflammation that triggers thrombosis or plaque rupture. Increasing baseline levels of the proinflammatory cytokine IL-6 also predict future cardiovascular events. Among patients presenting with acute coronary syndromes, an elevated serum level of IL-6 is a strong independent predictor of increased mortality and may be helpful in directing subsequent care. IL-6 is the primary determinant of CRP release from the liver and the only substance known to induce synthesis of all acute-phase proteins.

Cpn has been found in 16% to 71% of plaques in coronary, carotid, and femoral arteries. Two recent clinical trials showed there was no observed reduction in cardiovascular events during intermittent treatment with gatifloxacin, a drug known to be effective against Cpn. However, this infectious pathogen may set in motion an inflammatory process that contributes to plaque inflammation and vascular disease progression. Yet, it remains unknown whether Cpn causes atherosclerosis or is simply an innocent bystander.

In a previous study of patients undergoing elective endarterectomy, we found that serum CRP levels were significantly elevated among those whose carotid arteries were infected with viable Cpn compared with those without infection. However, there are no studies that have quantitatively examined the association between plaque infection and proinflammatory cytokines. We examined this association by measuring serum levels of IL-6 and CRP, and quantifying IL-6 expression in Cpn negative and positive carotid plaques using immunohistochemistry (IHC) and real-time quantitative (k)RT-PCR.

**Methods**

**Study Population and Carotid Specimens**

The target population consisted of patients scheduled for elective carotid endarterectomy who consented to the study and underwent nonurgent carotid endarterectomy at the University of California, San Francisco, had a preoperative clinic evaluation, and had surgical specimens available, as described in detail previously. The protocol...
was approved by the University of California, San Francisco Institutional Review Board.

The carotid artery plaque was obtained at the time of surgery, and handled steriley.13 These plaques had a high grade of atherosclerosis. Plaques were sectioned, placed in OCT, and snap-frozen in liquid nitrogen for IHC. An adjacent carotid plaque section was used for DNA and RNA extraction for detection of Cpn and for IL-6 quantitation. Plaque sections were stored at −80°C until processed. All experiments were performed in a blinded fashion.

**Immunohistochemistry**

Tissue was cross-sectioned at 6 μm. For IL-6, adjacent 6-μm sections were placed on Superfrost-Plus slides (Fisher Scientific). Each slide included positive controls prepared identically to carotid sections and included a section of human tonsil and a carotid sample positive for IL-6 by monoclonal antibody (MAb) staining (see below). Negative controls were sections of tonsil reacted with secondary IgG antibody with chromogen and a carotid sample known to be negative for IL-6. Duplicates were made for all sections.

IL-6 was detected using IL-6–specific MAb (Biosource) with alkaline phosphatase–conjugated secondary antibody and Fast Red (K-Med). Horseradish peroxidase–conjugated secondary antibody with chromogen DAB reacted against a Cpn-specific MAb (R & D Systems) and 100 μL of each primary antibody. An adjacent section was probed with a Cpn-specific MAb (R & D Systems) and 100 μL of each primary antibody. Sections were placed on Superfrost-Plus slides (Fisher Scientific). Staining was assessed under light microscopy at 40× and 100× oil using a Nikon EclipseTE200 with charge-coupled device–SpotII camera and a fixed measurement grid. Nuclei were counted to normalize the staining for IL-6 against cell number in 20 nonoverlapping fields of the same section. Results were averaged across duplicate slides.

To localize IL-6 in relation to Cpn, an IL-6–stained section was probed with a chlamydial-specific heat shock protein 60 (CHsp60) MAb (Affinity Bioreagents), and a duplicate slide was probed with a chlamydial lipopolysaccharide–genus-specific MAb (Cortex Biochem). Horseradish peroxidase–conjugated secondary antibody with diaminobenzidine (DAB) as the chromogen (Biocare) was used for each chlamydial MAb. Positive controls were: (1) a CHsp60- and lipopolysaccharide-positive sample by IHC, and chlamydial DNA-positive sample for Cpn by monoclonal antibody (MAb) staining (see below). Negative controls were sections of tonsil reacted with secondary IgG antibody with chromogen and a carotid sample known to be negative for IL-6. Duplicates were made for all sections.

