Detection of *Chlamydia pneumoniae* But Not Cytomegalovirus in Occluded Saphenous Vein Coronary Artery Bypass Grafts

Claus Bartels, MD; Matthias Maass, MD; Gregor Bein, MD; Rainer Malisius, MD; Nicole Brill; J.F. Matthias Bechtel, MD; Friedhelm Sayk; Alfred C. Feller, MD; Hans-Hinrich Sievers, MD

**Background**—A causal relation between atherosclerosis and chronic infection with *Chlamydia pneumoniae* and/or cytomegalovirus (CMV) has been suggested. Whether the unresolved problem of venous coronary artery bypass graft occlusion is related to infection with *C pneumoniae* and/or CMV has not been addressed.

**Methods and Results**—Thirty-eight occluded coronary artery vein grafts and 20 native saphenous veins were examined. Detection of *C pneumoniae* DNA was performed by use of nested polymerase chain reaction (PCR). Homogenisates from the specimen were cultured for identification of viable *C pneumoniae*. Both conventional PCR and quantitative PCR for detection of CMV DNA were applied. Differential pathological changes (degree of inflammation, smooth muscle cell proliferation [MIB-1]) were determined and correlated to the detection of both microorganisms. *C pneumoniae* DNA could be detected in 25% of occluded vein grafts. Viable *C pneumoniae* was recovered from 16% of occluded vein grafts. Except for 1 native saphenous vein, all control vessels were negative for both *C pneumoniae* detection and culture. All pathological and control specimens were negative for CMV DNA detection. Pathological changes did not correlate with *C pneumoniae* detection.

**Conclusions**—Occluded aorto-coronary venous grafts harbor *C pneumoniae* but not CMV. The detection of *C pneumoniae* in occluded vein grafts warrants further investigation. (*Circulation*. 1999;99:879-882.)

**Key Words:** viruses □ bypass □ atherosclerosis □ coronary disease

A utologous saphenous veins remain the most widely used graft for myocardic revascularization. Control of risk factors and various antiplatelet regimen improved graft patency. (for review see Ip et al, 1990). However, accelerated graft sclerosis and graft failure remain an unresolved problem (for review see Motwani and Topol). Angiographic studies demonstrated that 41% of venous coronary artery bypass grafts (CABG) were occluded 10 years after implantation. Seventy-five percent of remnant patent venous grafts showed atherosclerotic changes; thus only 17% of implanted CABG revealed no angiographically detectable atherosclerotic changes 10 years after implantation. Oclusion of CABG results in deterioration of clinical status and a subsequent high reoperation rate of 15% to 20% that is associated with increased mortality and morbidity rates. A causal relation between chronic infection with *Chlamydia pneumoniae* and/or cytomegalovirus (CMV) and coronary atherosclerosis has been suggested. *C pneumoniae* as well as CMV induce production of several cytokines. These cytokines may stimulate fibroblast and smooth muscle cell proliferation. (for review see Mehta et al 1998). Whether infection with *C pneumoniae* and/or CMV plays a role in the pathogenesis of coronary artery venous graft occlusion has not been adequately investigated. Histomorphological changes and smooth muscle cell proliferation in occluded vein grafts have never been correlated with the endovascular presence of both microorganisms. Therefore, the presence of *C pneumoniae* and/or CMV in occluded vein grafts and its correlation with specific pathological changes were examined.

**Methods**

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

**Pathological and Control Specimens**

A total of 38 occluded vein grafts from 32 consecutive patients undergoing reoperations were collected (6 patients had 2 grafts removed). Each patient contributed only once to the analysis of demographic variables; in no case of repeat observations there was any inconsistency regarding the detection of *C pneumoniae*/CMV in the 2 different vein grafts obtained from the same patient.

Only occluded vein grafts showing typical thickening of the venous wall, indicative for late graft occlusion, were selected for further investigation. Vein grafts were dissected free from the
adjacent tissue as far as technically possible. A representative specimen of the occluded vein graft was selected for further analysis. Mean interval from primary surgery to reoperation was 105±50 months. Patient characteristics for C pneumoniae positive and negative results are summarized in the Table. Twenty native saphenous veins served as controls: 16 veins collected during primary surgery, and 4 veins obtained during reoperation.

**C pneumoniae Detection**

Three different segments of the occluded vein grafts were selected for analysis. Tissue was cut into 0.3-cm segments, ground, and suspended in cell culture medium. 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. Briefly, DNA was purified from the plaque suspensions by protease K digestion and cetyltrimethylammonium bromide treatment. Nested PCR was then performed with the species-specific HL-1/HR-1 primer pair (438 bp). The nested primer pair IN-1/2 that yields a 128 bp product. For confirmation, nonradioactive DNA hybridization was performed with oligonucleotide HM-1 3’-labeled with digoxigenin-dUTP (Boehringer Mannheim) as the probe.

**C pneumoniae Culture**

Serum-free cell culture was performed as previously described. Essentially, suspensions were centrifuged onto HEP-2 host cell monolayers and incubated for 3 days at 35°C, 5% CO2 in isolation medium (Eagle’s Minimal Essential Medium, GIBCO/BRL) supplemented with 1 μg/mL cycloheximide (Sigma). Productive C pneumoniae growth was identified by staining inclusions with FITC-conjugated C pneumoniae–specific mouse monoclonal antibody (Cellabs).

