**Chlamydia pneumoniae** Does Not Influence Atherosclerotic Plaque Behavior in Patients With Established Carotid Artery Stenosis

R.G.J. Gibbs, FRCS; M. Sian, MPhil; A.W.M. Mitchell, FRCR; R.M. Greenhalgh, FRCS; A.H. Davies, FRCS; N. Carey, PhD

**Background and Purpose**—Research for infectious agents in the etiology of atherosclerosis has identified *Chlamydia pneumoniae* as a possible candidate. While there is evidence of an association between presence of this microorganism and atherosclerosis, it is unclear whether infection has a genuinely etiologic role in this disease, whether its presence influences clinical outcomes, and, if so, at which stages of disease this occurs. We have approached this issue in patients with advanced carotid artery atherosclerosis using molecular biological detection methods and clinically relevant indicators of pathology in carotid artery atheroma to determine whether the presence of *C pneumoniae* correlates with plaque instability.

**Methods**—*C pneumoniae* was detected with the use of a sensitive nested polymerase chain reaction. Preoperative embolization and preoperative infarcts were recorded with the use of transcranial Doppler insonation of the middle cerebral artery and cerebral CT, respectively.

**Results**—*C pneumoniae* DNA was detected in 25.5% of a cohort of 98 symptomatic patients. There was no significant difference in plaque stability as measured by embolization rates between the chlamydial-positive and -negative specimens. There was also no correlation between the number of ipsilateral hemispheric infarcts in the territory of the middle cerebral artery and chlamydial status.

**Conclusions**—This study confirms that *C pneumoniae* is a common finding in atherosclerotic plaques of the carotid artery but suggests that the presence of the infectious organism has little detectable impact on plaque instability when measured by clinically significant markers. This raises important questions for the rationale of antibiotic therapy in atherosclerosis. (Stroke. 2000;31:2930-2935.)

**Key Words:** atherosclerosis ▪ carotid arteries ▪ embolism ▪ infection

Established risk factors for atherosclerotic arterial disease, eg, serum lipids, smoking, hypertension, diabetes, age, sex, and family history, account for <50% of detectable variation in the occurrence of these disorders between groups (reviewed in Reference 1).1 Epidemiological evidence suggested a possible role for infectious organisms, and the gram-negative bacterium *Chlamydia pneumoniae* has been implicated by serological studies,2–7 immunohistochemical analyses of excised lesions with *C pneumoniae*–specific antibodies,8,9 and molecular detection with the use of the polymerase chain reaction (PCR).10,11 It has been postulated by a number of authors that the frequently reported association between the presence of *C pneumoniae* and atherosclerosis indicates a pathogenetic mechanism involving this microorganism, a suggestion that has found support from a small number of animal studies.12–14 The most usual mechanism postulated is that infection with *C pneumoniae* provokes an inflammatory immune response, triggering and possibly sustaining the inflammatory atherosclerotic lesion.

In carotid artery atherosclerotic disease it is clear that while degree of stenosis is regarded as the best predictor of outcome, other factors may be important in determining whether the lesion will give rise to the symptoms of amaurosis fugax, transient ischemic attack, or stroke or whether it will remain asymptomatic. Different plaques causing the same degree of luminal stenosis have a different propensity to undergo the pathologically critical processes of thrombosis followed by embolization, but it is not known what causes this differential plaque instability. If *C pneumoniae* influences the thrombotic or embolic processes, as has been suggested by a number of experimental models, it would have major implications for clinical progression of the plaque.

