Chlamydia pneumoniae in Carotid Artery Atherosclerosis

A Comparison of Its Presence in Atherosclerotic Plaque, Healthy Vessels, and Circulating Leukocytes From the Same Individuals

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Background and Purpose—There is growing clinical and experimental evidence that infections with Chlamydia pneumoniae might contribute to the development and progression of atherosclerosis. However, studies detecting the pathogen in atherosclerotic lesions examined either only atherosclerotic vessels or control vessels without atherosclerosis obtained from a different group of individuals. We analyzed atherosclerotic plaques of the carotid artery, samples of apparently healthy greater saphenous veins, and circulating leukocytes from the same individual patients for the presence of C pneumoniae.

Methods—From each of 46 patients undergoing carotid endarterectomy for symptomatic carotid artery stenosis, these samples were analyzed by nested polymerase chain reaction for C pneumoniae–specific DNA. Furthermore, we determined IgA and IgG titers specific for the pathogen and plasma levels of C-reactive protein in these patients.

Results—C pneumoniae DNA was detected in 86.9% of the leukocytes and in 82.6% of the atherosclerotic plaques but in only 6.5% of the saphenous veins. In 85% of patients who also had leukocytes positive for C pneumoniae, the atherosclerotic plaques were positive and the saphenous veins were negative. The presence of C pneumoniae–specific DNA in leukocytes significantly coincided with the presence of the respective DNA in the plaques of the carotid arteries (P=0.0002). No association between the presence of C pneumoniae and specific IgA or IgG levels was seen. C-reactive protein levels were significantly higher in patients with chlamydia-positive atherosclerotic plaques and with positive leukocytes than in patients with negative plaques of the carotid arteries or negative leukocytes, respectively (P<0.01, P<0.05).

Conclusions—Our observation of >80% incidence of C pneumoniae in atherosclerotic plaques of the carotid artery does not prove causality between an infection with the pathogen and the development of atherosclerosis. It must be emphasized, however, that >90% of apparently healthy saphenous veins were negative for C pneumoniae. Given the structural and functional differences between veins and arteries, careful interpretation of our results regarding a possible causative role of C pneumoniae seems warranted. (Stroke. 2002;33:2756-2761.)

Key Words: atherosclerosis ■ bacteria ■ coronary vessels ■ leukocytes

Inflammation seems to be a key event in the development and progression of atherosclerosis.1 There is growing evidence that infections with certain pathogens by initiating an inflammatory response might contribute to this disease process. Among other infectious agents such as Helicobacter pylori and cytomegalovirus, Chlamydia pneumoniae, an obligate intracellular bacterium, has been implicated in the initiation and progression of atherosclerosis.2,3 An association between antibodies against C pneumoniae and ischemic heart disease was first described in a case-control study from Finland.4 Since then, further evidence for a role of C pneumoniae in atherosclerosis has accumulated from seroepidemiological studies linking the presence of antibodies against C pneumoniae to myocardial infarction, coronary heart disease, femoral and carotid atherosclerosis, and stroke.5 On the other hand, several in vitro investigations have shown that C pneumoniae is capable of infecting vascular endothelial cells and smooth muscle cells and thereby initiates inflammatory activation of these cells via the nuclear factor-κB pathway, resulting in increased expression of adhesion molecules, tissue factor, and inflammatory cytokines.5 In addition, support for this hypothesis comes from animal studies that showed that C pneumoniae can accelerate the development of atherosclerosis in mice and rabbits, which can be prevented

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by antibiotics, and from pilot anti-chlamydial antibiotic intervention trials. Viable strains of *C. pneumoniae* have been isolated and successfully cultured from atherosclerotic plaques. Furthermore, *C. pneumoniae* has been detected in atherosclerotic lesions in coronary arteries, in abdominal aortic aneurysms, in atherosclerotic carotid arteries, and most recently in specimens of atherosclerotic middle cerebral artery by immunohistochemistry, in situ hybridization, identification of genomic material by polymerase chain reaction (PCR), or electron microscopy. It is noteworthy, however, that in some of these studies, only material from vascular lesions was used to detect *C. pneumoniae*. Alternatively, in a second group of investigations, in addition to vascular lesions, healthy vessels obtained from a different group of individuals were also analyzed for the presence of *C. pneumoniae*. Thus, on the basis of these studies, it is not possible to evaluate whether the presence of *C. pneumoniae* is restricted to the vascular lesion or if the pathogen is also found in healthy parts of the vasculature.

