Circulating Nucleic Acids of Chlamydia pneumoniae and Cytomegalovirus in Patients Undergoing Coronary Angiography

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Peripheral blood mononuclear cells from 208 consecutive patients undergoing elective coronary angiography or angioplasty were collected before, immediately after, and 4 h after the procedure. Nucleic acids of Chlamydia pneumoniae and of cytomegalovirus (CMV) were detected by PCR and confirmed by hybridization. Circulating C. pneumoniae DNA was identified in 24 patients (11.5%) and was associated with current smoking (odds ratio [OR] = 4.5, 95% confidence interval [CI] = 1.6 to 12.2, P = 0.004) but not with arterial narrowing on coronary angiogram or with serological results positive for C. pneumoniae. Circulating CMV DNA was identified in 36 patients (17.3%) and was associated with anti-CMV immunoglobulin G (OR = 2.7, 95% CI = 1.2 to 6.3, P = 0.02) but not with angiographic arterial narrowing or with the need for revascularization. Neither C. pneumoniae nor CMV DNA detection increased after angioplasty, a procedure in which endothelium is disrupted. Larger prospective studies are needed to determine the prognostic significance of DNA detection.

Previous exposure to Chlamydia pneumoniae and cytomegalovirus (CMV) has been associated with heart disease (6). C. pneumoniae antigen and DNA have been detected in coronary and carotid atheroma and in aortic aneurysms, and culture of C. pneumoniae from atheroma has been reported previously (10, 19). However, recent large prospective studies have not confirmed an association between anti-C. pneumoniae immunoglobulin G (IgG) serology results and vascular events (16, 20, 21), and there was poor correlation between serology results and the presence of C. pneumoniae antigen or DNA in tissue (5).

The detection of C. pneumoniae DNA circulating in peripheral blood mononuclear cells (PBMC) has been reported, although estimates of prevalence varied widely. In one study, 59% of 101 heart disease patients and 46% of 52 blood donor controls were positive for C. pneumoniae DNA (3). Among 804 men undergoing coronary angiography, the prevalence of C. pneumoniae DNA was 8.8% in those with heart disease versus 2.9% in those without heart disease (23). In 41 aortic aneurysm patients, detection of C. pneumoniae DNA in PBMC correlated with the isolation of C. pneumoniae DNA from aortic aneurysms (1). Potentially, detection of C. pneumoniae DNA in PBMC could enable large-scale epidemiological studies to clarify the role of C. pneumoniae in atherosclerotic heart disease and its complications.

CMV is associated with accelerated atherosclerosis of cardiac transplants and may be associated with coronary artery restenosis or thrombosis after angioplasty or atherectomy (7, 15). In a rat model, rat CMV increases neointimal cell proliferation after balloon injury to the carotid artery (18). A role in human disease remains unproven, and prospective studies of CMV serology have not confirmed a relationship with vascular events (20).

This study had three primary objectives: first, to determine the prevalence of circulating C. pneumoniae DNA and CMV DNA in patients undergoing coronary angiography; second, to determine if C. pneumoniae DNA detection increased after coronary angioplasty, on the assumption that disrupted endothelium would release C. pneumoniae (but not CMV) DNA into the bloodstream; and third, to determine whether DNA isolation was prognostically important.

MATERIALS AND METHODS

Patients. Consecutive elective outpatients were recruited from the Hamilton Regional Angiography Suite, Hamilton Health Sciences Corporation, Hamilton, Ontario, Canada, between February and October 1999. Information regarding age, gender, and a history of previous cardiac disease, smoking, diabetes mellitus, hyperlipidemia, and hypertension was obtained. Sample size calculations required 100 patients in the angioplasty stratum for an 80% probability of detecting an increase in DNA prevalence from 10 to 20%. Angiography and angioplasty patients were enrolled until predetermined strata of 100 patients each were filled, with recruitment of angiography patients being complete by April 1999 and recruitment of angioplasty patients continuing until October 1999. The angiogram report was scored by the presence of any arterial narrowing (>25%) and by the number of epicardial coronary arteries with at least 50% narrowing in two orthogonal views or at least 70% narrowing in one view by visual assessment. Six-month clinical outcomes (cardiac hospitalization, repeat angiogram or angioplasty, myocardial infarction, coronary artery bypass surgery, or death) were obtained by telephone calls to the patient and by hospital chart review. All clinical data were collected by study nurses blinded to laboratory data. All participating patients gave written consent, and the study protocol was approved by research ethics boards at St. Joseph’s Hospital (Hamilton, Ontario, Canada), Hamilton Health Sciences Corporation, and McMaster University (Hamilton, Ontario, Canada).