**CMV Detection**

DNA isolation and purification were performed (Qui amp Tissue kit, Quiagen). 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 dATP, dCTP, dGTP, dTTP, and 50 pmol of each primer. PCR was performed by 40 cycles after activation of AmpliTaq Gold: each cycle consisted of denaturation at 95°C for 10 seconds, annealing 30 seconds, 62°C, and extension 30 seconds, 72°C (352 bp).

The reaction mixture was then subjected to agarose gel electrophoresis, Southern blotting, and nonradioactive hybridization with digoxigenin-labeled oligonucleotide probe as previously described.

**Quantitative CMV PCR**

To obtain PCR conditions with reduced variability and contamination, quantitative PCR was performed with real-time Taqman (ABI PRISM 7700 Sequence Detector Systems, PE Applied Biosystems). Premixes containing all reagents except for targets were prepared, aliquoted into PCR tubes, and analyzed. No template controls and positive CMV controls (0.5 ng CMV, total virus genome, Keatech) paralleled each sample analysis. 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. PCR conditions were optimized for oligonucleotide and MgCl2 concentrations. The following composition of PCR assay (total volume 50 μL) was used: 100 ng purified sample DNA, 15 pmol of each primer, 200 μmol/L of each of dATP, dCTP, dGTP, and dTTP. The reaction conditions for 50 cycles after activation of AmpliTaq Gold were 95°C, 15 seconds (denaturation) and 55°C, 1.5 minutes (annealing) (101 bp). Serial dilution of CMV DNA for identification of CMV DNA detection threshold depicted positive CMV DNA down to 12 copies.

**Histomorphological Studies**

The occluded vein grafts were investigated in consecutive series by 2 independent investigators. The pathologists were unaware of clinical data or detection of microorganisms. Thrombotic changes, inflammation with respect to the extent of inflammation of vessel wall circumference, differential analysis of contributing cells (macrophages, lymphocytes, granulocytes, plasma cells, foam cells, fibroblasts), and fibrotic changes within the neointima were documented by histological examination (paraffin section, hematoxylin–eosin staining).

Smooth muscle cell proliferation was examined by immunohistochemical staining, with the monoclonal antibody MIB-1 (Ki 67 antigen, Dianova). The antigen was unmasked by incubating sections in 10 mmol/L citrate buffer, pH 6.0, and boiling in a microwave oven for 20 minutes at a power level of 159 W (Miele supratonic 752). As an immunohistochemical detection system, the ABC technique in accordance to Hsu et al was used. One vessel wall circumference or 400 smooth muscle cells were examined for positive staining.

**Statistical Analysis**

Statistical analysis was performed with Minitab (Release 10). Continuous variables were analyzed with the 2-sample t test (in the case of normal distribution) or Mann-Whitney U rank-sum test. Binary data were analyzed with Fisher’s exact test. All tests were 2 tailed. A value of P<0.05 was considered to indicate statistical significance.

**Results**

**C pneumoniae Detection**

Prevalence of positive C pneumoniae DNA detection in occluded vein grafts was 25% (n=8/32); viable C pneumoniae could be cultured from 16% (n=5/32). All specimens containing viable C pneumoniae depicted positive PCR results. Four different occluded vein grafts collected from 2 patients (2 grafts from each patient) contained viable C pneumoniae. One native saphenous vein was positive for PCR but not for culture. This control vein was obtained during a patient’s reoperation, in which occluded CABG turned out to be positive for C pneumoniae. All other examined control veins were negative for both PCR and culture.

Demographic variables did not correlate with the positive detection of C pneumoniae by PCR or culture.
CMV Detection
All examined pathological and control specimens were negative for CMV DNA detection by conventional and quantitative PCR.

Histomorphological Studies
No differences regarding histomorphological changes were detected comparing C pneumoniae positive or negative occluded vein grafts.

Proliferation rate of neointimal smooth muscle cells showed no association to the detection of C pneumoniae genome or culture.

Discussion
This study documents for the first time that occluded venous coronary-artery bypass grafts but not native control veins harbor viable C pneumoniae and C pneumoniae DNA. No correlation between demographic characteristics and the positive detection of C pneumoniae could be observed. CMV DNA could not be detected in occluded vein grafts or control veins. Histomorphological changes did not allow discrimination between C pneumoniae infected and noninfected vein grafts. Smooth muscle cell proliferation rate could not be related to the detection of C pneumoniae.

Cytomegalovirus
Restenosis after coronary angioplasty has been suggested to be causally related to reactivated CMV infection. Intimal thickening of CABG presents a unique form of accelerated atherosclerosis that shares some similarities to restenosis following coronary angioplasty. In this study neither in occluded CABG nor in native saphenous veins could CMV DNA be detected. This negative result was somewhat surprising because of the reported high prevalence of positive CMV DNA detection in atherosclerotic tissues. Two groups reported negative CMV genome detection in atherosclerotic lesions: Benditt et al could not detect CMV viral mRNA in atherosclerotic arterial wall tissue. Kol et al searched for the CMV major immediate early gene mRNA (PCR) in tissue obtained from 20 patients with unstable angina undergoing coronary atherectomy. As the CMV major immediate early gene is indicative for 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. Therefore, in the present study we did not investigate the detection of CMV gene products indicating viral replication but the presence of CMV DNA itself.

