Frequent Occult Infection with Cytomegalovirus in Cardiac Transplant Recipients despite Antiviral Prophylaxis

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Cytomegalovirus (CMV) remains an important cause of morbidity and mortality in solid-organ transplantation. Ganciclovir- and valganciclovir-based antiviral prophylaxis strategies suppress viral replication, and, as a consequence, acute CMV disease is largely suppressed (4, 7, 17). Complementary strategies that monitor for active viral replication above a threshold level and then employ preemptive antiviral therapy are also effective in reducing acute CMV disease (13). The long-term consequence of subclinical CMV infection in either setting has remained a concern (13) because of the association of this virus with allograft rejection and cardiac allograft vasculopathy (CAV; also known as transplant arteriopathy or transplant vascular disease) (14, 17, 22, 23). Recent concern about CMV as well as other chronic virus infections has been focused on chronic or indirect viral damage to the graft, leading to acute rejection and CAV (17, 23). Consistent with this, the frequency of CAV appeared to be greater in the pre-ganciclovir era, suggesting a benefit of antiviral prophylaxis in reducing the incidence of CAV (24). We have reported that heart transplant recipients receiving more aggressive anti-CMV prophylaxis exhibit reduced acute rejection and CAV (14), and our work has suggested an important role for the preexisting CMV-specific CD4 T-cell response in suppressing viral infection that predisposes to acute rejection and CAV (22).

Pathogenesis of chronic vascular disease generally assumes persistent viral infection within the transplanted organ that causes damage and promotes disease (8, 23). Current prophylaxis regimens suppress acute disease and CMV replication levels, although subclinical infection has consistently been detected despite prophylaxis (4, 7, 11, 16, 17). The contribution of subclinical viral infection to chronic vascular disease would become better understood through more thorough characterization of the incidence, sites, and level of active, subclinical CMV infection in transplant patients receiving anti-CMV prophylaxis. CMV reactivation following heart transplantation may originate from the donor (R+/D+), from the recipient (R+/D+), or from either source (R+/D+). The reservoir of latent CMV includes myelomonocytic progenitors, peripheral blood mononuclear cells (PBMCs), and tissue macrophages or dendritic cells that arise from progenitors (12, 19). While mononuclear cells acquire permissiveness for virus as they differentiate, polymorphonuclear leukocytes (PMNs) are nonpermissive but acquire CMV through phagocytosis. Active infection may be monitored by detecting virus, viral antigens, or viral DNA levels in plasma, peripheral blood (PB) leukocytes, PBMCs, or PMNs (5, 6, 15). There has been little direct investigation of the relative timing of viral infection detected in the circulating cell types or present within endomyocardial biopsy (EMB) samples collected from cardiac allografts, particularly in patients receiving antiviral prophylaxis to suppress CMV replication.

Risk of CMV disease depends on the CMV serostatus of the donor and recipient, and this has influenced the choice of anti-CMV prophylaxis regimen applied in different patient/donor combinations. A CMV-seropositive recipient (R+) of a heart from either a seropositive (D+) or seronegative (D−) donor has a lower risk of CMV disease than a seronegative
PMN and 610 PBMC samples from 66 at-risk patients as well as 349 EMB samples from 55 at-risk patients were analyzed for CMV DNA by a two-stage analysis, starting with qualitative nested PCR (qualPCR). PMN, PBMC, or EMB samples positive by qualPCR for CMV DNA were subjected to a second-stage quantitative real-time (TaqMan) PCR. Our approach is based on many years of experience studying CMV latency (12, 20), where aliquots of 10^6 bone marrow-derived mononuclear cells from healthy CMV-seropositive donors are consistently positive, whereas peripheral blood mononuclear cells from such individuals are consistently negative (data not shown). Screening of EMB specimens for adenovirus DNA was performed using real-time PCR.