However, it is far from proven that in human atherosclerosis the organism genuinely plays this etiologic role or that it truly influences disease progression. It is possible that *C pneumoniae* is merely an “innocent bystander,” whose presence in atherosclerotic lesions simply reflects an affinity...
TABLE 1. Demographic Parameters and Cardiovascular Comorbidity for the Patient Cohort, Divided into 2 Groups Dependent on the Presence or Absence of C pneumoniae in the Carotid Plaques (n=98)

<table>
<thead>
<tr>
<th></th>
<th>CP+</th>
<th>CP−</th>
<th>P (χ²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>26% (25)</td>
<td>74% (73)</td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td>71</td>
<td>70</td>
<td>NS</td>
</tr>
<tr>
<td>Sex, M:F ratio</td>
<td>4:1</td>
<td>2.6:1</td>
<td>NS</td>
</tr>
<tr>
<td>MI</td>
<td>17%</td>
<td>27%</td>
<td>NS</td>
</tr>
<tr>
<td>Angina</td>
<td>28%</td>
<td>30%</td>
<td>NS</td>
</tr>
<tr>
<td>Hypertension</td>
<td>58%</td>
<td>70%</td>
<td>NS</td>
</tr>
<tr>
<td>PVD</td>
<td>36%</td>
<td>36%</td>
<td>NS</td>
</tr>
<tr>
<td>NIDDM</td>
<td>16%</td>
<td>0.1%</td>
<td>NS</td>
</tr>
<tr>
<td>IDDM</td>
<td>0%</td>
<td>0.1%</td>
<td>NS</td>
</tr>
<tr>
<td>Smoker</td>
<td>83%</td>
<td>82%</td>
<td>NS</td>
</tr>
<tr>
<td>Amaurosis fugax</td>
<td>37%</td>
<td>28%</td>
<td>NS</td>
</tr>
<tr>
<td>TIA</td>
<td>37%</td>
<td>51%</td>
<td>NS</td>
</tr>
<tr>
<td>Stroke</td>
<td>26%</td>
<td>21%</td>
<td>NS</td>
</tr>
</tbody>
</table>

CP+ indicates C pneumoniae present; CP−, C pneumoniae absent; MI, myocardial infarction; PVD, peripheral vascular disease; NIDDM, non–insulin-dependent diabetes mellitus; IDDM, insulin-dependent diabetes mellitus; TIA, transient ischemic attack; NS, nonsignificant difference, ie, P>0.05.

for inflammatory environments. It is also possible that C pneumoniae can act as an initiating factor for atherosclerosis in some patients, but that once the pathological mechanisms are established they are maintained independently of infection status. It is important to resolve this issue because this will determine whether there is a sustainable rationale for the increasing numbers of antibiotic trials proposed for atherosclerotic disease.

We have investigated the hypothesis that the presence of C pneumoniae influences the stability of internal carotid artery (ICA) plaque in a large cohort of patients undergoing carotid endarterectomy for symptomatic atherosclerotic disease. We chose this patient group as a clinically significant high-risk cohort, for whom the development of effective interventions to minimize the risk of serious pathological events is a priority. Direct detection of C pneumoniae by molecular biological methods was coupled with functional assessment of plaque stability in vivo to determine whether there is a justifiable basis for proposing antibacterial treatment in patients in whom advanced atherosclerotic lesions are present.

In Situ Analysis of Plaque Stability

Plaque stability was assessed by preoperative embolic signals captured by transcranial Doppler (TCD) recording, an established technique for the detection of intracranial emboli, and evidence of ipsilateral hemispheric infarction was assessed by preoperative cerebral CT scans.

To assess the degree of embolic activity of the plaques in situ, all patients underwent TCD analysis (Sci-Med PC Dop 842) of their middle cerebral artery (MCA), ipsilateral to the diseased carotid artery, for 30 minutes to 48 hours before surgery. Preoperative monitoring of the contralateral MCA was also recorded. Intraoperative TCD of the ipsilateral MCA was also undertaken, and any emboli present before ICA clamping were recorded. Preoperative probe fixation was performed with the use of the manufacturer’s headband, and during surgery a head guard designed in-house was used to prevent movement. Gain and power settings were established for each patient to maximize the quality of the tracing. Accepted criteria for embolic signals were used, ie, short duration with a frequency or velocity focused intensity increase and unidirectional signals in the direction of flow accompanied by a characteristic clicking sound. Signals were recorded by the researchers during monitoring, and all embolic signals were also recorded onto digital audiotape and verified by an independent observer. Interobserver agreement for embolic signals exceeded 90%.