Quantification of Plaque Cpn DNA and RNA

DNA and RNA were extracted from plaques using DNA tissue extraction and RNeasy Protect Kits (Qiagen), respectively, immediately after obtaining carotid tissue. For RNA, residual DNA was removed by treating the column with RNase-free DNase. cDNA was generated by reverse transcription using TaqMan Gold RT-PCR Kit (Applied Biosystems). The presence of cDNA was confirmed by spectrophotometry.

Primer Express (Applied Biosystems) was used to design primers (Table 1).15 Each primer pair was designed against all sequences from all public databases for the respective gene and subjected to BLAST (Basic Local Alignment Search Tool) to ensure specificity. A plasmid for each of the 3 genes was constructed to create a standard curve for kPCR and kRT-PCR as described previously.15 PCR was performed1 using the primers (Table 1) to generate a PCR product for ligation into pCRII (TOPO TA cloning kit; Invitrogen). TOP10 Escherichia coli was transformed, and the plasmid was purified (GenElute HP; Sigma) and verified by sequencing. A standard formula was used to determine plasmid copy number per milliliter: y = Avagadro’s number × concentration (g/mL)/molecular weight (g) = molecules/mL.

kPCR or kRT-PCR using SYBR Green chemistry in an ABI7000 provided the copy number of DNA for each gene examined, as we have described previously.15 kRT-PCR was used to quantify number of viable Cpn genes. The following samples were run together in the same kPCR or kRT-PCR: triplicates of patient DNA or cDNA for each of the 3 primer pairs, 8 serial 2-fold dilutions of each plasmid (containing Cpn or β-actin DNA insert) to generate standard curves for each gene, and 2 negative controls. Negative controls were: a no-template control (all reaction reagents except for cDNA); and no reverse transcription to test for residual DNA when cDNA was the template. Reactions consisted of 1X SYBR Green PCR master mix, 300 mmol/L of each primer, DNA, cDNA (patient sample), or DNA (plasmid), water to 50 μL, and thermocycling of 10 minutes at 95°C, and 40 cycles of 15 s at 95°C and 1 minute at 60°C. Data were analyzed using Sequence Detection Software (ABI7000). DNA or cDNA copy number was determined using standard curves for the respective gene. Gene copy number was divided by β-actin copy number so that the pathogen gene copy number could be expressed per eukaryotic cell as the pathogen burden. Melting curve analysis was used to verify that the primers detected the correct gene. kPCR or kRT-PCR products were run on 3% to 4% SeaPlaque agarose gels using ethidium bromide with molecular weight markers to verify the size product.

Expression of IL-6 by Quantitative Real-Time RT-PCR

To detect expression of IL-6, we performed kRT-PCR using the same patient cDNA (as above) and our previously published techniques.15 Experiments were performed in triplicate; data were analyzed as above except that quantitation for IL-6 gene expression was normalized against 18s rRNA gene expression for each kRT-PCR as described.15

Serum CRP and IL-6 Levels

CRP levels were initially determined using fixed-rate nephelometry (Quest Diagnostics) at a sensitivity of 0.4 mg/L. Levels below this were defined as not detectable. For all samples below this level, high-sensitivity CRP (hsCRP; Quest) was performed, which has a sensitivity level of 0.175 mg/L. A commercial ELISA ( Biosource) using biotin streptavidin horseradish peroxidase was used to measure IL-6 serum levels. Briefly, 8 standards (0 to 500 ng/L) and patient sera diluted 1:100 in triplicate were applied to a 96-well plate, incubated, and washed. An ELISA plate reader at 450 nm was used for determining substrate conversion. The end point dilution was OD >0.1 above the level of controls. Background was subtracted to normalize data; results were compared with the standard curve to compute concentrations and SEM for each of the 3 values for each sample.

Statistical Analysis

All continuous and categorical variables were not normally distributed, so we used nonparametric statistics for analyses. Univariate
Results
Fifty-one endarterectomy samples were obtained from 49 patients (age mean±SD 72±9 years), 15 (29%) of whom were women. Treated carotid arteries were associated with symptoms attributable to stroke or transient ischemic attack in 14 patients (27%), a median of 56 days before surgery (range 1 to 1130 days); the remaining patients were asymptomatic, including both samples from the subjects with 2 sections with or without upregulation of IL-6 by kRT-PCR or IHC for CRP.