To address this question, we collected samples from 46 patients suffering from symptomatic carotid artery disease. We used nested PCR to identify *C. pneumoniae* DNA for the first time—at least to the best of our knowledge—in atherosclerotic plaques of the carotid artery, in circulating leukocytes, and in an apparently healthy vessel, namely the greater saphenous vein (GSV) obtained from each individual patient.

### Subjects and Methods

#### Subjects

Between December 1999 and December 2001, a total of 46 consecutive patients (28 men; mean age, 73 years; range, 52 to 86 years; 18 women; mean age, 70 years; range, 55 to 86 years) underwent carotid endarterectomy for symptomatic carotid artery stenosis. No exclusion criteria were applied, and all initially recruited patients were included in the analysis. These patients were representative of the underlying population of symptomatic patients. Patient details, symptoms, and risk factors are shown in Table 1. Nicotine consumption was diagnosed if the patients were currently active smokers or if smoking cessation dates back to ≤4 years. This definition is based on an observation that a smoker’s risk to suffer from cardiovascular disease approaches that of a person who had never smoked within 3 to 4 years after cessation. Patients were assigned to the hypertension risk group if they had stage 1 to 4 hypertension based on an average of ≥3 readings during the preoperative stay at the surgical ward. Patients were estimated as hypertensive if the average systolic blood pressure was ≥140 mm Hg and average diastolic blood pressure was ≥90 mm Hg or if they were taking antihypertensive medication. Dyslipidemia was diagnosed if total cholesterol was >200 mg/dL regardless of serum triglyceride concentration. Isolated hypertriglycerideremia was not included in the risk factor profile. Patients with diabetes mellitus either had dietary therapy or took oral medication. Dyslipidemia was diagnosed if total cholesterol was >200 mg/dL regardless of serum triglyceride concentration. Isolated hypertriglycerideremia was not included in the risk factor profile. Patients with diabetes mellitus either had dietary therapy or took oral medication. Dyslipidemia was diagnosed if total cholesterol was >200 mg/dL regardless of serum triglyceride concentration. Isolated hypertriglycerideremia was not included in the risk factor profile.

#### DNA Isolation

DNA from circulating leukocytes was prepared according to Miller et al. Alternatively, DNA was isolated from the same blood samples with a commercially available DNA extraction and purification kit using FTA cards for blood collection and storage (Life Technologies). No difference was seen when DNA analyzed by either method was further processed. DNA was isolated from ∼100 mm^3^ of either the carotid atherosclerotic plaque or pieces of healthy GSV by digestion with 1 mL of a solution containing 50 mmol/L Tris-HCl, 100 mmol/L EDTA, 100 mmol/L NaCl, 1% sodium dodecyl sulfate, and 0.5 mg/mL proteinase K, pH 7.2 at 55°C overnight. After addition of 410 μL of 5 mol/L NaCl and centrifugation for 10 minutes at 13,000 rpm, the DNA was precipitated with twice the volume of 96% ethanol. The DNA was washed 4 times with 70% ethanol and resuspended in 1 mL of a buffer containing 10 mmol/L Tris, 1 mmol/L EDTA, pH 8.0 at 4°C. Care was taken to maintain aseptic handling of the blood and tissue samples during the DNA isolation procedure. Patient samples and PCR reaction assembly were kept in separate laboratories to prevent contamination of the samples by PCR products. Each sample was analyzed in duplicate.

