Increased CD8\(^+\) T Cells Associated With Chlamydia pneumoniae in Symptomatic Carotid Plaque

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Background and Purpose—The presence of Chlamydia pneumoniae has been reported in carotid atheroma, but its causative effect in the activation of an atherosclerotic plaque to a prothrombotic state remains unproved. Antigen-mediated activation of T lymphocytes within plaque may represent a mechanism by which infection can result in plaque conversion. The goal of the present study was to characterize the T-cell subtype profile related to the presence of C pneumoniae in patients with symptomatic versus asymptomatic carotid atherosclerosis.

Methods—We studied 14 plaques (5 symptomatic and 9 asymptomatic) positive for C pneumoniae confirmed by polymerase chain reaction and 14 plaques (6 symptomatic and 8 asymptomatic) from age- and stenosis-matched patients negative for C pneumoniae by polymerase chain reaction. T-cell subpopulations of T-helper, T-cytotoxic, and T-memory lymphocytes were identified through indirect enzyme immunohistochemistry with anti-CD3\(^+\), anti-CD4\(^+\), anti-CD8\(^+\), and anti-CD45RO\(^+\) monoclonal antibodies, respectively. Results are expressed as the number of positive cells per millimeter squared.

Results—In the absence of C pneumoniae, symptomatic plaques had a modest but significant increase of CD3\(^+\) (89.6 versus 55.3, \(P=0.013\)), CD4\(^+\) (57.3 versus 32.7, \(P=0.01\)), and CD45RO\(^+\) (82.8 versus 43.7, \(P=0.007\)), but not CD8\(^+\) T cells (28.5 versus 25.5, \(P=0.245\)) compared with asymptomatic. However, in the presence of C pneumoniae, there was significant increase of all T-lymphocyte subtypes in symptomatic plaques, including CD8\(^+\) (76.8 versus 30.3, \(P=0.03\)), CD3\(^+\) (192.1 versus 80.4, \(P=0.004\)), CD4\(^+\) (111.9 versus 37.9, \(P=0.003\)), and CD45RO\(^+\) (120.2 versus 72.9, \(P=0.003\)) cells compared with asymptomatic plaques. With use of 2-way ANOVA, both the presence of chlamydia and symptoms were associated with significantly higher T-cell counts (\(P<0.005\) for all subtypes).

Conclusions—Although all patients with symptomatic disease show a modest elevation in the concentration of intraplaque lymphocytes, a preferential increase in CD8\(^+\) class I–restricted T cells is observed in symptomatic carotid plaque positive for C pneumoniae. These data provide incentive to further explore the role of Chlamydia in the modification of immune-mediated mechanisms in active atherosclerotic plaque.

Key Words: carotid artery diseases □ Chlamydia pneumoniae □ leukocytes □ plaque

The role of inflammation is emerging as a pivotal component in the initiation, maturation, and activation of atherosclerotic plaque.\(^1\)\(^,\)\(^2\) Evidence indicates that plaques associated with thrombotic and thromboembolic events in the coronary and carotid vessels have an increased expression of inflammatory mediators, including leukocyte adhesion molecules,\(^3\) and of inflammatory cells, including macrophages and T lymphocytes.\(^4\)\(^,\)\(^5\) Activation of these inflammatory cells is believed to result in plaque wall destabilization and increased prothrombotic character of the plaque, leading to increased risk for stroke and heart attack.\(^4\)\(^,\)\(^6\)–\(^8\)

Recently, attention has been given to T lymphocytes as a mediator of activation of the atherosclerotic plaque. T cells can be recruited to the inflammatory environment of the arterial atherosclerotic plaque wall regardless of their anti-genic specificit.\(^9\) The majority of the T cells are believed to be a heterogeneous population made up predominantly of memory T cells that are quiescent, but can be activated by exposure to specific antigen or mimic antigens to T-cell receptors.\(^9\)–\(^11\) Oligoclonal expansion of T cells in atherosclerotic plaque suggests the occurrence of an antigen driven T-cell proliferation in atherosclerosis.\(^12\) T lymphocytes that can be stimulated with antigens such as oxidized, low-density lipoprotein\(^13\) have been suggested as possible triggers for plaque activation. However, to date, evidence is insufficient to demonstrate specific antigen activation of an atherosclerotic plaque in association with symptomatic disease.

