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Quantitative Analysis of Cytomegalovirus (CMV) Viremia Using the pp65 Antigenemia Assay and the COBAS AMPLICOR CMV MONITOR PCR Test after Blood and Marrow Allogeneic Transplantation

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The performance of a commercially available qualitative PCR test for plasma (AMPLICOR CMV Test; Roche Diagnostics) and a quantitative PCR test for plasma and leukocytes (COBAS AMPLICOR CMV MONITOR Test; Roche Diagnostics) was evaluated with samples from 50 blood or marrow allogeneic transplant recipients who received short courses of sequential ganciclovir therapy (2 weeks intravenously followed by 2 weeks orally) based on a positive cytomegalovirus (CMV) pp65 antigenemia (AG) assay. The number of persons with a positive CMV test was significantly higher for leukocyte-based assays (AG, 67.5%; PCR, 62.5%) compared to both quantitative and qualitative PCR tests of plasma (42.5 and 35%, respectively). One person developed CMV disease during the study despite a negative AG assay; in this particular case, all PCR assays were found to be positive 10 days before his death. There was a trend for earlier positivity after transplantation and more rapid negativity after initiation of ganciclovir for the tests performed on leukocytes. The mean number of CMV copies as assessed by PCR was significantly higher in leukocytes than in plasma ($P = 0.02$). Overall, excellent agreement (kappa coefficient, >0.75) was found only between the two PCR assays (qualitative and quantitative) based on plasma. These results suggest that either the pp65 AG assay or the COBAS AMPLICOR CMV MONITOR Test using leukocytes could be used to safely monitor CMV viremia in related allogeneic blood or marrow transplant recipients. Such a strategy will result in preemptive treatment for about two-thirds of the persons with a relatively low rate (<33%) of secondary viremic episodes following short courses of ganciclovir therapy.

Cytomegalovirus (CMV) has been recognized as the most important viral pathogen in persons undergoing bone marrow transplantation (BMT) (16, 17). Two basic strategies are currently in use to prevent the development of CMV disease in this patient population. The first consists of the administration of an effective antiviral agent, such as ganciclovir, to all recipients at risk of CMV reactivation (so-called universal prophylaxis), whereas the second strategy uses antivirals only in persons with proven viral reactivation but before occurrence of disease (preemptive therapy) (4, 20, 24). In theory, a more selective use of ganciclovir for a short period of time in high-risk patients would avoid unnecessary side effects related to prolonged antiviral therapy, such as myelosuppression and subsequent secondary infections, and it could accelerate CMV-specific immune reconstitution resulting in a lower risk of "late" CMV disease (4, 14). However, preemptive therapy must rely on the use of an early and sensitive marker of CMV reactivation. In that regard, previous trials of preemptive ganciclovir therapy based on shell vial cultures of blood and other biological fluids (12) or on high levels of CMV pp65 antigenemia (AG) (4) have resulted in unacceptably high rates of CMV disease (in excess of 12%).

More-sensitive, PCR-based methods have been recently evaluated for monitoring of CMV reactivation after BMT (3, 7, 13, 15, 25). However, it remains difficult to evaluate the exact role of the previously reported PCR assays in guiding preemptive CMV therapy in the allogeneic BMT population due to differences in the origin of samples (leukocytes or plasma) and the type of PCR procedures (qualitative versus quantitative) used for monitoring. In this study, we performed a longitudinal evaluation of two commercially available PCR assays (qualitative AMPLICOR CMV Test for plasma, quantitative COBAS AMPLICOR CMV MONITOR Test for plasma and leukocytes) with samples from blood and marrow recipients who were eligible for early ganciclovir therapy based on a positive pp65 AG assay result.