#### PCR and Nested PCR

To detect *C. pneumoniae* at the required level of sensitivity, a portion of the 16S rRNA gene was amplified with a PCR protocol published by Gaydos et al using the following primers: sense, 5'-TGCAACTCCTGAGAAATACACGC-3', and antisense, 5'-GGGTCCTCTCTCTCCTATAAAT-3'. To confirm the results obtained by PCR, we performed nested PCR using 2 sets of primers (external: sense, 5'-ATAATGAGTTTGCTCTTCCTATAAAT-3'; and antisense, 5'-TAAAATATGGTGGATGCACAC-3'; internal: sense, 5'-AGTGTTAATAGGCGCTCATTAATAT-3'; and antisense, 5'-GCGTTATTTCTACAGTTG-3') designed to detect a fragment of the 16S rRNA gene of *C. pneumoniae* as described by Black et al. In all samples tested for the presence of *C. pneumoniae*-specific DNA, the same results were obtained with PCR and nested PCR technology.
Serological Testing and CRP Determination
Specific IgA and IgG antibodies against C pneumoniae in the serum of the patients were determined by use of commercially available enzyme-linked immunosorbent assays (ELISAs; SeroCP Quant IgA and SeroCP Quant IgG, Savyon Diagnostics) and microimmunofluorescence assays (MIFs; Chlamydia pneumoniae IgA MIF Test Kit and Chlamydia pneumoniae IgG MIF Test Kit, Labsystems) according to the manufacturer’s protocol. C-reactive protein (CRP) was determined in the plasma samples of the patients using a highly sensitive assay obtained from Dade Behring.

Statistical Analysis
Data are represented as mean and SD. The significance of any differences in proportions was tested by Fisher’s exact test. The association of the presence of C pneumoniae in leukocytes (CPL) and plaques (CPP) was adjusted by use of a multiple logistic regression model with CPP as a dependent variable. The model was restricted to 3 independent variables because of the small sample size. Therefore, as independent variables in addition to CPL, we chose the 2 prognostic factors from age, sex, CRP, nicotine consumption, dyslipidemia, hypertension, and diabetes mellitus showing the highest correlation with CPP and CPL. The significance of any difference in mean CRP levels was tested by use of Student’s t test. The significance of any difference in antibody titers was tested by use of Student’s t test after log transformation. Multiple comparisons were accounted for by the Bonferroni-Holm method. Antibody titers measured with different methods were compared by Spearman’s correlation. All probability values were 2 tailed, and confidence intervals were calculated at the 95% level. All calculations were performed with a computer program (SPSS for Windows 10.0.1).

Results
Through nested PCR, C pneumoniae DNA was detected in the leukocytes of 40 of 46 patients (86.9%) and in the atherosclerotic plaques of 38 of 46 patients (82.6%). In contrast, C pneumoniae DNA was found only in the saphenous veins in 3 of 46 patients (6.5%). In 85% of patients who had leukocytes positive for C pneumoniae as determined by PCR, the atherosclerotic plaques also were positive, whereas the saphenous veins were negative. In 7.5% of the patients, C pneumoniae DNA was exclusively found in circulating leukocytes. In the remaining 7.5% of patients with positive leukocytes, the plaque and the saphenous vein were positive. Only leukocytes from 6 patients were negative for C pneumoniae as determined by PCR. In 5 of these patients, the atherosclerotic plaque and saphenous vein also were negative, whereas in 1 patient, C pneumoniae DNA was detected in the plaque. The presence of C pneumoniae–specific DNA in leukocytes significantly coincided with the presence of the respective DNA in the plaques of the carotid arteries (P=0.0002). This association remained highly significant after adjustment for the factors showing the highest correlation with the presence of C pneumoniae–specific DNA in leukocytes and in the plaques of the carotid arteries (age and CRP, P=0.0005). There was no significant association between the presence of C pneumoniae DNA in veins and leukocytes (P=1.0) or arteries (P=1.0), respectively (Table 2). C pneumoniae–specific IgA and IgG levels determined in serum samples by an MIF assay showed a high correlation to IgA (R=0.883, P<0.00001) and IgG (R=0.876, P<0.00001) antibody levels determined by ELISA. In both assays, no significant difference in antibody titers was seen after adjustment for multiple comparisons between patients whose atherosclerotic plaques were tested positive for C pneumoniae DNA compared with patients whose plaques were tested negative for C pneumoniae (Table 3). CRP levels were significantly higher in patients with chlamydia-positive atherosclerotic plaques than in patients with negative plaques of the carotid arteries (P<0.01) and in patients with positive leukocytes compared with patients with negative leukocytes (P<0.05), whereas no significant difference was seen when patients with positive saphenous veins were compared with patients with negative veins (P=0.840; Table 4).