Chlamydia pneumoniae, a common pathogen in human respiratory tract infections, is a potential candidate antigen
that has been repeatedly identified in atherosclerotic plaque of the coronary and carotid vessels.\textsuperscript{14–20} \textit{C pneumoniae} has been identified as an antigen that can activate T cells cultured from the peripheral blood of patients with atherosclerotic disease in addition to T cells cultured from carotid atherosclerotic plaque.\textsuperscript{9,11,21} In addition, \textit{C pneumoniae} has been reported to stimulate a cell-mediated immune (CMI) response\textsuperscript{8,11,22} that could potentially activate both the CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells through antigenic peptide binding to HLA.

Chlamydia has been identified as an antigen that can activate T cells cultured from carotid atherosclerotic plaques.\textsuperscript{9,11,21} In addition, \textit{C pneumoniae}–positive and \textit{C pneumoniae}–negative groups revealed no differences in the medication profile with respect to aspirin, angiotensin-converting enzyme inhibitors, \(\beta\)-blockers, or statins. No patient was taking steroids.

Carotid plaques were obtained from patients undergoing carotid endarterectomy at the National Naval Medical Center (Bethesda, Md) who had stenotic atheromatous lesions of \(\geq 50\%\), as measured by North American Symptomatic Carotid Endarterectomy Trial criteria.\textsuperscript{26} All patients gave consent for use of their plaque and blood samples for research analysis per the hospital’s institutional review board.

History and neurological examination were obtained by a neurologist from the Stroke Branch of the National Institute of Neurological Disorders and Stroke before surgery for all patients to determine symptomatic and asymptomatic group classification. Stroke risk factors of hypertension (blood pressure \(>140/90\) mm Hg for \(\geq 1\) year), past history of smoking (\(\geq 5\) pack-year history), diabetes (oral agent or insulin dependent for \(>1\) year), and hypercholesterolemia (LDL \(>160\) mg/dL untreated, fasting triglycerides \(>200\) mg/dL, or on cholesterol-lowering medication for \(>1\) year), current medications including use of antplatelet agents, and time from last ischemic event were recorded. Patients with atrial fibrillation were excluded from the symptomatic group to avoid the possible confusion between a cardiac and carotid source of embolism. A CT scan of the head was obtained for all patients and used as supporting evidence of the history and physical examination in the asymptomatic population to rule out silent infarction. Patients were classified as symptomatic if they had a prior transient ischemic attack (TIA) or stroke within 4 months or asymptomatic if they had no prior history of cerebral ischemic event (TIA or stroke) and no evidence of ischemic lesion on the CT scan performed before surgery. All 5 patients with \textit{C pneumoniae}–positive symptomatic plaques had TIAs, and of the 6 symptomatic patients with \textit{C pneumoniae}–negative plaques, 4 had strokes and 2 had TIsAs.

Carotid plaque specimens were collected in the operating room, with care to remove the plaque in a single piece. The highest-grade region of the plaque was identified on angiography. Using a correction factor of 15\% magnification of the angiographic image, measurements from the bifurcation to the area of highest-grade stenosis was performed and a 3- to 5-mm cross-sectional segment was obtained at that point, with visual inspection of the dissected specimen to confirm that the high-grade region of the plaque was obtained. All preparation of the tissue was performed under sterile conditions. These frozen specimens were snap-frozen in liquid nitrogen and stored at \(-70^\circ\)C and used for PCR analysis. Crystal sections directly adjacent to the material used for PCR analysis were mounted for immunohistochemical staining.

**PCR Protocol**

The presence of \textit{C pneumoniae}–specific DNA within the carotid plaque was confirmed by PCR according to previously described protocol.\textsuperscript{14,27}
DNA was isolated from approximately 5 to 10 serial sections with the QIAamp Tissue Kit (Qiagen) 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 the 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. This primary laboratory was supplied separately from the main laboratory. Each sample was analyzed in duplicate to insure reliability.