Serum CRP levels before endarterectomy ranged from undetectable to 86 mg/L, with a median below the detection threshold. CRP levels that were undetectable remained so on repeat testing with the hsCRP test. IL-6 serum levels were measured before endarterectomy and ranged from 3.1 to 333.2 pg/μL (median 6.6 pg/μL). CRP and IL-6 serum levels were correlated (R=0.77; P<0.0001).

Serum CRP levels were significantly associated with Cpn infection by all tests and with Cpn burden (Tables 3 and 4; R=0.56; P<0.0001; Figure 1B). IL-6 serum levels were associated with Cpn burden only by kPCR. Serum levels of

organisms in 19 (37%). All plaques with viable organisms were also positive by kPCR and PCR (Table 2). Cpn was present in 28 (62%) of 45 plaques by IHC; 15 (83%) of 18 kRT-PCR-positive plaques were also positive by IHC. Cpn plaque burden (copy number by kPCR) was directly associated with plaque IL-6 expression as measured by kRT-PCR of IL-6 mRNA (Tables 2 and 3; nonparametric Spearman R=0.55; P<0.0001; Figure 1A). Although Cpn was not associated with plaque IL-6 by IHC (Table 2), Cpn plaque burden was correlated with degree of IL-6 IHC staining (Table 3).

IL-6 colocalized with Cpn antigen staining by IHC, primarily in the endothelium (Figure 2D); the carotid plaque revealed Cpn (arrows; brown staining with DAB) and colocalization with IL-6 (arrowheads; staining with Fast Red) in the same cells in the endothelium. Figure 2A shows the carotid section from a patient with atherosclerosis; Cpn was stained with CHsp60-specific MAb, and the nuclear counter stain was hematoxylin. Figure 2B shows a positive control for Cpn, where the organism was cultured in U-937 macrophages, pelleted, snap-frozen, sectioned, and stained with CHsp60-specific MAb (arrows). As a negative control, we used a carotid section with no evidence for Cpn by IHC staining (kPCR and kRT-PCR; Figure 2C).

We found evidence for CRP staining in injured vascular endothelial tissue. However, there were no differences in overall staining among the sections and therefore no statistically significant difference between those sections with or without Cpn by IHC, kPCR, or kRT-PCR, or between those sections with or without upregulation of IL-6 by kRT-PCR or IHC for CRP.


table 2. Characteristics of Carotid Plaques According to Presence or Absence of Cpn

<table>
<thead>
<tr>
<th></th>
<th>Cpn by kPCR</th>
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<tbody>
<tr>
<td></td>
<td>Present (n=22)</td>
<td>Absent (n=29)</td>
<td>P value</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cpn by PCR</td>
<td>20 (91%)</td>
<td>0 (0%)</td>
<td>&lt;0.0001</td>
<td></td>
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<tr>
<td>Viable Cpn by kRT-PCR</td>
<td>19 (86%)</td>
<td>0 (0%)</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cpn by immunohistochemistry*</td>
<td>18 (86%)</td>
<td>10 (42%)</td>
<td>0.005</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6 expression, median (interquartile range)</td>
<td>1.8 (0.3–3.3)</td>
<td>0.3 (0.1–1.6)</td>
<td>0.002</td>
<td></td>
<td></td>
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<tr>
<td>Plaque immunohistochemistry for IL-6</td>
<td>12 (55%)</td>
<td>9 (31%)</td>
<td>0.15</td>
<td></td>
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</table>

P values derived from Wilcoxon rank-sum test for continuous variables and Fisher exact test for others; *n=45.

analysis was performed with Fisher exact test for dichotomous variables, Wilcoxon rank-sum test for comparison of categories of continuous variables, and Spearman rank correlation coefficient (R) for associations between continuous variables using Stata (Version 8.0).


table 3. Spearman Rank Correlations (nonparametric) Among Plaque Characteristics and Serum Levels of Inflammatory Markers