All patients routinely underwent preoperative cerebral CT (Siemans Somatom +4) scans before carotid endarterectomy, of which 70 were available for analysis. This analysis was performed by an independent consultant radiologist who was blinded to both the side of surgery and the chlamydial status of the plaque. All infarcts in both hemispheres, as well as the subgroup of infarcts lying within the territory of the MCA ipsilateral to the carotid stenosis, were counted. Acute CT scans from the time of symptom presentation were unavailable.

Tissue

Carotid endarterectomy atherectomy specimens obtained at surgery were immediately divided into 2 fragments down the longitudinal axis of the specimen. One fragment was snap-frozen in liquid nitrogen and stored at −70°C for DNA extraction. The second fragment was formalin fixed, processed to paraffin blocks, sectioned, and stained for elastin with an elastic van Gieson stain and with hematoxylin and eosin. The snap-frozen endarterectomy samples were ground to powder under liquid nitrogen with a cooled pestle and mortar and resuspended to a final volume of 700 μL in 50 mM Tris (pH 8.0), 100 mM EDTA, 100 mM NaCl, and 1% (wt/vol) SDS. Proteinase K was added to a final concentration of 100 μg/mL, and the samples were incubated at 55°C overnight. DNA was extracted with a phenol-chloroform mixture and precipitated with isopropanol by standard methods. The extracted DNA was purified with the use of guanidinium thiocyanate and diatomaceous earth, as previously described. Purified DNA was stored in 100 μL of TE (10 mM Tris, pH 7.5, 1 mM EDTA, pH 7.5) at −20°C.

Detection of C pneumoniae DNA in Tissue Samples

A nested PCR was developed with the use of 2 sets of primers to maximize the sensitivity of the assay. The HL1/H11 primer pair amplifying a 437-bp species-specific target sequence from the C pneumoniae genome (OMP gene) was used for the first round. Primers for the second round were designed with the use of the PRIMER program to give a 207-bp product (forward primer 5’TT-TAGATCAGTTGGTGTCATTCCG3’; reverse primer 5’AAGGT-TCATCCTTGAAGGCA3’). The reaction mix comprised 10 pmol of each primer, 200 μmol/L dNTPs, 1.5 mM/L MgCl₂, 1 U Taq DNA polymerase (Promega), 1× buffer (Promega), and 10 μL of each DNA sample in a 50-μL reaction volume. Ten microliters of the first round product was used in the second round reaction mix. Amplification was 40 cycles for round 1 (1 cycle of 94°C for 1 minute, 52°C for 1 minute, and 72°C for 1 minute, with a final extension cycle of 5 minutes) and...
Agarose gel electrophoresis of nested PCR products after amplification of the specific C pneumoniae OMP gene fragment. Lane 1: molecular weight markers, with representative bands marked (bp). Lanes 2 and 9: negative controls (no DNA). Lane 3: positive control; purified plasmid DNA template. Lanes 4 to 8: representative clinical samples.

30 cycles for round 2 (as for round 1, but with a 60°C annealing step). To avoid contamination of sample preparation, reagent preparation and product analysis were performed in 3 separate areas, with the use of filter-tipped pipettes. Positive and negative controls were included in all PCRs.

To optimize and quantify the PCR, we cloned the first round PCR product (using chlamydial genomic DNA supplied by Dr Ong, St Mary’s Hospital, London, as a template) into the vector pMOS Blue (Amersham Life Science) and selected transformed bacterial colonies according to the manufacturer’s instructions. Pure plasmid DNA was isolated with the use of the Wizard Plus Plasmid DNA extraction kit (Promega), following liquid culture of the required clone in selective antibiotics. The identity of the cloned insert was confirmed by sequencing on an ABI 373 automated sequencer. The sensitivity limit of the nested PCR was calculated by serial dilutions of the cloned construct.