**Qualitative PCR.** We developed methods based on earlier work (see the reference list in reference 12) to amplify viral DNA from clinical samples with a low background and a sensitivity of 3 to 5 CMV genomes/10^5 cell equivalents. Aliquots of 5 x 10^6 freshly separated PB cells were lysed overnight at 60°C in 250 µl buffer containing 50 mM KCl, 10 mM Tris-HEPES (pH 8.0), 2 mM MgCl2, 0.45% NP-40, 0.45% Tween 20 (Siga-Aldrich Inc., St. Louis, MO), and 100 µg proteinase K (InnovoVir Corp., Carlshad, CA). Following lysis, samples were boiled for 5 min, cooled on ice, and stored at −80°C until use. DNA was isolated from EMB samples using the QIAEXP DNeasy Tissue kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's instructions. The initial amplification of DNA was with primers IE2AII (5' ATCGAGCTCTGCTGCAAAGGACAAAGATTCGAG3' and IE2BIBI (5' CAATACATACATCTCTTGCGAAGGG'). Following by IE3B (5' TCCTGCGAGCAGAGCTTTC3' and IE3P3A (5' GTGACCAAGGCAGCACGTT3'). For the initial qualPCR, 5 µl (1 µg cell equivalents) of extracted PBMC and PMN DNA or 50 ng EMB DNA was added to 45- µl reaction mixtures containing 10 mM Tris-HEPES (pH 8.3), 50 mM KCl, 1.5 mM MgCl2 (Roche Diagnostics Corporation, Indianapolis, IN), 200 µM of each of the dNTPs, and 0.45% NP-40, 0.45% Tween 20 (Roche Diagnostics Corporation), and 1 µl of each primer. For the second qualPCR, 5 µl of the first reaction mixture was added to a 45- µl reaction mixture as used for the initial qualPCR. qualPCR mixtures were layered with 50 µl of mineral oil and subjected to one cycle of 94°C for 3 min, followed by 30 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 62°C, and 2 min of extension at 72°C, and a final extension step of 7 min at 72°C. Positive and negative controls were included in each run, and, after amplification, each positive sample was electrophoresed through a 2% agarose gel containing 0.2 µg/ml ethidium bromide with appropriate DNA size markers. Amplicons were visualized on a transilluminator and photographed.

**Quantitative PCR.** We developed methods to quantify viral load in the qualPCR-positive samples using SYBR green detection of real-time PCR products able to detect a range of 3 to 100,000 CMV genome copies/10^5 cells. Real-time PCR was performed in a GeneAmp 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). Five microliters of sample was added to 45 µl of reaction mix containing 25 µl SYBR green PCR Master Mix (Applied Biosystems) and 10 pmol of each primer. The primers used for the real-time PCR were also located in the gene encoding the IE1 protein, exon 2 (IE1-2F, 5' CTGCTGGAATCTCCTGATGTA3'; and IE1-2R, 5' GCCGCGAAATGATCCCCCTAAA3'). A specific CMV detection primer mix (51 ng of each primer) and reaction volume containing 45 µl of each hexon-specific primer (5' GCCGAC GTGGGGTTTCTTACACAT3' and 5' GCCGACGTGGTTCTTACAGCACACT3') and 5 µM probe (6-carboxyfluorescein-5' AACCCACGAGCCCGTCAAGTAGGTTTCACCG3') were used. PCR detection was by SYBR green detection of real-time PCR products using a 58°C melt curve analysis to ensure that all products had an expected, uniform melting temperature. In addition, the specificity of the amplified products was assessed on each run by dissociation curve analysis to ensure that all products had an expected, uniform melting temperature. In addition, a detection of CMV-positive and CMV-negative samples was subjected to quantitative PCR analysis for CMV, Epstein-Barr virus, and human herpesvirus type 6 in the laboratory of Lawrence Cory and Meei-Li Hi at the University of Washington, Seattle, confirming the CMV DNA levels in positive samples but failing to detect any correlation between CMV DNA detection and the lack of detection of Epstein-Barr virus or human herpesvirus type 6 (data not shown).