Blood collection. Serum was collected prior to angiography or angioplasty. Circulating PBMC were obtained by venipuncture into an 8-ml Vacutainer CPT cell preparation tube (BD Vacutainer Systems, Franklin Lakes, N.J.) prior to, immediately after, and 4 h after the procedure for a total of three tubes. Specimens obtained prior to the procedure were obtained in a precatheterization outpatient clinic days to weeks before the procedure. CPT tubes contain a blood separation medium composed of a thixotropic polyether gel and a density gradient liquid solution. Laboratory personnel processed CPT tubes essentially according to the manufacturer’s instructions, except for a second centrifugation.

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Briefly, CPT tubes were centrifuged in a Beckman GPR centrifuge at 1,500 g for 30 min and refrigerated. After transport to the research laboratory (generally within 24 h), the specimens were mixed by inversion and recentrifuged, and the mononuclear cell layer (if visible) or 1 ml of plasma directly above the gel was aspirated and frozen at −70°C. In batches, mononuclear cell preparations were thawed and 200-μl aliquots were extracted using QIAamp DNA minikits (Qiagen, Mississauga, Ontario, Canada) into 100 μl of elution buffer.

**Detection of DNA.** Laboratory staff were blinded to all clinical data. A 2.5-μl aliquot was amplified by a nested PCR (22), consisting of 40 amplification cycles for a 233-bp product (external primers CP1 and CP2) and 30 cycles for a 207-bp product (internal primers CPC and CPD), followed by separation on a 2.0% (wt/vol) agarose gel containing ethidium bromide and UV light visualization. The 207-bp product was confirmed as *C. pneumoniae* by hybridization with an in-house specific fluorescein-labeled oligonucleotide probe (5′ TAC GGA GAC TTT TGA CCT 3′, GenBank accession no. AF131889, positions 196 to 215).

**Amplification for CMV DNA** was performed by a nonnested PCR procedure targeting an immediate-early gene (17), with primers P1 and P3, with confirmation by agarose gel electrophoresis, visualization of a 123-bp product, and hybridization with a specific in-house oligonucleotide probe (5′ TTT TGA CCT CCA TAG AAG AC 3′, GenBank accession no. HEICMVGC, positions 173700 to 173681). CMV strain AD 169 was used as a control. All positive results were independently confirmed by a second staff in separate laboratories, with blinding to other laboratory results.

**RESULTS**

**Description of patients.** One hundred eighteen patients undergoing angiography and 90 patients undergoing angioplasty were enrolled. The median age was 60.4 years (minimum to maximum, 21 to 85 years, respectively), and 75.4% of patients were male. Medical history included the following: diabetes mellitus, 21.6% of patients; hypertension, 56.8%; hypercholesterolemia, 66.1%; current smokers, 18.1%; former smokers, 59.9%; and lifelong nonsmokers, 22.1%. A history of myocardial infarction was present in 46.2%, one of unstable angina was present in 74.1%, one of previous angioplasty was present in 21.6%, and one of coronary artery bypass grafting was present in 10.1% of patients.