To detect *C pneumoniae* DNA at the required level of sensitivity, a 2-step nested PCR protocol was implemented. Both the inner and outer sets of PCR primers were designed with the Lasergene software (DNASTar) on an LCIII computer (Apple) to maximize PCR performance. The sequence of the 474-bp inv/R fragment was used as the basis for this PCR since it has previously been shown to have little sequence similarity to the other members of this genus by dot blot hybridization. The outer primers for the primary PCR step were designated CPl-(5’-TTATTACCCGTCCTACAGCAGAAA-3’), and CP2-R (5’-GCGGGITCAGGGATCTTGTG-3’) and produce a 404-bp sequence that corresponds to chlamydial polymerase. The inner primers (nested) contained within the 404 bp sequence were designated CPN1-L (5’-TTACGAAACCGCCATTACACCGCTA-GAAATCAAT-3’), and CPN1-R (5’-TATGGCATATCCGCT-TCGGGAACGAT-3’), which result in a 214-bp product. After an initial 9 minutes at 95°C to activate the PCR enzyme, the outer PCR was carried out using 40 cycles of: 95°C for 30 seconds to melt, 60°C for 50 seconds to anneal, and 72°C for 30 seconds to extend the primers in the same PCR buffer as given above.

Samples were amplified in 25 μL of 100 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 2 mmol/L MgCl2, 100 μmol/L concentration of each dNTP, and 50 nmol/L of each Chlamydia primer (5’-ATCG-3’, 5’-ATCG-3’), 0.25 U AmpliTag Gold (Perkin Elmer), templated with 1 μL of the DNA isolated from the patient samples. This first-step PCR was carried out in individual thin-wall reaction tubes (Molecular Bio-Products), and when the reaction tubes were inoculated with the template DNA, only 1 tube was opened at a time in a further step to avoid cross-contamination of samples.

The inner, nested PCR was carried out with 25 to 30 cycles at 94°C for 15 seconds to melt, 60°C for 1 minute to anneal, and 72°C for 15 seconds to extend the primers in the same PCR buffer as given above except that the primers were 5’-ATCG-3’ and 5’-ATCG-3’, and the enzyme used was AmpliTaq Gold. The nested reaction was templated using 1 μL of the primary PCR product hot started at 50°C. PCR products were visualized under 300-nm UV transillumination (Fotodyne) after electrophoresis for 20 minutes at 5 V/cm in 2% ultraPURE agarose (Life Technologies), 45 mmol/L Tris, 45 mmol/L borate, and 5 mmol/L EDTA, pH 8.3 (Biofluids), and 1:10 000 SYBR Green II (Molecular Probes). Images were photographed using Polapan type 57 film (Polaroid Corp). Specific nested PCR product was identified using 5 to 15 ng dX74 DNA restricted with *HaeIII* as a size marker. All PCRs were carried out in a Perkin Elmer 9700 Gene Amp PCR System (PE Applied Biosystems).

**Immunohistochemistry**

In sections adjacent to the regions of *C pneumoniae* identification, total T-lymphocyte count (CD3+), T-helper cells (CD4+), T-cytotoxic cells (CD8+), and T-memory cells (CD45RO+) were studied using single indirect enzyme immunohistochemistry according to previously described method by Frosterard et al.28 Sections of tissue directly adjacent to the regions of immunohistological correlation and characterization with the regions of the plaque identified to have *C pneumoniae*–positive DNA were log transformed for 2-way ANOVA analyses to compare means for continuous variables, but if normality and/or equal variance tests failed, the Mann-Whitney *U* test was used for comparison of median values. We used 2-way ANOVA, including an interaction term, to compare means between 2 classifications. Logistic regression was used to model symptomatic outcome as the dependent variable, with other possible risk factors as covariates; the logistic regression was used to model symptomatic outcome as the dependent variable, with other possible risk factors as covariates; the logistic likelihood ratio test was used to determine significance. T-cell counts were log transformed for 2-way ANOVA analyses to compare means and for use in logistic regression analyses. For all tests, a 2-tailed *P* value of ≤0.05 was considered significant.