**Data analysis.** Continuous variables are expressed as means ± standard deviations of the means or as medians with 25th to 75th percentiles given in parentheses, as appropriate, if not otherwise specified. Student's t-test was used for...
to compare continuous variables that showed a normal distribution. Chi-square or Fisher's exact analyses were applied to test differences in categorical variables. In order to compare patient's survival free from CMV detection in PMN versus PBMC serologically dictated subgroups, we applied a nonparametric test for paired data (Wilcoxon signed-rank test). The estimated incidence of CMV detection in PBMCs, PMNs, and EMB specimens was displayed using Kaplan-Meier plots. Differences in incidence of the first CMV detection between serological subgroups of patients were tested with the log-rank test. Differences in the time from transplant to peak CMV titer were analyzed by using a Mann-Whitney test.

In order to distinguish differences in CMV load among positive samples, we assigned each patient sample to a quartile and considered samples above the 75th percentile of this distribution to represent high-grade infection. This study was performed with approval of the Human Subjects Institutional Review Board at Stanford University.

RESULTS

CMV DNA detection in the study population. Although collected and assayed at the same time as for patients at risk of CMV infection, CMV DNA was not detected in any samples collected from the R⁺/D⁻ subgroup of nine patients, reinforcing the importance of serology in predicting risk of CMV infection. Conversely, most of the 66 at-risk patients receiving antiviral prophylaxis had an active CMV infection based on detection of viral DNA in PB leukocytes over the first 16 months after transplant (Fig. 1). In the PMN fraction, which becomes positive for CMV DNA only during active infection, 58 patients presented at least one CMV-positive sample, an estimated cumulative incidence of 91% ± 4% of patients with active CMV infection. CMV was detected in the PBMC fraction in a pattern similar to that of PMNs, although viral DNA in PBMCs may represent latency or productive infection (8, 12). Sixty patients became positive, accounting for an estimated cumulative incidence of 92% ± 4%. Although detection in PMNs was considered an unambiguous indicator of active CMV replication (5), both fractions became positive for viral DNA in most at-risk patients (54 patients; 82%). CMV DNA was detected in about 200 samples regardless of leukocyte fraction, with four (6%) patients positive only in PMNs and six (9%) positive only in PBMCs. This extends earlier observations on high-risk heart transplant recipients (15), from whom comparable numbers of PBMC and total PB leukocytes had

FIG. 1. Kaplan-Meier analysis of the incidence of the first CMV DNA detection in PB cells and cardiac EMB samples from heart transplant recipients receiving anti-CMV prophylaxis. Results of qualitative PCR detection in PMNs (gray line), PBMCs (black line), and EMB specimens (dashed line) are shown.

FIG. 2. Differences in length of time to the first detection of CMV DNA based on cell type and CMV serostatus at transplant. (A) Time to first CMV detection in PBMCs versus PMNs in R⁺/D⁻ patients (P = 0.016 by Wilcoxon signed-rank paired analysis). (B) Time to first CMV detection in PBMCs versus PMNs in R⁻/D⁻ patients (P = 0.9 by Wilcoxon signed-rank paired analysis). (C) Time to first CMV detection in PBMCs versus PMNs in R⁺/D⁻ patients (P = 0.5 by Wilcoxon signed-rank paired analysis). The thick line indicates the median, the lower and upper sides of the rectangles indicate 25th and 75th percentiles, respectively, and the bars indicate the 90th and 10th percentile limits.
been shown to be CMV DNA positive. Acute CMV disease occurred in three patients, one R+/-D+, one R+/D+, and one R+/-D-, during the study period, a frequency (5%) that was in line with previous reports (11). CMV detection was less frequent in the R+/-D+ subgroup than in the subgroup receiving standard antiviral prophylaxis, as previously reported (14). Thus, despite achieving the expected level of suppression of acute CMV disease, neither standard anti-CMV prophylaxis in the R+ subgroup nor aggressive anti-CMV prophylaxis in the R+/-D+ subgroup eliminated viral reactivation and replication. Prophylaxis achieved the goal of reducing CMV replication levels but had little apparent impact on the incidence of reactivation.

