AUTHOR’S CORRECTION

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 non-permissive 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
recipient (R) of a D+ heart (13, 17, 25), and a difference has been ascribed to the benefit of preexisting immunity as well as the burden of primary infection and difficulty of mounting a primary antiviral immune response during the posttransplant period. A standard 28-day course of intravenous ganciclovir significantly reduces acute CMV disease when the recipient is seropositive (R+/D+ and R+/D- settings) but fails to adequately control disease in the R-/D+ setting (11, 17). This has led to the empirical application of aggressive prophylaxis in high-risk patients, typically employing intravenous ganciclovir starting 1 day posttransplant followed by long-term oral ganciclovir or valganciclovir, sometimes in combination with CMV hyperimmune gammaglobulin for several months (14, 17, 25). Studies of long-term oral ganciclovir or oral valganciclovir (4) and ganciclovir with long-term CMV hyperimmune gammaglobulin (14, 25) have shown a benefit in high-risk (R+/D+) heart transplant recipients, and evidence suggests that this benefit extends to acute rejection and CAV (14). The extent to which systemic replication in the bloodstream and localized replication in the transplanted heart are affected in any of these prophylaxis regimens remains unknown.

We have found a surprisingly rapid time course and frequent CMV detection in circulating leukocytes from heart transplant recipients treated to suppress CMV disease. Our analysis reveals a high frequency of occult infection and defines the interplay between systemic CMV infection, donor/recipient serology, prophylaxis regimen, and direct CMV infection of the allograft.

This research was presented at the 8th Cytomegalovirus and Second Betaherpesvirus Workshop, April 2005, Williamsburg, VA [abstract 5.06], and the European Society for Cytovirology Congress, April 2005, Geneva, Switzerland.)

MATERIALS AND METHODS

Patients. Seventy-five first heart transplant recipients (age, 50 ± 12 years; 54 males) surviving at least 6 months after transplant and at risk of CMV infection (11 R+/D-, 34 R+/D+, 21 R-/D+, and 9 R-/D-) were consecutively enrolled between 24 January 2002 and 19 August 2004 and monitored for up to 16 months posttransplantation. Immunosuppressive therapy has been described in detail previously (14) and involved induction with daclizumab (1) as well as maintenance with oral cyclosporine and prednisone together with either mycophenolate mofetil or sirolimus. All patients who were at risk of CMV infection (R+/D-, R+/D+, and R-/D+) received a standard course of intravenous ganciclovir at 5 mg/kg of body weight twice a day for the first 2 weeks after transplantation and 6 mg/kg daily for the following 2 weeks. Because of a higher risk for CMV disease, CMV-naive patients receiving a heart from a CMV-seropositive donor (R+/D-) were subjected to more aggressive prophylaxis, including intravenous hyperimmune gammaglobulin (14, 25). Patients. Seventy-five first heart transplant recipients (age, 50 ± 12 years; 54 males) surviving at least 6 months after transplant and at risk of CMV infection (11 R+/D-, 34 R+/D+, 21 R-/D+, and 9 R-/D-) were consecutively enrolled between 24 January 2002 and 19 August 2004 and monitored for up to 16 months posttransplantation. Immunosuppressive therapy has been described in detail previously (14) and involved induction with daclizumab (1) as well as maintenance with oral cyclosporine and prednisone together with either mycophenolate mofetil or sirolimus. 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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 qPCR 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 PBMCs 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 PBMCs was significant only in the 40 R⁺ (21 to 79) versus 60 [30 to 124] days; P = 0.048) patients and not in the 21 R⁻/D⁺ (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 PBMCs 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⁻ (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/10⁵ 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/10⁵ leukocytes in PMNs and 17 (5 to 57) copies/10⁵ leukocytes in PBMCs. Overall, CMV load was significantly higher in PMNs than PBMCs (P < 0.01), consistent with active viral replication in these patients. These levels were substantially lower than has been observed in untreated immunocompromised patients (5, 6, 15) and suggests that prophylaxis reduces infection levels without altering the reactivation dynamics. Time course analysis revealed that CMV DNA was detected less frequently and remained at lower median levels during prophylaxis than afterwards (11), and this was evident in both the PMN and PBMC fractions. In particular, during and after prophylaxis the incidence of CMV-positive PMN samples was 19% and 36% (P < 0.01), and the median genome copy numbers were 23 and 67 per 10⁵ PMNs (P = 0.05), respectively. Similarly, after prophylaxis the incidence of CMV-positive samples in PBMCs rose to 38% (P < 0.01) and the median DNA copy number rose to 34 genomes/10⁵ PBMCs (P < 0.01). We also compared the time course of CMV detection in CMV-naïve patients (R⁻/D⁻) and CMV-seropositive patients (R⁺). First, we analyzed differences in cumulative incidence of CMV DNA detection. We then compared the times to peak CMV DNA levels in PMNs and PBMCs. CMV DNA was detected significantly earlier and more often in PMN or PBMC fractions from the R⁺ subgroup than in those of the R⁻/D⁻ subgroup (Fig. 2 and 3A and B), although the time to peak CMV DNA level and the frequency of CMV DNA-positive patients were the same in the two patient subgroups regardless of the PB leukocyte fraction that was assessed (data not shown). The incidence of CMV detection began to diverge at about 1 month posttransplantation, at the time when prophylaxis of the R⁺ subgroup was terminated.