**Results**

We analyzed T-cell subsets in 14 plaques (5 symptomatic and 9 asymptomatic) from patients positive for *C pneumoniae*–specific DNA confirmed by PCR and 14 plaques (6 symptomatic and 8 asymptomatic) from stenosis-matched patients negative for *C pneumoniae*. Table 2 shows demographic characteristics of patients with *C pneumoniae*–positive and *C pneumoniae*–negative carotid atherosclerotic plaques. There was no significant difference between the groups with respect to age, sex, degree of stenosis, occurrence of ischemic symptoms, and risk-factor profile, except for the presence of diabetes. A total of 8293 images (a median of 53 images per section for each monoclonal antibody) that covered an area 556.2 mm² of 112 sections from 28 plaque specimens were analyzed.
To explore the coeffect of symptoms and the presence of Chlamydia on counts of T-cell populations, 2-way ANOVA was performed, including an interaction term. For each T-cell subset, CD3$^+$, CD4$^+$, CD8$^+$, and CD45RO$^+$, both presence of chlamydia and symptoms were significantly associated with higher cell counts (all $P<0.005$).

In carotid atherosclerotic plaques, in the absence of C pneumonieae, there was a significant increase in the total T-lymphocyte count (median, cells per millimeter squared) in symptomatic versus asymptomatic patients (CD3$^+$ 89.6 versus 55.3, $P=0.013$). The predominant T-cell subtypes in symptomatic plaque were CD4$^+$ helper T cells (57.3 versus 32.7, $P=0.01$) and CD45RO$^+$ memory T cells (82.9 versus 43.7, $P=0.007$). CD8$^+$ cytotoxic T cells were at relatively lower levels and not significantly different between symptomatic and asymptomatic patients (28.6 versus 25.5, $P=0.25$; Figure 2).

However, in the presence of C pneumonieae, a significant elevation of CD8$^+$ cytotoxic T cells was observed in the symptomatic versus asymptomatic patients (76.8 versus 30.3, $P=0.03$). In addition, CD4$^+$ cells (111.9 versus 37.9, $P=0.003$), CD45RO$^+$ cells (120.2 versus 72.9, $P=0.003$), and total T-cell count (CD3$^+$ T cells 192.1 versus 80.4, $P=0.004$) were elevated in C pneumonieae–positive symptomatic plaque (Figure 3).

Comparison of symptomatic plaques with and without evidence of C pneumonieae presence revealed a significant increase in all T-cell populations in plaque positive for C pneumonieae (Figure 4).

Logistic regression was used to explore the effect of presence of Chlamydia and each T-cell subset count (log transformed) on the presence of symptoms. Each T-cell subset count was statistically significant as a predictor of symptoms (all $P=0.003$), and chlamydia presence was also significant for all subsets $P=0.008$, except for CD8$^+$ ($P=0.08$). Other demographic and risk factors were explored in a univariate logistic regression to see whether they were associated with symptoms. Smoking, high cholesterol, and diabetes were significant (all $P=0.04$). When these covariates
were entered into the logistic models singly and in any combination, with presence of Chlamydia and each of the T-cell subsets, each T-cell subset still remained a significant predictor of symptoms (all $P < 0.04$).

