Association of Serology With the Endovascular Presence of \textit{Chlamydia pneumoniae} and Cytomegalovirus in Coronary Artery and Vein Graft Disease

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**Background**—Chemotherapeutic treatment for patients with symptomatic coronary artery disease to reduce cardiovascular events may be initiated in response to elevated antibody titers against \textit{Chlamydia pneumoniae} or cytomegalovirus. How antibody titers are associated with the endovascular presence of these microorganisms is still unclear.

**Methods and Results**—Antibody titers against \textit{C pneumoniae} (microimmunofluorescence) and cytomegalovirus (ELISA) in patients undergoing primary (coronary desobliterates, n=80) or repeated CABG (occluded vein grafts, n=45) were correlated with the endovascular presence of the 2 microorganisms. \textit{C pneumoniae} was detected by means of a nested polymerase chain reaction (PCR) and by culturing. Both conventional PCR and quantitative PCR were applied for detection of cytomegalovirus. \textit{C pneumoniae} (PCR/culture) was detected in 19/9% (15/80 and 7/80) of coronary desobliterates and in 18/11% (8/45 and 5/45) of occluded vein grafts. There was no statistical evidence that IgG values differed between patients with or without \textit{C pneumoniae} detection who were undergoing primary CABG. In contrast, repeated-CABG patients with a positive PCR (\(P=0.0027\)) or \textit{C pneumoniae} culture (\(P=0.0018\)) had distinctly elevated IgG titers compared with patients in whom \textit{C pneumoniae} was not detected. Cytomegalovirus could not be detected in the examined specimens.

**Conclusions**—Cytomegalovirus infection does not seem to be associated with advanced coronary artery lesions. \textit{C pneumoniae} antibody titers are not associated with the endovascular presence of \textit{C pneumoniae} in patients with coronary artery disease. The observed strong association between elevated IgG titers and the detection of \textit{C pneumoniae} in occluded vein grafts warrants further investigation. (\textit{Circulation}. 2000;101:137-141.)

**Key Words:** \textit{Chlamydia pneumoniae} \textbullet; viruses \textbullet; coronary disease

Several studies have reported an association between chronic infections with \textit{Chlamydia pneumoniae} and/or cytomegalovirus (CMV) and atherosclerosis by means of both seroepidemiology and demonstration of the microorganisms in the atherosclerotic lesions (for review, see articles by Danesh et al\(^1\) and Libby et al\(^2\)). Increasing evidence of a causal relationship between chronic infections and atherosclerosis led to clinical studies examining the effect of macrolide treatment in patients with symptomatic coronary artery disease for secondary prevention of cardiovascular events.\(^3,4\) The optimum parameter for identification of patients who might be candidates for chemotherapeutic treatment has yet to be determined.\(^5\) Elevated antibody titers against the mentioned microorganisms might be considered indicators of patients who are at greater risk for cardiovascular events.\(^4,5\) However, whether serological parameters are appropriate selection criteria for chemotherapeutic treatment is a subject of controversy.\(^5\) Recently, our group reported the detection of \textit{C pneumoniae} in occluded vein grafts; these results may indicate a possible relation between chronic \textit{C pneumoniae} infection and vein graft disease.\(^6,7\) This study examines how antibody titers against \textit{C pneumoniae} and CMV in patients with coronary artery and vein graft disease are associated with the endovascular presence of the 2 microorganisms.

**Methods**

The study was approved by the local ethics committee, and informed consent was obtained from all patients.

**Pathological and Control Specimens**

Occluded vein grafts from 45 patients undergoing reoperation were collected. Only occluded vein grafts showing typical thickening of the venous wall indicative of late graft occlusion were selected for further investigation.\(^8\) The mean interval from initial surgery to reoperation was 112±53 months. In 80 patients, coronary desobliterates (dissecting the atherosclerotic cylinder from the adventitial layer of the coronary artery) were obtained from occluded or...
Patient Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Primary CABG (n=80)</th>
<th>Repeated CABG (n=45)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean±SD), y</td>
<td>63.1±8.7</td>
<td>60.7±15.4</td>
</tr>
<tr>
<td>Sex, M/F</td>
<td>66/14</td>
<td>36/9</td>
</tr>
<tr>
<td>Body mass index (mean±SD), kg/m²</td>
<td>26.8±3.6</td>
<td>26.1±3.0</td>
</tr>
<tr>
<td>History of smoking, n (%)</td>
<td>44 (59)</td>
<td>20 (49)</td>
</tr>
<tr>
<td>Current</td>
<td>20 (27)</td>
<td>7 (17)</td>
</tr>
<tr>
<td>Previous</td>
<td>24 (32)</td>
<td>13 (32)</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>26 (35)</td>
<td>13 (31)</td>
</tr>
<tr>
<td>Hyperlipidemia, n (%)</td>
<td>42 (57)</td>
<td>28 (67)</td>
</tr>
<tr>
<td>Median MIF IgG</td>
<td>64</td>
<td>64</td>
</tr>
</tbody>
</table>

MIF indicates microimmunofluorescence.

severely stenotic coronary arteries. Patient characteristics are given in the Table. There was no evidence of a difference between patients undergoing primary or repeated CABG with regard to epidemiological parameters or risk factors.