**Time course and prevalence of high CMV DNA levels.** Aiming to analyze the effect of preexisting CMV infection (based on serostatus) and the influence of prophylaxis regimen on the kinetics of high CMV DNA levels, we studied the time course of detection of CMV DNA above the 75th percentile of the entire cohort of samples, levels greater than 110 CMV DNA copies/10⁵ PMNs. As shown in Fig. 4A, we found that R⁺ patients had a higher frequency of detection of CMV DNA at or above this level than R⁻/D⁻ patients. This difference was particularly evident at month 4 after transplant, when prophylaxis was ongoing in the R⁻/D⁻ subgroup but was 2 to 3 months after prophylaxis had been discontinued in the R⁺ subgroup. Consistent with the frequency of high CMV load being detected following termination of prophylaxis, the fre
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 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.

Over a third (39%) of the R+ subgroup experienced high-level infection, whereas only 15% (P = 0.04) of the R-/D+ subgroup had CMV DNA levels in this range. Thus, the overwhelming majority (85%) of patients who experienced high-grade infection were CMV seropositive at the time of transplant. Together with our recent report examining the impact of CMV infection on clinical endpoints in an overlapping cohort of these patients (14), this study demonstrates that viral levels are suppressed by the aggressive antiviral regimen in use on R-/D+ patients (Fig. 4B) and that this prophylaxis regimen effectively reversed the traditional higher risk for CMV infection than that of R+ patients (Fig. 2C). Note that demographic and baseline characteristics of patients with versus those without high-grade CMV infection were similar (Fig. 4), limiting the possibility that factors other than serology and prophylaxis regimen influenced high-grade CMV occurrence.

CMV DNA detection in EMB samples. Over 80% of patients consented to provide EMB samples for viral DNA analysis to compare infection in the transplanted heart to infection of PB cell fractions. We tested 349 EMB samples from 55 of the patients distributed across the high- and low-grade infection subgroups. CMV DNA was detected in only two allograft samples, each from a different patient. EMB samples from high-risk patients (R-/D+) receiving aggressive prophylaxis were uniformly negative, consistent with the lower CMV DNA levels in leukocytes from this subgroup (Table 1; Fig. 4). The only patients with positive EMB samples were CMV seropositive at the time of transplant (one R+/D+ and one R-/D-), and these infections were coincident with high-grade systemic infection in PB cells and followed termination of anti-CMV prophylaxis. CMV DNA levels were at the highest levels recorded for these patients, whether evaluated in PBMCs (346 and 2,152 CMV genome copies/10^5 PBMCs, respectively) or PMNs (524 and 936 CMV genome copies/10^5 PMNs, respectively) on the day EMB samples tested positive. Although neither patient had signs of clinically evident CMV disease or cellular acute rejection at the time the positive samples were collected (2), one had a high incidence of other infections (bacterial as well as herpes zoster) and an acute rejection episode requiring increased immunosuppressive therapy during the previous month, and the other experienced a rejection episode requiring additional immunosuppressive treatment 1 month afterwards. In addition to CMV, adenovirus has been implicated in chronic vascular disease in pediatric heart transplant patients (24), suggesting that this virus may contribute to disease independently or in combination with CMV. We detected adeno-

![FIG. 4. Frequency of high-level CMV DNA in heart transplant recipients receiving anti-CMV prophylaxis. (A) Rate of samples showing >110 CMV DNA copies/10^5 PMNs (high-grade infection) in the R-/D+ (open circles) and R+ (filled circles) patient groups over 16 months posttransplant. (Time periods were as follows: month 1, 14 to 45 days; month 2, 46 to 76 days; month 3, 77 to 105 days; month 4, 106 to 161 days; months 5 to 7, 162 to 208 days; months 8 to 10, 209 to 304 days; months 11 to 13, 305 to 426 days; months 14 to 16, 427 to 486 days). The standard prophylaxis period used for R+ patients is indicated by dark gray shading, and the aggressive prophylaxis period used for R-/D+ patients is indicated by light gray shading. (B) Distribution of patients developing low-grade (≤110 copies/10^5 cells) and high-grade (>110 copies/10^5 cells) infection according to CMV serological status.](image)

| TABLE 1. Baseline patient characteristics according to CMV DNA levels |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| No. of CMV DNA copies | Recipient age (yr; P = 0.96)^a,b | Recipient gender (males/females; P = 0.63)^c | Donor age (yr; P = 0.95)^c | Donor gender (males/females; P = 0.77)^c | Cold ischemic time (min; P = 0.67)^c | Prophylaxis protocol (P = 0.04)^c |
| ≤110 (n = 46) | 49 ± 14 | 35/11 | 33 ± 14 | 33/13 | 203 ± 44 | 28 (61) |
| >110 (n = 20) | 48 ± 15 | 14/6 | 32 ± 12 | 15/5 | 212 ± 57 | 17 (85) |
| Prophylaxis protocol (P = 0.04)^c | Standard (no. [%] of R+/D+ and R+/D- patients; P = 0.04)^c | Aggressive (no. [%] of R-/D+ patients) |
| R+/D+ patients | 0.77 |
| R-/D+ patients | 0.77 |

^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 427 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/10⁵ PMNs) in R⁺ than in R⁻/D⁻ patients. Previous observations have shown that CMV can be detected on average 40 days earlier in R⁺ 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 [0 to 106] days versus 51 [4 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-genemia 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.

**ACKNOWLEDGMENTS**

We appreciate the statistical advice of Balasubramanian Narasimhan as well as the continued encouragement and comments from members of the Institute of Cardiology of the University of Bologna. This work was supported by a PHS program project grant (PO1 AI50153) to E.S.M., J.P.C., and H.A.V. as well as by a fellowship from the Italian Society of Cardiology (to L.P.).

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