**C. pneumoniae DNA detection.** A total of 547 samples from 208 patients had DNA extracted and amplified for *C. pneumoniae*, including 207 samples from before, 182 samples taken immediately after, and 158 samples taken 4 h after the angiogram or angioplasty. Twenty-five of 547 PBMC samples (4.6%) from 24 of 208 patients (11.5%) were positive for *C. pneumoniae* DNA (Table 1). For all but one patient, a single sample from the three time periods was positive. When the positive samples were reextracted and PCR tested in triplicate, there were 20 replicate sets with one of three positive, two replicates with two of three positive, and three replicates with all three positive (for a total of 33 positive in 75 PCR tests). Of these original 25 *C. pneumoniae*-positive samples, 24 were tested a third time, in triplicate.
cate, and 18 of the 24 were positive. By comparison, in a random sample of 30 previously negative patient PBMC samples, 1 of 30 samples (1 of 90 PCR tests) was positive. Among 49 plasma samples (taken from the same CPT tube from which mononuclear cell fractions were obtained) tested in triplicate, including all 25 samples with concurrent C. pneumoniae DNA-positive PBMC, no C. pneumoniae DNA was identified (0 of 147 PCR tests). Ten of the 25 (40.0%) C. pneumoniae DNA positives were also positive by at least one of three PCRs targeting alternative chlamydial sequences. In addition, 12 amplification products from different patients were sequenced, and all matched C. pneumoniae exactly.

C. pneumoniae serology assays were performed for 203 of the 208 patients (Table 2). Anti-C. pneumoniae IgG serology results were positive for 16 of 23 patients (70.0%) with C. pneumoniae DNA-positive PBMC, versus 142 of 180 patients (78.9%) who were DNA negative (odds ratio [OR] = 0.6, 95% confidence interval [CI] = 0.2 to 1.8, P = 0.3). Anti-C. pneumoniae IgA serology results were positive for 13 of 23 (56.5%) patients with DNA detected, versus 115 of 180 (63.9%) patients who were DNA negative (OR = 0.7, 95% CI = 0.3 to 1.9, P = 0.49).

By multiple logistic regression modeling, the presence of C. pneumoniae DNA in PBMC was associated with current smoking (OR = 4.5, 95% CI = 1.6 to 12.2, P = 0.004), the months of February to April compared with May to October (OR = 4.6, 95% CI = 1.3 to 16.6, P = 0.02), and concurrent CMV DNA detection (OR = 3.9, 95% CI = 1.4 to 10.6, P = 0.008). Specifically, circulating C. pneumoniae DNA was detected in 9 of 37 (24.3%) current smokers, compared with 10 of 121 (8.3%) former smokers and 5 of 48 (10.4%) lifelong nonsmokers. There was no association with gender, cardiac history, or other cardiovascular risk factors. There were 21 patients with no coronary artery disease at angiography, and 13, 53, 76, and 45 patients with mild or one-, two-, or three-vessel disease, respectively (Table 3). C. pneumoniae DNA status was not associated with the presence (OR = 1.3, 95% CI = 0.3 to 11.9, P = 1.00) or degree (P = 0.56, test for trend) of coronary artery stenosis at angiography.

There was no increased detection of C. pneumoniae DNA in PBMC in samples obtained after coronary angiography or angioplasty (Table 1). C. pneumoniae DNA was detected in 5.1% of samples taken before angiography, 5.8% of samples taken immediately after angiography, and 8.3% of samples taken 4 h after angiography (P = 1.00 for each before-after comparison, McNemar test). For angioplasty patients, DNA was detected in 2.2% of samples taken before the procedure and up to 2.7% of samples taken afterward (P = 1.00 for each before-after comparison).

Among the 58 patients who had the composite end point of revascularization or a clinical cardiac event (coronary artery bypass surgery, repeat angiogram, cardiac hospitalization, myocardial infarction, or death) during 6 months of follow-up after the procedure, there was no association with PBMC C. pneumoniae DNA status (OR = 0.8, 95% CI = 0.3 to 2.5, P = 0.7) (Table 4).