**Discussion**

In this study, we examined the association between the cellular immune response and the presence of C pneumoniae in carotid plaque specimens from patients with symptomatic and asymptomatic atherosclerotic disease. Our study confirms the growing body of literature that reports symptomatic atherosclerotic disease is associated with a proinflammatory profile, including an increase in T lymphocytes.2,4 In the absence of C pneumoniae in our study, a significant increase in CD3$, CD4$, and CD45RO$ cells was seen in patients with symptomatic versus those with asymptomatic plaque. No increase in CD8$ cells was seen, and the HLA class II–dependent CD4$-restricted cell immune response appears to be the dominant cell type. Previous studies21,29 have reported CD4$ cells as the dominant type in atherosclerotic plaque, although the studies did not compare patients with symptomatic to those with asymptomatic plaque. In the present study, there is an increase in CD4$ and CD45RO$ subsets in the symptomatic versus the asymptomatic patients. This CD4$ predominance could represent either a T-cell recruitment to the site of inflammatory tissue in the atherosclerotic plaque or a major histocompatibility complex class II response to antigen, such as oxidized LDL13 or heat-shock protein-60.21 These antigens are capable of initiating an inflammatory cascade within the carotid atherosclerotic plaque via the CD4$ pathway.28

However, our data show a strong association between the presence of C pneumoniae and the accumulation of T lymphocytes, including CD8$ cytotoxic T cells in addition to CD4$ T cells in symptomatic versus asymptomatic patients. These data raise the possibility that in the presence of C pneumoniae, an antigen-specific CD8$-restricted activation of the cellular immune response occurs within symptomatic plaque. Further, the findings support our hypothesis that a specific antigen can activate resting atherosclerotic plaque via both the CD4$ and CD8$ pathways of cell-mediated immunity. These data have to be justified against data which suggest that the simple presence of C pneumoniae alone is insufficient to result in plaque destabilization.14,30 Given that not all plaques with C pneumoniae became symptomatic, our data also suggest that the presence of that organism is insufficient for symptomaticity and that another factor, such as reactive Chlamydia-specific T cells, is also required. Memory T-lymphocyte predisposition for a specific antigen may play a role in the differential activation of a plaque.

**TABLE 2. Demographics and Distribution of Risk Factors in the Study Population**

<table>
<thead>
<tr>
<th></th>
<th>C pneumoniae Positive (n=14)</th>
<th>C pneumoniae Negative (n=14)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age,* mean±SD y</td>
<td>72±8</td>
<td>68±7</td>
<td>0.15</td>
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<tr>
<td>Female, %</td>
<td>14</td>
<td>36</td>
<td>0.38</td>
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<tr>
<td>Hypertension, %</td>
<td>86</td>
<td>86</td>
<td>1.0</td>
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<tr>
<td>Diabetes, %</td>
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<td>36</td>
<td>0.06</td>
</tr>
<tr>
<td>High cholesterol, %</td>
<td>71</td>
<td>71</td>
<td>1.0</td>
</tr>
<tr>
<td>Smoking, %</td>
<td>71</td>
<td>93</td>
<td>0.33</td>
</tr>
<tr>
<td>Stenosis,* mean±SD %</td>
<td>76±14</td>
<td>77±13</td>
<td>0.74</td>
</tr>
<tr>
<td>Symptomatic, %</td>
<td>36</td>
<td>43</td>
<td>1.00</td>
</tr>
<tr>
<td>Time from symptoms to surgery,* d</td>
<td>32±49</td>
<td>49±49</td>
<td>0.728</td>
</tr>
</tbody>
</table>

*Student’s $t$ test. In all other cases, $P$ was calculated using Fisher’s exact test.

**Figure 2.** T cells in symptomatic and asymptomatic patients with C pneumoniae–negative carotid atherosclerotic plaque.

**Figure 3.** T cells in symptomatic and asymptomatic patients with C pneumoniae–positive carotid atherosclerotic plaque.

**Figure 4.** T cells in symptomatic patients with C pneumoniae–positive [Chlam (+)] and C pneumoniae–negative [Chlam (−)] carotid atherosclerotic plaques.
It is known that Chlamydia spp can induce both CD4+ and CD8+ T-cell response.22,31 The CD4+-recognized antigens are derived from vacuolar content bound to HLA class II molecules expressed by antigen presenting cells.32 The CD8+ T cells recognized antigen bound to HLA class I molecules expressed on nucleated cells in the cytoplasmic compartments.33 Induction of HLA class I CD8+ cytotoxic T-lymphocyte response can be induced by several potential antigens expressed by C pneumoniae.23,31 The lack of increased CD8+ T-cell levels in asymptomatic patients with Chlamydia indicates that Chlamydia can be present without necessarily triggering a cytotoxic T-cell response. This could be due either to an insufficient antigen load or a potential lack of resting memory T cells for Chlamydia in the plaque. Either possibility could explain the findings seen in previously reported studies that failed to identify a correlation between PCR-defined C pneumoniae presence and symptoms.14,30