For analysis, all products were electrophoresed on 2% agarose (wt/vol)/1×TBE (0.9 mmol/L Tris-borate, 0.002 mmol/L EDTA, pH 8.0) gels containing 0.1 μg/mL ethidium bromide and visualized on a UV transilluminator.

Results
Presence of C pneumoniae in Carotid Artery Atherosclerotic Lesions
Using a nested PCR on serial dilutions of the cloned chlamydial OMP gene fragment, we demonstrated that we could detect a minimum of 70 chlamydial genomes in our assay (data not shown). The Figure is a representative agarose gel, in which C pneumoniae DNA was detected in patient samples. With the use of the nested PCR described in Subjects and Methods, only single bands were detected in positive samples. Nested PCR data were only considered acceptable for inclusion when the negative controls for the first and second rounds were both completely free from contamination. As an added precaution, when ≥2 consecutive samples tested positive for the presence of C pneumoniae DNA by PCR, the samples were repeated but separated from one another to confirm genuine positivity. Our data showed that C pneumoniae DNA could be unequivocally detected in 25 of 98 atherosclerotic carotid lesions (25.5%).

Pathological Indicators and Chlamydial Status of Lesions
Table 1 shows the demographic analysis of our patient set, divided into 2 groups according to the presence or absence of the infectious organism. Chlamydial status in the carotid lesion was independent of the recorded demographic factors and also failed to correlate with presenting symptoms in this cohort of patients.

Closer examination of functional pathological parameters in this patient cohort also failed to demonstrate any correlation with plaque infection status, as shown in Table 2. The degree of ICA stenosis was almost identical between the C pneumoniae–positive and –negative groups. We found that 36.8% of patients had evidence of plaque embolization (based on any signals recorded either in the 30 minutes of scanning before surgery or recorded intraoperatively before ICA clamping). All patients who had preoperative embolization also embolized during the dissection phase of endarterectomy. Because no embolic signals were ever detected during monitoring of the contralateral MCA and all patients with atrial fibrillation were excluded from the study, we can be confident that the embolic events detected arose from the ipsilateral carotid lesion. In the 87 patients for whom we have TCD recordings, there was no significant difference in the percentage of patients embolizing in the C pneumoniae–positive and –negative groups. Recording was not possible in 11 of the patient cohort because of an inadequate transtemporal window. The mean time from symptom presentation to detection of emboli in our study was 2.7 months (range, 1 week to 12 months). Patients in whom emboli were detected had a mean time from symptoms of 2.07 months, compared with 3.06 months in patients in whom no emboli were detected (P=0.05, 2-tailed t test).

Analysis of CT scans (same time gap from initial symptom presentation to scans for TCD) demonstrated that 57% of the chlamydial-positive group had evidence of cerebral infarction compared with 69% of the chlamydial-negative group. There was no significant correlation between the chlamydial status of the plaque and either the number of ipsilateral MCA infarcts, the number of ipsilateral hemispheric infarcts, or total number of cerebral infarcts in these patients. The mean number of infarcts in the territory of the ipsilateral MCA in the plaque-positive group was 0.81 versus 0.91 in the plaque-negative group. In terms of the total number of cerebral

| TABLE 2. Embolization and Infarction Data for the Patient Cohort, Divided into 2 Groups Dependent on the Presence or Absence of C pneumoniae in the Carotid Plaques |
|---|---|---|
| Infarction | CP+ | CP− | P |
| No. | 21 | 49 | 0.32* |
| ICA stenosis | 86.3% | 87.6% | NS* |
| Embolization | 39% | 36% | NS* |
| Cerebral infarction | 12 (57%) | 34 (69%) | 0.32* |
| Mean No. of infarcts | 0.81 | 0.94 | 0.57† |
| Ipsilateral MCA territory | 0.81 | 0.91 | 0.54† |
| Ipsilateral hemispheric | 1.19 | 1.63 | 0.09† |