Serology

Sera were tested for *C pneumoniae*—specific antibodies by use of a commercially available microimmunofluorescence assay (IgG, IgM; Labsystem). Sera were screened for CMV antibodies (ELISA; IgG, IgM; Enzygnost, Dade-Behring). An anti-*C pneumoniae* IgG titer ≥16 was considered to indicate previous *C pneumoniae* infection. For CMV, IgG titers ≥230 were considered indicative of previous CMV infection.

*C pneumoniae* Detection

Tissue was cut into 0.3-cm segments, ground, and suspended in cell culture medium. The suspensions were then divided for polymerase chain reaction (PCR) and culture.

*C pneumoniae* PCR

Genomic *C pneumoniae* DNA was detected by a nested PCR protocol, as described elsewhere.6,7 Briefly, DNA was purified from the suspensions by proteinase K digestion and cetyltrimethylammonium bromide treatment. The DNA extraction protocol has been optimized to provide very pure DNA preparations with minimal inhibitory activity in the amplification procedure, as previously described.9 Furthermore, 2 types of inhibitor controls to detect potential PCR inhibitors in the samples were used: addition of the extracted DNA of 10 inclusion-forming units or addition of 10⁶ copies of a plasmid containing the PCR target sequence, a Psrl fragment of *C pneumoniae* genomic DNA (kindly provided by T. Miethke, Medical Microbiology, Technical University of Munich). Nested PCR was then performed by use of the species-specific HL-1/HR-1 primer pair for the first round of 32 amplifications. The nested primer pair IN-1/2, which yields a 128-bp product, was then used in the subsequent 32 amplification cycles. For confirmation, nonradioactive DNA hybridization was performed with oligonucleotide HM-1–3 labeled with digoxigenin-ddUTP (Boehringer) used as the probe.

*C pneumoniae* Culture

Serum-free cell culture was performed as previously described.6,7 Briefly, suspensions were centrifuged onto HEp-2 host-cell monolayers and incubated for 3 days at 35°C in 5% CO₂ in isolation medium (Eagle’s minimal essential medium, GIBCO/BRL) supple-mented with 1 μg/mL cycloheximide (Sigma) in ≥10 serial passages. Productive *C pneumoniae* growth was identified by staining inclusions with FITC-conjugated *C pneumoniae*-specific mouse monoclonal antibody (Cellabs). The strains were first identified in the second to fifth subculture. The cell culture monolayer of the first passage could not be judged by immunofluorescence staining because it contained too much autofluorescent material from the homogenized tissue. Because staining kills *C pneumoniae*, a substantial portion of the monolayer had to be retained for subsequent cultures until growth was established. In a compromise between early visualization of *C pneumoniae* and establishment of culture growth, about one third of the monolayer was used for staining purposes; the rest was used for subsequent cultures.

CMV Detection

Oligonucleotide primers (TIB MOLBIOL) and probes (PE Applied Biosystems) complementary to the pp65 gene were used.

Conventional CMV PCR

PCR was performed in a total volume of 50 μL consisting of 1× PCR buffer, 2.5 U AmpliTaq Gold (PE Applied Biosystems), 200 μmol/L of each dNTP, and 50 pmol of each primer. PCR was performed for 40 cycles after activation of AmpliTaq Gold. The reaction mixture was then subjected to agarose gel electrophoresis, Southern blotting, and nonradioactive hybridization with a digoxigenin-labeled oligonucleotide probe, as previously described.6,10

Quantitative CMV PCR

To obtain PCR conditions with reduced variability and contamination, quantitative PCR was performed by use of a real-time Taqman PCR system (ABI PRISM 7700 Sequence Detector Systems, PE Applied Biosystems).6,11,12 The hybridization probe that binds to both PCR products was labeled with a reporter dye, FAM, on the 5' nucleotide and a quenching dye, TAMRA, on the 3' nucleotide.13 The following composition of PCR assay (total volume, 50 μL) was used: 100 ng purified sample DNA, 15 pmol of each primer, 10 pmol probe, and 200 μmol/L of each dNTP. This PCR protocol had a sensitivity of 10 CMV DNA copies, as determined by serial dilution.