**CMV DNA detection.** Forty of 559 samples (7.2%) from 36 of 208 patients (17.3%) were positive for CMV DNA (Table 1). For all but four patients, a single sample from the three time periods was positive. Seven amplification products from different patients were sequenced, and all matched CMV sequences exactly. In a test of 49 plasma samples (31 of which had CMV in the corresponding mononuclear cell fraction), 9 were positive for CMV DNA. Five of these nine were CMV DNA positive in both plasma and mononuclear cell fractions, and eight of these nine were anti-CMV IgG positive.

Anti-CMV IgG status was positive for 124 of 207 patients (59.6%) and for 28 of 36 patients (77.8%) with concurrent CMV DNA detection. Specifically, circulating C. pneumoniae DNA was detected in 9 of 37 (24.3%) current smokers, compared with 10 of 121 (8.3%) former smokers and 5 of 48 (10.4%) lifelong nonsmokers. There was no association with gender, cardiac history, or other cardiovascular risk factors. There were 21 patients with no coronary artery disease at angiography, and 13, 53, 76, and 45 patients with mild or one-, two-, or three-vessel disease, respectively (Table 3). C. pneumoniae DNA status was not associated with the presence (OR = 1.3, 95% CI = 0.3 to 11.9, P = 1.00) or degree (P = 0.56, test for trend) of coronary artery stenosis at angiography.

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TABLE 4. Association of C. pneumoniae and CMV DNA in PBMC with subsequent cardiac procedures and events

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Total no. of patients</th>
<th>No. (%) of patients with 6-month clinical endpoint&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. with type of DNA/total no. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Revascularization</td>
<td>Other events</td>
</tr>
<tr>
<td>Angiogram</td>
<td>118</td>
<td>48 (40.7)</td>
<td>6 (5.1)</td>
</tr>
<tr>
<td>Angioplasty</td>
<td>90</td>
<td>5 (5.6)</td>
<td>3 (3.3)</td>
</tr>
<tr>
<td>Total</td>
<td>208</td>
<td>53 (25.5)</td>
<td>9 (4.3)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Revascularization means coronary artery bypass graft surgery or coronary angioplasty; other events mean repeat angiogram or angioplasty, myocardial infarction, or death; total means number of patients reaching a 6-month end point of revascularization or a clinical event.

<sup>b</sup> C. pneumoniae DNA association with 6-month clinical end points was as follows: OR = 8.9, 95% CI = 3.0 to 2.5, and P = 0.07 by logistic regression (SPSS).

<sup>c</sup> CMV DNA association with 6-month clinical endpoints was as follows: OR = 1.4, 95% CI = 3.0 to 3.0, and P = 0.04 by logistic regression.

not associated with the presence (OR = 4.6, 95% CI = 0.6 to 35.5, P = 0.14) or the degree (P = 0.83, test for trend) of coronary artery stenosis at angiography (Table 3).

There was no increased detection of CMV DNA in PBMC following angiography or angioplasty (Table 1). CMV DNA was detected in 9.5% of samples taken before, 8.7% of samples taken immediately after, and 4.2% of samples taken 4 h after angiography. By contrast, CMV DNA was detected in 6.7% of samples taken before, 8.9% of samples taken immediately after, and 4.1% of samples taken 4 h after angioplasty (P = 1.0 for before-after comparisons; P = 0.88 for angioplasty versus angiography).

The presence of CMV DNA in PBMC did not predict the 58 patients who subsequently required revascularization or had a cardiac event (OR = 1.4, 95% CI = 0.6 to 3.0, P = 0.02) (Table 4). Similarly, anti-CMV IgG serological status did not predict these clinical events (OR = 0.9, 95% CI = 0.5 to 1.7, P = 0.81).

DISCUSSION

This study addressed several issues of direct relevance for the development and validation of molecular tests for cardiovascular studies. First, we examined the prevalence of C. pneumoniae DNA in PBMC from patients undergoing angiography, most of whom had documented atherosclerotic heart disease. The estimate of 11.5% for C. pneumoniae DNA prevalence was similar to the results of one recent study (23) and may be more representative than the higher prevalence of 59% reported in another study (3). The discrepancy may represent interlaboratory variation or a different population being sampled during a typical centrifugation and may facilitate the undertaking of similar studies by other laboratories. The finding that mononuclear cell-associated and found within the mononuclear cell fraction.