Although an association between C pneumoniae and elevated CD8+ T-cell count in symptomatic plaque was identified, the limitation of this study is that it remains to be proved that the T-cell response was C pneumoniae antigen-specific and that this cellular response was causative for plaque destabilization. Work is ongoing in our laboratory to examine the antigen-specific T-cell response in the setting of symptomatic and asymptomatic atherosclerotic disease. Additionally, the labor-intensive nature of this study prevents examination of the entire plaque, and therefore the sampling may not represent all regions of a heterogeneous plaque. However, the intent of this study was to characterize T-cell subtype in regions of the plaque conventionally identified as being associated with symptoms and in which the status of C pneumoniae presence was known.

In conclusion, CD4+ T lymphocytes were significantly elevated in all patients with symptomatic atherosclerotic plaque versus those with asymptomatic plaque. However, there was a significant and preferential increase of CD8+ class I–restricted T cells in symptomatic plaque positive for C pneumoniae. These data provide intriguing information that warrants further exploration of C pneumoniae as a potential modifier of the immune system in atherosclerotic plaque. Further proof is needed to draw a direct link to C pneumoniae presence and plaque destabilization.

Acknowledgment

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References

6. Van der Wal AC, Becker AE, Van der Loos CM, Das PK. Site of intimal rupture or erosion of thrombosed coronary atherosclerotic plaques is characterized by an inflammatory profile irrespective of the dominant plaque morphology. Circulation. 1994;89:36–44.
The annual risk of stroke due to high-grade stenoses of the internal carotid artery can be as high as 13% after the recent occurrence of transient cerebral or retinal ischemia or as low as 1% to 2% in clinically asymptomatic patients.1–2 The mechanisms that convert a stable plaque into “unstable internal carotid artery disease” are incompletely understood but have obvious implications for the treatment of cerebrovascular disease patients at risk of stroke. Advanced atherosclerotic plaques exhibit abundant infiltration by immune cells such as macrophages and T cells.3 This local inflammatory response has been proposed to play an important role in the process of plaque destabilization manifesting clinically as unstable angina or ischemic stroke.3–6 In symptomatic patients with high-grade carotid stenosis, increased expression of matrix metalloproteinase-9 and thrombogenic tissue factor at sites of plaque inflammation may provide key effector mechanisms underlying plaque rupture and luminal thrombosis, respectively.7,8 Numerous studies have recently reported the detection of C pneumoniae in atherosclerotic plaques, raising the possibility that this microorganism may be one of the factors driving T-cell–mediated inflammation during atherogenesis.9 In their interesting study, Nadareishvili and colleagues show a preferential increase of T cells in symptomatic carotid artery plaques positive for C pneumoniae.10 The results of this study are important because they suggest a potential mechanism by which C pneumoniae may modulate the local inflammatory microenvironment in the plaques. However, it should be stressed that at the present stage, conclusions with respect to a causal role of C pneumoniae in plaque destabilization are still circumstantial and not directly supported by the data presented. Of note, previous findings reported by both LaBiche et al10 and Gibbs et al11 showed that the presence of chlamydia DNA in plaque material is not directly correlated with clinical features of plaque destabilization. Thus, specific T-cell–mediated immune responses rather than the mere presence of the microorganism itself may predispose to plaque rupture and ischemic complications. In this regard, future studies addressing the antigen specificity of the observed CD8+ T-cell response will be of particular importance.

**References**


5. van der Wal AC, Becker AE, van der Loos CM, Das PK. Site of intimal rupture or erosion of thrombosed coronary atherosclerotic plaques is characterized by an inflammatory process irrespective of the dominant plaque morphology. Circulation. 1994;89:36–44.


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