To exclude false-negative PCR results, the following inhibitor control for both CMV PCR assays was applied. Each single CMV PCR assay consisted of 3 parallel PCR experiments: (1) PCR mix and 100 ng sample DNA; (2) PCR mix and water (negative control); and (3) PCR mix, 100 ng sample DNA, and 0.5 ng leukocyte DNA from a known CMV-infected patient (positive control). The test was valid only if the negative control was negative and the positive control revealed a clear positive signal.

Statistical Analysis

Except as otherwise stated, variables are reported as geometric mean (GM) and 95% CI or total numbers and relative frequencies (in the case of dichotomous variables). For comparison between groups, Student’s t test or Mann-Whitney’s U rank-sum test and the χ² test or Fisher’s exact test (for case of dichotomous variables) were used as appropriate. All tests were 2 tailed. A value of P<0.05 was considered statistically significant. The analyses were performed with Minitab software (release 10).

Results

*C pneumoniae* Detection

*C pneumoniae* DNA was detected in 19% of coronary desobliterates (15 of 80) and 18% of occluded vein grafts (8 of 45); viable *C pneumoniae* could be cultured from coronary desobliterates in 9% (7 of 80) and from occluded vein grafts in 11% (5 of 45) of cases. There was no evidence of a systematic difference in graft age between *C pneumoniae*–
positive and *C pneumoniae*-negative grafts. All specimens with positive cultures also showed positive PCR results. Except for a single native saphenous vein (collected during reoperation in a patient with *C pneumoniae* in the occluded vein grafts), all control specimens were negative for *C pneumoniae*.

**CMV Detection**

Pathological and control specimens were negative for CMV DNA in both conventional and quantitative PCR.

**Serology**

*Cytomegalovirus*

IgG CMV antibody titers $\geq 230$, indicating prior CMV infection, were determined in 58.3% of the patients. Elevated IgM titers were not detected.

*C pneumoniae*

No elevated *C pneumoniae* IgM antibodies were observed in any patients. With the raw data, there was no statistical evidence that the IgG values differed in relation to a positive or negative *C pneumoniae* PCR or culture result ($P = 0.63$ and $P = 1.0$, respectively) for patients undergoing primary revascularization. In detail, in negative cultures, the GM was 56.0 (95% CI, 36.0 to 87.4); in positive cultures, the GM was 32.1 (95% CI, 2.2 to 464.0); in negative PCRs, the GM was 53.2 (95% CI, 32.5 to 87.1); and in positive PCRs, the GM was 53.2 (95% CI, 16.6 to 170.5).

In contrast, patients with repeated CABG and a positive PCR ($P = 0.0018$), *C pneumoniae* culture ($P = 0.0027$) result had distinctly elevated IgG titers compared with patients in whom *C pneumoniae* was not detected. Because of the limited number of positive specimens, a cutoff titer (IgG value) that would predict the vascular presence of *C pneumoniae* with reasonable CIs could not be calculated. In detail, in negative cultures, the GM was 32.0 (95% CI, 16.2 to 63.4); in positive cultures, the GM was 294.1 (95% CI, 95.8 to 903.3); in negative PCRs, the GM was 29.1 (95% CI, 13.9 to 61.1); and in positive PCRs, the GM was 181.1 (95% CI, 71.5 to 458.5).

**Discussion**

This study has several new findings. For patients undergoing primary coronary revascularization, IgG values did not differ between patients with or without endovascular presence of *C pneumoniae*. In contrast, patients with repeated CABG and positive PCR or *C pneumoniae* culture results had distinctly elevated IgG titers compared with patients without *C pneumoniae*.

CMV was not associated with advanced coronary artery or vein graft disease in this study.

*C pneumoniae*

We observed a *C pneumoniae* PCR positivity rate of 19% in patients undergoing first-time CABG and 18% for patients undergoing reoperation. Only a few studies have examined coronary arteries by means of PCR; the *C pneumoniae* rates were 2%, 16%, 17%, and 32%, respectively, in 4 studies.$^{14-17}$ Therefore, our positivity rates are within the expected range.