**Time course of CMV in PMNs and PBMCs.** When the time to first CMV detection was calculated for the patient subgroup that became CMV positive in both leukocyte fractions, the median time (25th to 75th percentile) to CMV DNA detection was 54 (21 to 79) days in PMBCs and 95 (33 to 166) days in PMNs (P = 0.02 by Wilcoxon signed-rank test). Overall, CMV DNA was detected earlier in PMBCs than in PMNs. More specifically, paired data analysis revealed that the earlier appearance in PMBCs was significant only in the 40 R+ patients (55 [21 to 79] versus 60 [30 to 124] days; P = 0.048) patients and not in the 21 R+/-D+ patients (53 [20 to 138] versus 87 [18 to 143] days; P = 0.9) patients. Within the two R+ patient subgroups, the time to first CMV detection was significantly shorter in PMBCs than in PMNs only in the 27 R+/D+ (48 [20 to 79] versus 70 [30 to 105] days; P = 0.02) patients (Fig. 2). This relationship was not observed in the 13 R+/-D patients (58 [21 to 96] versus 53 [30 to 141] days; P = 0.5) patients (Fig. 2). However, the small size of this subgroup remains a confounding factor.

Subsequent quantification with real-time PCR revealed higher peak levels in PMNs than in PBMCs on a background that varied from a minimum of 5 (the cutoff) to a maximum of >4,000 DNA copies/10^5 leukocytes. The majority of positive samples had low CMV DNA levels, such that overall median (25th to 75th percentile) levels were 34 (8 to 110) CMV genome copies/10^5 leukocytes in PMNs and 17 (5 to 57) copies/10^5 leukocytes in PMBCs. Overall, CMV load was significantly higher in PMNs than PBMCs (P < 0.01), consistent with active viral replication in these patients. These levels were substanti-
frequency was highest in R−/D+ patients during the 5- to 7-month period following discontinuation of prophylaxis after the fourth month. Thus, in either the R+ or R−/D+ subgroups, high-grade infection frequency peaked 2 to 3 months after prophylaxis was discontinued.

Table 1 shows the demographic and clinical characteristics of the 20 patients who reached levels above 110 CMV genome DNA copies/10^5 PMNs and the 46 patients who remained at or below 110 CMV genome copies. The frequency of high-level samples in R+ patients was twice that in the R−/D+ subgroup.

Table 1. Baseline patient characteristics according to CMV DNA levels

<table>
<thead>
<tr>
<th>No. of CMV DNA copies</th>
<th>Recipient age (yr; P = 0.96)^ab</th>
<th>Recipient gender (males/females; P = 0.61)^c</th>
<th>Donor age (yr; P = 0.95)^d</th>
<th>Donor gender (males/females; P = 0.77)^d</th>
<th>Cold ischemic time (min; P = 0.67)^d</th>
<th>Standard (no. [%] of R+; P = 0.04)^c</th>
<th>Aggressive (no. [%] of R−/D+ patients; P = 0.04)^d</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤110 (n = 46)</td>
<td>49 ± 14</td>
<td>35/11</td>
<td>33 ± 14</td>
<td>33/13</td>
<td>203 ± 44</td>
<td>28 (61)</td>
<td>18 (39)</td>
</tr>
<tr>
<td>&gt;110 (n = 20)</td>
<td>48 ± 15</td>
<td>14/6</td>
<td>32 ± 12</td>
<td>15/5</td>
<td>212 ± 57</td>
<td>17 (85)</td>
<td>3 (15)</td>
</tr>
</tbody>
</table>

^a Means ± standard deviations.
^b Student’s t analysis.
^c Chi-square analysis.
virus DNA in two patients, three times in one patient (16, 66, and 142 days posttransplant) and twice in another patient (78 and 427 days posttransplant), suggesting that infection with this virus was also infrequent within the transplanted heart. The detection of adenovirus did not coincide with CMV detection, acute rejection, adverse events, or other infections.