*Pearson χ². †Mann-Whitney.
infarcts, there was a mean of 1.19 in the plaque-positive group versus 1.63 in the plaque-negative group (Table 2). Unsurprisingly, our data show that patients with evidence of embolization were significantly more likely to have CT evidence of cerebral infarction and a greater number of ipsilateral hemispheric infarcts (mean = 1.33) compared with patients without evidence of embolization (mean = 0.66) (Table 3).

Histological examination of the excised plaques showed no gross differences in morphology between the chlamydial-positive and -negative samples, with the majority of samples showing evidence consistent with a complex inflammatory lesion (data not shown).

**Discussion**

Arterial disease is a huge burden on healthcare services, and the identification of a putative association between atherosclerosis and infection raised the tantalizing possibility of developing an antibiotic intervention for this disorder. There is an obvious precedent for this in the now-common prescription of antibiotics to eradicate the *Helicobacter pylori* infections that cause gastric ulcers, but it appears increasingly likely that for atherosclerosis more complex pharmaceutical strategies may be required. There is interesting preliminary evidence from animal studies that *C pneumoniae* may be a potential etiologic agent in atherosclerosis, but it is unclear how well this translates to the human disease condition.

The prevalence of *C pneumoniae* DNA in the advanced atherosclerotic lesions examined in this study was 25.5%. This figure is lower than has been reported by some other authors, and there are a number of potential reasons for this apparent discrepancy. In some cases in which detection of very low numbers of bacterial genomes has been reported, PCR has been followed by Southern blots to increase sensitivity. This often results in the detection of multiple bands or smears, raising questions about the specificity of the assay. In contrast, we used an extremely specific nested PCR that generated only a single product, the identity of which was confirmed by DNA sequencing during the initial establishment of the assay. A potential lack of specificity is also a problem with immunohistochemical detection systems for *C pneumoniae*, in which it can be very difficult to confirm that a positive reaction on a tissue section is genuinely specific to the pathogen and not to a cross-reacting cellular epitope. Therefore, while our analysis of the samples may appear to be less sensitive than some other screening, it is highly specific and less likely to introduce false-positives into our data sets. Additionally, analysis of randomly selected samples indicated no detectable inhibition of our PCR-based assay (data not shown).

In our study there was no correlation between the degree of carotid artery stenosis and the presence of *C pneumoniae*. Additionally, our data also strongly suggest that in advanced atherosclerotic lesions of the carotid artery, the presence of the infectious organism has no detectable impact on plaque stability, as defined by the functionally significant end points of microembolization and infarction. It is unlikely that the lack of association is an artifact caused by difficulties of in vivo assessment of plaque instability. While it is true that TCD detection of cerebral microemboli is a time-dependent observation (the longer monitoring is undertaken, presumably the greater the number of embolic events that can be detected), there was an association between plaques demonstrably embolizing and a significantly greater likelihood of cerebral infarction. This would suggest that TCD recording over the time interval used in this research functioned as a pathologically significant indicator of plaque instability (Table 3). Interestingly, patients in the group with TCD evidence of embolization and more ipsilateral hemispheric infarcts were also significantly younger than those in the group without TCD evidence of embolization.

Our data would therefore suggest that in advanced lesions the presence of *C pneumoniae* does not alter the in vivo stability of the atherosclerotic plaque. This might seem at odds with the growing body of cellular evidence that suggests a pathogenetic role for this microorganism, which, for example, has been shown to be capable of infecting and multiplying within the cells of the arterial wall. Additionally, *C pneumoniae* infection of human endothelial cells promotes migration of inflammatory cells and stimulates proinflammatory cytokine release (including tumor necrosis factor, interleukin-1β, and interleukin-6 release) from human monocytes as part of a cell-mediated immune response and possibly also from vascular smooth muscle cells. Additionally, antigens on the lipopolysaccharide cell wall of the bacteria cause local macrophage activation, and the organism has also been shown to stimulate an increase in expression of tissue factor from infected human endothelial cells, which would increase the risk of thrombosis on the plaque.