Discussion
There is growing and intriguing evidence from clinical and experimental studies linking the pathogen C pneumoniae to the development and progression of atherosclerosis.2–5 However, doubts about a role that C pneumoniae plays in the development of atherosclerosis are raised by studies that could not find an association between the bacterium and the disease and by other studies that found positive correlation only with total pathogen burden consisting of multiple infections with various agents.20–23 Similarly, the results of antibiotic trials conflict with results of some studies finding survival benefit and prevention of progression of cardiovascular disease.24,25 Other studies and first reports of the WIZARD and AZACS trials do not indicate risk reduction in myocardial infarction and ischemic stroke related to the use of antibiotics.26–28 On the other hand, C pneumoniae has been detected and in some cases successfully isolated in or from atherosclerotic lesions at various locations, including coronary arteries, aortic aneurysms, carotid arteries, and middle cerebral arteries.5,6–13 Thus far, however, most of these studies have analyzed only atherosclerotic vessels for the presence of the pathogen. In some of these studies, control vessels without atherosclerosis were obtained from a different

### Table 2. Distribution of C pneumoniae DNA in Atherosclerotic Plaques of the Carotid Arteries and in Saphenous Veins Obtained From Patients Whose Leukocytes Tested Either Positive or Negative for the Presence of C pneumoniae–Specific DNA as Determined by Nested PCR

<table>
<thead>
<tr>
<th></th>
<th>P pos V pos</th>
<th>P pos V neg</th>
<th>P neg V pos</th>
<th>P neg V neg</th>
</tr>
</thead>
<tbody>
<tr>
<td>L pos (n=40)</td>
<td>3 (7.5)</td>
<td>34 (85.0)</td>
<td>0</td>
<td>3 (7.5)</td>
</tr>
<tr>
<td>L neg (n=6)</td>
<td>0</td>
<td>1 (16.7)</td>
<td>0</td>
<td>5 (83.3)</td>
</tr>
</tbody>
</table>

L indicates leukocytes; P, atherosclerotic plaques of the carotid artery; V, saphenous veins; pos, tested positive for the presence of C pneumoniae–specific DNA by nested PCR; neg, tested negative for the presence of C pneumoniae–specific DNA by nested PCR.
In the present study, we have collected samples from atherosclerotic plaques of the carotid artery, samples of a group of individuals who were mostly not age matched, which led to the concern that differences in prevalence of the bacterium were due to the differences in age between the study group and the younger control group.29 To investigate possible links between the presence of C pneumoniae and the development of atherosclerotic lesions, it would be appropriate to study the localization of the pathogen within individuals. Taking this approach, a recent study has analyzed different arterial locations in individuals postmortem for the presence of C pneumoniae by immunocytochemistry. The investigators found an association between immunoreactivity to C pneumoniae and the distribution of atherosclerosis within the arterial segments.30

In the present study, we have collected samples from atherosclerotic plaques of the carotid artery, samples of apparently healthy GSVs and circulating leukocytes from each of 46 patients suffering from symptomatic carotid artery diseases. To the best of our knowledge, we have for the first time analyzed this material by nested PCR for the presence of C pneumoniae DNA. In 41 patients (89.1%), C pneumoniae-specific DNA was found in ≥1 samples, whereas in only 5 patients (10.9%), no evidence for the presence of the bacterium based on negative PCR results was found. We could show that in 40 patients (86.9%), C pneumoniae-specific DNA was present in circulating leukocytes; in 38 patients (82.6%), such DNA was found in the atherosclerotic plaque; and only in 3 patients (6.5%) was the saphenous vein positive for C pneumoniae. It should be mentioned that the high percentage of C pneumoniae-positive leukocytes and atherosclerotic plaques found in our group of patients is in contrast to data published in other studies.8,11–13,31 There was no recent epidemic in the country, and the samples have been collected randomly over the whole year. On the other hand, a recent study found C pneumoniae DNA in 95% of carotid artery plaques in smokers but in only 36% of the plaques obtained from nonsmokers.33 Thus, the high prevalence of smoking in our group of patients might, at least in part, help to explain our findings. A further confounding factor might be the socioeconomic status of the patients.33,34 Unfortunately, we can provide only limited information on this parameter (see also the Study Limitations section): All our patients had basic medical insurance probably indirectly indicating a low to middle class economic status. All patients were white and lived in the eastern part of Austria. No homeless individuals were present in our group of patients. Concerning the methodology used, it should be emphasized that we used 2 different