<table>
<thead>
<tr>
<th></th>
<th>Cpn by Quantitative PCR</th>
<th>Cpn by PCR</th>
<th>Viable Cpn by IHC</th>
<th>Cpn by kPCR</th>
<th>IL-6 Expression</th>
<th>IL-6 by IHC</th>
<th>Serum CRP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quantitative plaque Cpn by kPCR</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plaque Cpn by PCR</td>
<td>0.83‡</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Plaque viable Cpn by kRT-PCR</td>
<td>0.81‡</td>
<td>0.96‡</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plaque Cpn by IHC</td>
<td>0.40‡</td>
<td>0.37*</td>
<td>0.34*</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plaque IL-6 expression</td>
<td>0.55‡</td>
<td>0.35*</td>
<td>0.39‡</td>
<td>0.54‡</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plaque IHC for IL-6</td>
<td>0.37‡</td>
<td>0.11</td>
<td>0.14</td>
<td>0.32‡</td>
<td>0.80‡</td>
<td>1.00</td>
<td></td>
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<tr>
<td>Serum CRP</td>
<td>0.57‡</td>
<td>0.30*</td>
<td>0.33*</td>
<td>0.40†</td>
<td>0.80‡</td>
<td>0.76‡</td>
<td>1.00</td>
</tr>
<tr>
<td>Serum IL-6</td>
<td>0.35*</td>
<td>0.10</td>
<td>0.13</td>
<td>0.26</td>
<td>0.56‡</td>
<td>0.69‡</td>
<td>0.77‡</td>
</tr>
</tbody>
</table>

*P<0.05; †P<0.01; ‡P<0.0001.
CRP and IL-6 were strongly associated with plaque IL-6 expression by kRT-PCR and IHC.

IL-6 plaque expression was associated with serum levels of CRP ($R=0.80; P<0.0001$; Figure 1C) and serum IL-6 ($R=0.56; P<0.0001$; Figure 1D). Similarly, IL-6 plaque levels by IHC were associated with serum levels of CRP and IL-6 (Table 3).

Symptomatic presentation was not associated with any serum or plaque measure. Serum inflammatory markers and plaque characteristics were similar for both tests done on the subjects with 2 specimens; results were nearly identical when only the first measure from each was used in subsequent analyses.

**Discussion**

We previously reported an association between Cpn in carotid plaques and serum levels of CRP. Our current study confirms our findings by demonstrating that serum CRP levels are associated with Cpn burden and quantified by kPCR and semiquantitative IHC. Cpn burden in carotid plaques was also associated with plaque IL-6 expression. Furthermore, cells with Cpn antigens tended to colocalize with IL-6. Higher IL-6 plaque expression was associated with higher serum CRP and, to a lesser degree, with higher serum IL-6 levels.

Cpn is a potent inducer of IL-6. In vitro infection of human vascular smooth muscle cells with Cpn upregulates IL-6 expression and basic fibroblast growth factor, suggesting a mechanism of fibrous plaque formation in arterial disease. Cpn-infected cells secret increased quantities of IL-6 in a time-dependent fashion 48 hours after inoculation, which is proportional to the Cpn burden. Furthermore, in vitro inhibition of Cpn protein synthesis by chloramphenicol prevents upregulation of IL-6 expression. These findings are consistent with our study in that Cpn burden, after controlling for the number of eukaryotic cells, was significantly associated with plaque IL-6 expression. Other inflammatory cytokines released by Cpn-infected cells such as IL-1 could also contribute to CRP production. Consequently, Cpn burden may directly influence the degree of plaque inflammation. Yet, it is important to note that the plaques in this study were of a high grade, which may promote an incubator effect for Cpn and a greater degree of inflammation that is not representative of atherosclerosis in general. Further, the nonsp-
specific presence of CRP in injured vasculature would be expected in high-grade plaques. Thus, although we found no association of Cpn with symptomatic vascular disease and would not expect to because most patients with atherosclerosis do not have symptoms from the disease, the data suggest that Cpn is associated with inflammation and atherogenesis via arterial tissue and systemic markers of inflammation.