Our results obtained by the use of highly sensitive and specific methods indicate that endovascular CMV presence does not seem to be associated with advanced vein graft disease.

Animal models demonstrated that CMV may induce atherosclerosis. Accelerated atherosclerosis, like vein graft disease, differs from spontaneous native atherosclerosis regarding vascular injury mechanism, pathological appearance, and duration of pathological process. Therefore, our negative CMV DNA detection in occluded vein grafts does not exclude an association between initiation of native coronary artery disease and CMV infection.

C pneumoniae
Preliminary results from our group reported detection of C pneumoniae in occluded vein grafts. This observation prompted us to systematically study the prevalence of C pneumoniae in occluded vein grafts and native saphenous veins. C pneumoniae detection was frequent in occluded CABG: PCR 25% and positive culture 16%. In 2 patients, C pneumoniae could be detected in 2 separate vein grafts, indicating that endovascular presence of C pneumoniae is not limited to 1 graft only. Except for 1 native saphenous vein collected during reoperation from a patient with positive C pneumoniae PCR in the occluded CABG, all control vessels were negative for culture and PCR. Thus it may be assumed that native saphenous veins do not harbor C pneumoniae at implantation but that colonization takes place in a relatively short time frame. This finding may be of significance for the unresolved problem of rapid vein graft occlusion.

Vein grafts are subjected to mechanical injury during harvesting that initiates a healing process involving macrophages. Occlusion of vein graft is thrombotic in origin leading to leukocyte migration in the organized thrombus. C pneumoniae replicate within alveolar cells and spread systematically using macrophages and monocytes as vectors. Thus macrophage migration into the healing vein wall or organized thrombus may account for the detection of C pneumoniae in occluded vein grafts. Whether chronic macrophage infection contributes to local inflammation and plaque initiation or progression remains unclear. It has been suggested that vein graft occlusion may be immune-mediated. C pneumoniae infection appears to induce chronic immune activation mediated by cytokines. Whether cytokine mediated, direct endothelial cell damage or systemic inflammatory response stimulating acute phase proteins (eg, fibrinogen, C-reactive protein) is responsible for the observed association between chronic infection and atherosclerosis remains to be determined.

Histomorphology
An interactive role of chronic infection with C pneumoniae and/or CMV, inflammation, and coronary artery disease has been suggested. However, it remains unclear whether the local presence of the mentioned microorganisms or systemic inflammatory changes are responsible for the observed association between chronic infection and atherosclerosis. Ocular infection with Chlamydia trachomatis causes conjunctival infiltration by macrophages and lymphocytes. Therefore, specific histological changes, including inflammatory cell subtypes correlated to the endovascular of C pneumoniae may enlighten the underlying mechanism of vessel wall damage. Both C pneumoniae and CMV may induce the induction of cytokines. Cytokines stimulate fibroblast and smooth muscle cell proliferation. More recently, Kol et al demonstrated that C pneumoniae heat shock protein 60 is localized in human atheroma and regulates tumor necrosis factor and metalloproteinase expression. In the present study, neither specific inflammatory changes nor extent of smooth muscle cell proliferation could be correlated to the endovas-
cular presence of *C. pneumoniae*. However, these results are based on a relatively small number of examined specimen.

**Limitations of the Study**

Local quantification of different cytokines in correlation to the endovascular presence of *C. pneumoniae*/CMV and possible consecutive increased smooth cell proliferation may help to clarify the underlying pathological mechanism. In the present study, cytokines were not examined and thus cannot be correlated to the observed level of smooth muscle cell proliferation. Further studies quantifying the level of cytokines (eg, TNF-α, -interleukin-1, -4, and -6) and comparing these levels with the proliferation rate of smooth muscle cells are warranted to further elucidate the underlying mechanism of vessel damage by *C. pneumoniae*.

Detection of both microorganisms and histomorphological analysis were performed in different parts of the occluded vein grafts. Therefore, no direct correlation of morphologic changes to the presence of *C. pneumoniae* can be performed. However, as *C. pneumoniae* nested PCR was reproducible positive in the analyzed three specimen from 1 occluded vein graft, it can be speculated that *C. pneumoniae* genome was present in the specimen selected for pathological examination.

Therefore, the following conclusions can be drawn from the our results: Occluded venous coronary-artery bypass grafts but not native control veins harbor viable *C. pneumoniae* and *C. pneumoniae* DNA. No correlation between demographic characteristics and the positive detection of *C. pneumoniae* could be observed. CMV does not seem to persist in occluded vein grafts; whether CMV initiates vein graft disease remains to be determined.

The observed detection of *C. pneumoniae* DNA and viable *C. pneumoniae* in occluded vein grafts warrants further investigation.

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**References**


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