**TABLE 3. Levels of C pneumoniae-Specific IgA and IgG in Serum Obtained From Patients Undergoing Carotid Endarterectomy for Symptomatic Carotid Artery Stenosis as Determined by Specific MIF Assays and ELISAs**

<table>
<thead>
<tr>
<th>MIF Assay</th>
<th>IgA Titer, n (% of total)</th>
<th>IgG Titer, n (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plaque Neg (n=8)</td>
<td>3:1</td>
<td>3:1</td>
</tr>
<tr>
<td>Plaque Pos (n=35)</td>
<td>1:32</td>
<td>1:64</td>
</tr>
<tr>
<td>ELISA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plaque nd</td>
<td>3:1</td>
<td>3:1</td>
</tr>
<tr>
<td>Plaque Pos (n=35)</td>
<td>1:32</td>
<td>1:64</td>
</tr>
</tbody>
</table>

Pos indicates tested positive for the presence of C pneumoniae-specific DNA by nested PCR; Neg, tested negative for the presence of C pneumoniae-specific DNA by nested PCR; nd, not determined.

No significant differences in antibody titers between patients with C pneumoniae positive and negative plaques were found when the Bonferroni Holm correction for multiple comparison was applied.

**TABLE 4. Levels of C-Reactive Protein in Plasma Obtained From Patients Undergoing Carotid Endarterectomy for Symptomatic Carotid Artery Stenosis as Determined by a Specific High-sensitive ELISA**

<table>
<thead>
<tr>
<th>C-Reactive Protein, n (mg/dL)</th>
<th>Pos</th>
<th>Neg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes</td>
<td>0.68±0.18 (37)</td>
<td>0.14±0.08* (6)</td>
</tr>
<tr>
<td>Plaques</td>
<td>0.71±0.21 (35)</td>
<td>0.13±0.07† (8)</td>
</tr>
<tr>
<td>Saphenous veins</td>
<td>0.41±0.40 (3)</td>
<td>0.62±0.15 (40)</td>
</tr>
</tbody>
</table>

Pos indicates tested positive for the presence of C pneumoniae-specific DNA by nested PCR; Neg, tested negative for the presence of C pneumoniae-specific DNA by nested PCR.

| *P<0.05. |
| †P<0.01. |
PCR methods and 2 different methods for preparation of DNA from peripheral blood leukocytes that gave identical results. Furthermore, it should be mentioned that contamination of the material as a reason for the high incidence of positive samples of atherosclerotic plaques and peripheral blood leukocytes can be ruled out because the vast majority of samples of saphenous veins, which were processed and tested in parallel to the plaque material and the leukocytes from each individual patient, were negative. The presence of \textit{C pneumoniae}--specific DNA in leukocytes significantly coincided with the presence of the respective DNA in the plaques of the carotid arteries. In 85% of patients whose leukocytes were positive for \textit{C pneumoniae}, the atherosclerotic plaque also was positive, whereas the respective saphenous vein was negative. In contrast, in 83.3% of patients with negative leukocytes, the atherosclerotic plaque and the respective saphenous vein also tested negative for the pathogen. Thus, the predictive value of the presence or absence of chlamydial DNA in the leukocytes for the plaques of the carotid artery and the saphenous vein was highly significant.