We used nested PCR, a modification of PCR that is optimized for specificity. However, there is an obvious difference between *C pneumoniae* PCR positivity rates and the positive results seen when immunological procedures are applied. Studies using immunocytochemistry demonstrated positive detection ranges from 39% to 45%.$^{15-17}$ Two authors compared PCR methods with immunocytochemistry; they observed a higher rate of *C pneumoniae* detection with immunocytochemistry compared with PCR.$^{15,16}$ The difference in *C pneumoniae* detection by PCR compared with immunocytochemistry is underlined by the rare discordant detection of *C pneumoniae* by both methods. In a report by Jackson and coworkers,$^{16}$ only 1 specimen was positive in both immunocytochemistry and PCR (overall number positive in PCR and/or immunocytochemistry, 13 of 34 coronary arteries).

The highest positivity rate (79%) was reported in a study using direct immunofluorescence staining of atherosclerotic tissue.$^{18}$ However, this result and the technique have not been reproduced by others. The other immunohistochemical studies have provided reproducible data, and it is difficult to explain the variations in the positivity rate other than by the methodological differences. However, immunohistochemical procedures may lead to some unspecific reactivity compared with PCR because unexpected antibody reactivity might occur. This has been discussed in a *Circulation* editorial.$^{19}$

Only a few studies have focused on the relation between elevated anti-*C pneumoniae* antibodies and the detection of the pathogen in the vessel wall. Campbell and associates$^{17}$ did not observe an association between the frequency of *C pneumoniae* detection in native or restenosed coronary artery lesions and *C pneumoniae*-specific antibodies. Puolakainen et al$^{20}$ reported more frequent detection of *C pneumoniae* genome in coronary artery lesions in subjects with low IgG titers (GM titers, 13.6 $\pm$ 4.2) than in those with no *C pneumoniae* in the atherosclerotic tissue (53.4 $\pm$ 4.3). In contrast to these findings, Blasi et al$^{21}$ detected an association between elevated anti-*C pneumoniae* antibodies and *C pneumoniae* genome in 51 specimens obtained from infrarenal aortic aneurysms. Davidson and coworkers$^{22}$ examined whether elevated *C pneumoniae* antibodies precede development of atherosclerotic changes and detection of *C pneumoniae* by immunocytochemistry and PCR. The authors reported a significant relationship ($P = 0.024$) between *C pneumoniae* detection and high levels of *C pneumoniae*-specific IgG antibodies (GM titers, 94.9 versus 54.9 for negative *C pneumoniae* results). However, they correlated high IgG levels with the combined detection of *C pneumoniae* by immunocytochemistry and PCR ($n = 12$) despite a substantial discordant *C pneumoniae* detection by immunocytochemistry and PCR ($n = 12$). No elevated IgM antibodies were observed in any patients. With the raw data, there was no statistical evidence that the IgG values differed in relation to a positive or negative *C pneumoniae* PCR or culture result ($P = 0.63$ and $P = 1.0$, respectively) for patients undergoing primary revascularization. In detail, in negative cultures, the GM was 56.0 (95% CI, 36.0 to 87.4); in positive cultures, the GM was 32.1 (95% CI, 2.2 to 464.0); in negative PCRs, the GM was 53.2 (95% CI, 32.5 to 87.1); and in positive PCRs, the GM was 53.2 (95% CI, 16.6 to 170.5).

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Cytomegalovirus

In this study, no CMV DNA was detected in the control or pathological specimens. This negative result is surprising because of the reported high prevalence of CMV DNA in atherosclerotic tissues. However, reports of CMV genome detection in atherosclerotic and nonatherosclerotic tissue are inconsistent and vary considerably. Small differences in CMV DNA detection rates between atherosclerotic and control specimens were reported by most contributors (57% versus 36%) (for review, see References 1 and 25 through 28). Two groups reported failure to detect CMV genome in atherosclerotic lesions. Benditt et al. did not detect CMV viral mRNA by means of in situ hybridization in atherosclerotic arterial wall tissue. It has been suggested that active CMV replication is responsible for plaque instability. Thus, Kol et al. investigated whether CMV major immediate early gene mRNA could be identified by PCR in tissues obtained from 20 patients with unstable angina undergoing coronary atherectomy. Because the CMV major immediate early gene is indicative of replicative CMV and the authors did not observe a single positive PCR result, they concluded that CMV replication is not a cause of unstable angina. More recently, the association between prior CMV infection and the risk of restenosis after balloon PTCA has been investigated. The authors did not observe an association between prior CMV infection and restenosis.

Conclusions

CMV does not appear to be associated with advanced coronary artery or vein graft disease. Elevated C pneumoniae antibody titers do not seem to be a relevant indicator for macrolide therapy in patients with coronary artery disease, considering the observed endovascular presence of C pneumoniae. The significant association between elevated IgG anti–C pneumoniae titers and the detection of this pathogen in occluded vein grafts warrants further investigation.

Acknowledgments

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References


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