**DISCUSSION**

This study focuses on the incidence of active CMV infection in patients receiving prophylaxis to prevent acute CMV disease (14, 22). Despite prophylaxis, CMV is commonly detected in white blood cells of most heart transplant recipients in a time course that appears to be influenced by donor and recipient CMV serostatus. Viral DNA is only rarely detected in the allograft itself. Antiviral prophylaxis does not appear to reduce the reactivation of virus from latency but does appear to reduce the amplification of virus that is responsible for acute CMV disease (5, 6, 15). Our failure to detect CMV DNA in any R/D patients is consistent with evidence that CMV infection originates from the host or the engrafted organ and may be readily predicted by pretransplant CMV serostatus, as in the pre-ganciclovir era (4, 7, 17). The subclinical CMV replication levels arise in recipients who are latently infected as well as in settings where latent virus is introduced with the allograft, just as during the pre-ganciclovir era.

CMV DNA PCR analysis of whole blood, plasma, or leukocytes can predict the risk of acute or chronic disease (5, 6, 14, 15, 22). Fractionation of PB leukocytes before PCR analysis revealed differences in the timing of CMV DNA detection. PBMCs are reservoirs of CMV latency and support reactivation (8, 12, 19), whereas PMNs are indicators of active viral replication, whether assessed for level of infectivity, antigenemia, or viral nucleic acids (5, 6). In our study, the earlier appearance of CMV DNA in PBMCs than in PMNs in R/D patients hints at differences that depend on CMV serostatus, effectiveness of prophylaxis, or other factors influencing infection that deserve further study.

CMV DNA levels were lower in the high-risk subgroup receiving more aggressive prophylaxis to reduce acute disease. Standard prophylaxis may not adequately suppress CMV replication that contributes to chronic disease (11, 17). Infection seems to start earlier and sustains higher DNA levels (i.e., >110 DNA copies/μL PMNs) in R patients than in R/D patients. Previous observations have shown that CMV can be detected on average 40 days earlier in R patients than in R/D patients, although the times to peak CMV levels following the termination of antiviral therapy are comparable (3). Viral doubling time is likely to be shorter in R/D patients than in R patients due to preexisting antiviral immunity. The lack of preexisting immunity may underlie a trend towards a shorter median time to peak viral DNA levels after termination of prophylaxis in R/D patients (35 to 106 days versus 51 to 140 days; P = 0.3). Although we cannot estimate CMV doubling time (3), the more aggressive prophylaxis seems to benefit these patients even in the absence of immunity.

CMV DNA is rarely in EMB samples in R settings but is not present at all in the R/D setting. Like previous reports (18), this is striking given the high incidence of systemic CMV infection. Despite expectations that CMV would target the transplanted heart via a tropism for endothelial cells (8, 23), little evidence has accumulated to support subclinical CMV replication in this tissue, even in patients with high-level anti-growth and acute disease symptoms together with subendothelial inflammation (10). Similar results have been reported previously for a study using animal models (21). The detection of CMV in EMB samples of two patients in our study was most consistent with spillover of high-level cell-associated systemic CMV infection, suggesting that leukocytes represent primary sites of viral replication in the transplant setting. Thus, systemic infection levels appear to be the critical factor in detecting virus in transplanted heart tissue.

This prospective study suggests that use of more aggressive prophylaxis regimens on all patients at low risk of acute CMV disease may be more effective in reducing levels of CMV replication and thereby reduce long-term chronic disease consequences, although further studies will be needed to clarify whether the levels of subclinical infection that we have described are a requisite component of progression to chronic allograft disease.

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AUTHOR’S CORRECTION

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