When we determined IgA and IgG antibodies against \textit{C pneumoniae} by specific ELISAs and specific MIF assays, we—in agreement with a recent study—found good correlation between these 2 methods (IgA: \( R=0.883, P<0.00001 \); IgG: \( R=0.876, P<0.00001 \)). However, in both assays, no significant difference in antibody titers was seen after adjustment for multiple comparisons between patients whose atherosclerotic plaques were tested positive for \textit{C pneumoniae} DNA compared with patients whose plaques were tested negative for \textit{C pneumoniae}. Thus, after careful statistical analysis of our data, we, like others, conclude that serology is unreliable in predicting the presence of \textit{C pneumoniae} DNA. In correlation with a previously published study, we were able to show that patients whose peripheral leukocytes or atherosclerotic plaques were positive for \textit{C pneumoniae} DNA had significantly higher plasma levels of CRP compared with patients with negative leukocytes and plaques. Thus, our results lend further support to the hypothesis put forward by the authors of the study mentioned previously that infections with \textit{C pneumoniae} are associated with increased levels of CRP and that this might contribute to the link between elevated CRP levels and the risk for stroke and cardiovascular disease.

In conclusion, we could show that in >80% of our patients with symptomatic carotid artery disease, \textit{C pneumoniae} DNA was present in the atherosclerotic plaque and in >85% of these patients was \textit{C pneumoniae} DNA present in circulating leukocytes. It should be emphasized that in >90% of the patients with positive leukocytes, \textit{C pneumoniae} DNA was also present in the atherosclerotic lesion. This correlation was highly significant, which is in agreement with other reports postulating a high predictive value of the detection of \textit{C pneumoniae} in peripheral blood for vascular infection with the pathogen. It is noteworthy that although the total number of patients is small and such statistical analysis should therefore be interpreted only with caution, this association remained highly significant after adjustment for the factors showing the highest correlation with the presence of \textit{C pneumoniae}--specific DNA in leukocytes and in the plaques of the carotid arteries, namely age and CRP levels.

Although our observation of >80% incidence of \textit{C pneumoniae} in atherosclerotic plaques of the carotid artery in our patients does not prove causality between an infection with the pathogen and the development of atherosclerosis, it should be emphasized that in >90% of the patients, samples of apparently healthy saphenous vein were negative for \textit{C pneumoniae}. Until now, concern has persisted that the presence of \textit{C pneumoniae} in patients with advanced disease may reflect only a late-onset “bystander” role for the bacterium. Recently, however, evidence for infection with the pathogen also in early atherosclerosis has been presented. Furthermore, a growing number of prospective studies has linked infection with \textit{C pneumoniae} and other infectious agents to the risk of carotid or coronary atherosclerosis. Considering our results showing a preferential localization of the pathogen at sites of vascular lesions, it is tempting to speculate about mechanisms leading to a selective transport of \textit{C pneumoniae} to and its deposition at probably minimally preinjured vascular sites. One could hypothesize that at such sites \textit{C pneumoniae}, by initiation of an inflammatory response (eg, via activation of the nuclear factor-\( \kappa \)B pathway in endothelial cells and smooth muscle cells, which has been shown in several in vitro studies), then might contribute to the progression of such a lesion.

**Study Limitations**

In our study, we compared the presence of \textit{C pneumoniae} in atherosclerotic plaques of the carotid artery to its presence in apparently healthy saphenous veins from the same individuals. It cannot be ruled out that the difference in infection rate with \textit{C pneumoniae} between atherosclerotic carotid arteries and apparently healthy saphenous vein is brought about by the different wall structures of arteries and veins (see elsewhere for a review). As mentioned, this difference does not prove causality between infection with the bacterium and atherosclerosis mainly because the veins normally do not show atherosclerotic lesions. In this regard, however, it should be emphasized that vein grafts transplanted into arterial vascular beds can develop atherosclerotic plaques similar to lesions seen in arteries. Furthermore, there is ample evidence that, at least in vitro, \textit{C pneumoniae} is able to infect venous endothelial cells, and a recent study has described the presence of the bacterium in diseased but not in healthy saphenous veins. Thus, in summary, because of the known functional and structural differences between arteries and veins, careful interpretation of our results is warranted.

As mentioned, an inverse correlation between socioeconomic status and infection with \textit{C pneumoniae} has been demonstrated in several studies. It is a limitation of our study that we can provide only limited information on socioeconomic factors in our group of patients. None of our patients had private health insurance, but all had basic health coverage. This fact might indirectly indicate a low to middle class socioeconomic status. Thus, because of the lack of detailed information on socioeconomic parameters, we cannot rule out the possibility that these are confounding factors in our study.

**Acknowledgments**

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References

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