The presence of CMV DNA in PBMC did not predict the 58 patients who subsequently required revascularization or had a cardiac event (OR = 1.4, 95% CI = 0.6 to 3.0, P = 0.02) (Table 4). Similarly, anti-CMV IgG serological status did not predict these clinical events (OR = 0.9, 95% CI = 0.5 to 1.7, P = 0.81).

Second, we examined the reproducibility of PCR results. Despite use of a very sensitive PCR, samples were usually positive in only one of three sampled time periods and on repeat testing were positive in only one of three replicates. Potential explanations for these results include nonspecific amplification, contamination of the PCR, between-sample variation (biological sampling), and within-sample variation (statistical sampling). The amplification products were C. pneumoniae, as evidenced by hybridization with a specific oligonucleotide probe and by molecular sequencing of 12 amplification products. We believe that contamination is not a likely explanation, given that all positives had been extracted on two separate occasions, 75% were positive when tested a third time, and 40% of the samples were confirmed by one or more PCRs targeting a different part of the genome. Furthermore, among the plasma samples and initially negative mononuclear cell samples, we detected only a single positive sample in 237 PCR runs. Finally, of over 200 C. pneumoniae-negative controls run using this PCR by our laboratory in the last year, including 40 negative controls which were open during the entire specimen addition step, there was not a single false positive. Biological variation is plausible, with intermittent shedding of infected mononuclear cells and clearance by the reticuloendothelial system. The most likely explanation for intermittent positivity is statistical sampling. We have recently demonstrated that the proportion of replicates which are positive is directly related to the concentration of C. pneumoniae organisms in a sample and that a positive proportion of one in three replicates can be predicted using probit analysis for samples with low DNA concentrations (M. Smiejka et al., unpublished data).

Third, this and a smaller study (1) have demonstrated that the Vacutainer CPT tube method is a simple and feasible method for obtaining mononuclear cell-associated DNA. The method eliminates the need for standard Ficoll-Hypaque gradient centrifugation and may facilitate the undertaking of similar studies by other laboratories. The finding that mononuclear cell fractions, but not the corresponding plasma fractions, were positive for C. pneumoniae DNA constitutes clear evidence for humans that circulating C. pneumoniae DNA is cell associated and found within the mononuclear cell fraction. This corroborates the recent finding of C. pneumoniae antigen in circulating mononuclear cells (2).

Fourth, we demonstrated the routine detection of CMV DNA in peripheral blood of nontransplant vascular patients. To our knowledge, this has not been previously reported. CMV was found in both the mononuclear cell fraction and plasma, although a greater number of positives was detected in the mononuclear cell fraction.

Fifth, we examined whether C. pneumoniae DNA or CMV...
DNA detection was augmented by coronary angioplasty. We reasoned that \textit{C. pneumoniae} in macrophages within the ath erosclerotic plaque would seed the bloodstream during and after the angioplasty, because of disruption of the endothelium and plaque. No such increase in \textit{C. pneumoniae} DNA or CMV DNA was observed, either immediately after or 4 h after angioplasty. Potential explanations include too low a concentration of organism in coronary lesions, incorrect sampling time, incorrect sampling fraction (mononuclear cells), or lack of organisms in the atheroma of most patients. Further improvements in the methods of detection, and other methods of sampling such as those with intra-coronary-artery catheters or before and after coronary thrombolysis, may help to resolve this issue.

Last, we examined whether detection of \textit{C. pneumoniae} or CMV DNA in PBMC had any prognostic significance. Neither CMV DNA nor \textit{C. pneumoniae} DNA was associated prospectively with subsequent revascularization or other clinical events. To examine whether the detection of \textit{C. pneumoniae} DNA and CMV DNA has prognostic importance, large, well-designed, prospective observational and treatment studies are needed. The measurement of DNA in PBMC using the methods described here may facilitate such studies and may be a more specific measure of recent exposure than serology.

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REFERENCES