Increasing evidence suggests that IL-6 is important in atherosclerotic progression. IL-6 is expressed by endothelium and macrophages,21 representing foam cells, which are an early marker of atherosclerosis. IL-6 also has procoagulant effects on platelets, activates monocytes in vascular tissue, increases endothelial release of adhesion molecules, inhibits lipoprotein lipase, and stimulates lipolysis, all of which contribute to the development of vascular disease.22 We found that plaque expression of IL-6 was associated with serum levels of IL-6. Serum levels of IL-6 have been strongly correlated with IL-6, but the association between IL-6 and cardiovascular disease risk persisted after adjustment for CRP levels.

IL-6 is the primary determinant of CRP release from the liver.23 Consequently, it is not surprising that we found a strong association between serum CRP and plaque IL-6 expression and serum IL-6 levels. Because plaque IL-6 expression is associated with serum IL-6, it is plausible that IL-6 stimulates CRP production, which indirectly reflects localized plaque inflammation. The high degree of IL-6 staining in plaques suggests that plaques may be the source of elevated serum IL-6. Although CRP levels increase via several mechanisms, chronic vascular wall infection and inflammation may be an important stimulus, with IL-6 acting as the primary messenger. Thus, Cpn may induce plaque production of IL-6, which could subsequently cause elevation in serum CRP and IL-6.

We found evidence for Cpn in 37% of carotid plaques. Others have reported Cpn in 0% to 100% of atherosclerotic plaques. In a review of 43 studies with 1852 specimens, an average of 46% had evidence for Cpn infection.24 This range likely reflects the variety of techniques used to identify Cpn and the samples studied. However, contamination or false positives, especially with PCR, may be an issue.25 The consistency of our findings with a high correlation of PCR, kPCR (DNA), kRT-PCR (RNA), and semiquantitative IHC results for Cpn in the plaques (P<0.0001) and independent performance of each test are inconsistent with contamination or false positives. Furthermore, the association of plaque Cpn with serum IL-6 and CRP cannot be explained by contamination because blood and tissue samples were handled completely separately.

Cpn may still be an innocent bystander in plaques because infected monocytes are recruited to these sites. CRP and plaque inflammation, indicated by plaque IL-6 expression, may correlate with upstream factors associated with risk or overall severity of atherosclerosis, whereas Cpn burden may simply be determined by the degree of cellular infiltration into the plaques. Arguing against this, the burden of Cpn was determined based on number of Cpn normalized against eukaryotic cells. Furthermore, within plaque sections, expression of IL-6 tended to cluster around cells with Cpn.

Trials of antibiotic treatment for Cpn in coronary artery disease have shown variable results, with no overall benefit.11,12,26 These studies failed either because Cpn is not a causative factor in atherosclerosis or the treatment was inadequate. Our study found that Cpn was associated with

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### TABLE 4. Characteristics of Carotid Plaques in Association With Serum CRP and IL-6 Levels*

<table>
<thead>
<tr>
<th>Plaque Characteristic</th>
<th>CRP</th>
<th>IL-6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Detectable (n=22)</td>
<td>Not Detectable (n=29)</td>
</tr>
<tr>
<td>Cpn presence by kPCR, median (interquartile range)</td>
<td>28.4 (0–231)</td>
<td>0 (0–0.1)</td>
</tr>
<tr>
<td>Cpn presence by immunohistochemistry, n (%)‡</td>
<td>17 (85%)</td>
<td>11 (44%)</td>
</tr>
<tr>
<td>IL-6 expression, median (interquartile range)</td>
<td>2.8 (1.9–3.5)</td>
<td>0.2 (0.1–0.4)</td>
</tr>
<tr>
<td>IL-6 presence by immunohistochemistry, n (%)</td>
<td>18 (82%)</td>
<td>3 (10%)</td>
</tr>
</tbody>
</table>

*P values derived from Wilcoxon rank-sum test for continuous variables and Fisher exact test for others. High and low IL-6 defined as above or below median; ‡n=45.
inflammation via inflammatory markers, which likely play a role in disease development and progression. Thus, our data are not inconsistent with previous findings. Treatment has reduced serum levels of CRP in some studies, but information about the reduction in plaque inflammation or Cpn eradication is not available and would be important in establishing a definitive role for Cpn in atherosclerosis.

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References
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