It has also recently been demonstrated that T-lymphocytes reactive to *C pneumoniae* can be detected in a subset of plaques from the human carotid artery, which the authors suggested indicated a role for cell-mediated immunity to this organism in the inflammatory lesion. It still remains to be established, however, that *C pneumoniae* is actually a pathological mediator of atherosclerotic processes rather than simply an “innocent bystander.” Despite this, the potential consequences of infection of atherosclerotic tissue by *C pneumoniae* have provided the rationale for trials of antibiotic therapy in the secondary prevention of athero-
sclerotic coronary arterial disease. A small number of antibiotic trials have been reported, of which the best known is the ROXIS randomized, prospective, double-blind, placebo-controlled study.27 This study showed that in a group of slightly more than 200 patients with unstable angina, roxithromycin appeared beneficial with end points of death or reinfarction. However, this study did not discriminate between patients positive or negative for C pneumoniae infection, and therefore it cannot be determined whether the apparently beneficial effects of roxithromycin were due to eradication of the microorganism or were a consequence of other actions of this macrolide antibiotic. These compounds have an anti-inflammatory action, which may independently improve plaque stability irrespective of antibiotic properties. Additionally, analysis of clinical end points at extended intervals after the initial administration suggested that the apparently beneficial effects may be short term.28 It has also been reported that treatment with roxithromycin reduced the bacterial burden of C pneumoniae in atherosclerotic carotid arteries, although it was not known whether this was associated with any clinical benefit.29 Nevertheless, further studies are planned or have started, presumably on the assumption that it is the antimicrobial effect of antibiotic therapy that is the critical parameter. If successful, such trials could potentially lead to large numbers of the population being treated with antibiotics. This will clearly have an ecological impact in terms of bacterial drug resistance in the community, which from a clinical perspective mandates careful selection of patients for this treatment.

Our data cannot determine whether C pneumoniae in an atherosclerotic carotid lesion influences early disease events because it is impossible to determine the likely duration of infection in our patients. Since our analysis of both the infection data and the embolization events was on an all-or-nothing basis, we cannot exclude the possibility of a graded response, eg, that above a certain bacterial load there is an effect on plaque stability, possibly mediated by, and additive with, the cellular responses outlined above, which cannot be detected by existing methodologies. However, it would appear that the presence of the microorganism is not a major influence on one of the most important mediators of disease presentation, ie, plaque stability. This raises further important caveats about the likely applicability of large-scale antibiotic intervention because our data suggest that in well-established "mature" plaques, the organism may potentially be neutral in impact. It is possible that antibiotic therapy may have a role to play in controlling the pathogenic development of relatively early lesions or, alternatively, vaccines may be of use in primary prevention. Our findings indicate that much more work may be required before the routine use of antibiotics in secondary intervention can be justified for the types of patients reported here. Much more detailed patient selection may be required for the rational application of antibiotic therapy. This may include both the size and complexity of the atherosclerotic plaque but also factors specific to the individual, eg, difference in their immune/inflammatory responsiveness or even defined genetic variability, as has been indicated with genetically engineered mice. It will also be vitally important to determine which is the functionally significant action of the macrolide drugs in this clinical context, ie, their antibiotic or their anti-inflammatory activities. Ultimately, it may be the burgeoning field of pharmacogenetics that has the most impact in treatment of atherosclerosis, but our current data support the view that much remains to be established in the area of infectious etiologies for this process and that the use of large-scale antibiotic intervention in well-established atherosclerotic lesions may be premature.

Acknowledgments

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