MATERIALS AND METHODS

Patients and preemptive therapy. Recipients of allogeneic peripheral blood or a marrow hematopoietic stem cell transplant from a matched sibling donor were recruited in two BMT units of the Province of Québec, Canada. Enrollment criteria included either recipient or donor CMV seropositivity prior to transplantation. A chemotherapy-based conditioning regimen with busulfan and cyclophosphamide was used for 80% of the persons, whereas the rest received high-dose cyclophosphamide and total-body irradiation. Samples used in this study were from consecutive patients enrolled in a new preemptive strategy for CMV as described below. Preemptive ganciclovir therapy was initiated at a time of a single positive pp65 AG test ($\geq$1 positive cell/2×10^5 leukocytes), which was performed at weekly intervals from the day prior to initiation of the conditioning regimen until day 98 after transplantation. The preemptive protocol consisted of an induction phase with intravenous (i.v.) ganciclovir (5 mg/kg twice a day, adjusted for renal function) for a minimum of 2 weeks or until a negative AG result was obtained, followed by a 2-week maintenance phase with oral ganciclovir (1 g three times a day, also adjusted for renal function). The sequential use

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TABLE 1. Times after transplantation before detection of first positive CMV tests of samples from 50 allogeneic blood or marrow transplant recipients

Test	No. positive/total (% positive)[a]	Time (no. of days to positivity)			SD
		Mean	Median	Range	
pp65 AG	27/50 (54.0)	41.7	42.0	14–84	16.8
PCR-Lquant	26/49 (53.1)[b]	41.2	38.5	21–70	12.7
PCR-Pquant	18/50 (36.0)	45.9	49.0	14–91	17.7
PCR-Pqual	15/50 (30.0)	43.4	49.0	7–77	17.8

[a] Number of persons with a positive test among the 40 persons surviving for ≥77 days after transplantation and the 10 patients who died before day 77.
[b] For one person, results of the PCR-Lquant assay were unavailable.

of i.v. ganciclovir followed by oral ganciclovir was repeated whenever a positive pp65 CMV AG assay result was obtained until posttransplantation day 98.

Qualitative and quantitative CMV assays. Blood samples were collected weekly in EDTA-treated tubes and processed within 6 h. Plasma and polymorphonuclear leukocytes (PMNLs) were separated using a standard dextran sedimentation procedure. An aliquot of 2×10^5 PMNLs was immediately spotted on a slide for the CMV AG assay (CINA pool test; Argene, Parc Technologique Delta Sud, France) by following the manufacturer's recommendations. An aliquot of 8×10^5 PMNLs and two aliquots of plasma (1 ml each) were frozen at −80°C for a maximum of 4 to 5 months, avoiding freeze-and-thaw cycles, before subsequent PCR studies. Qualitative detection of CMV DNA (PCR-Pqual) in 50 μl of plasma was performed using the AMPLICOR CMV Test (Roche Diagnostics, Laval, Québec, Canada) as previously described (13). The lower limit of detection of this assay is approximately 1,000 copies/ml of plasma. Quantitative assessment of the CMV DNA load was performed with the COBAS AMPLICOR CMV MONITOR Test and the automated COBAS System (Roche Diagnostics) using either 200 μl of plasma (PCR-Pquant) or 8×10^5 PMNLs (PCR-Lquant). The lower limit of detection of this assay is approximately 400 copies per ml of plasma or per 4×10^6 PMNLs, which represents an analytical sensitivity of about 10 copies per PCR (A. Williams, S. Adhikary, G. Boivin, A. Caliendo, M. Espy, J. Handfield, A. Keen, C. Lewinski, M. Forman, D. McNairn, C. Paya, T. Quinn, I. Sia, T. Smith, V. Tevere, B. Turck, and J. Spadoro, Abstr. 98th Gen. Meet. Am. Soc. Microbiol. 1998, abstr. C-2, p. 131, 1998).

Statistical analyses. Paired two-by-two frequency tables were prepared for the various CMV detection tests, and comparisons were evaluated using McNemar's exact test for the following variables: incidence of subjects with a positive test, rate of negativity during ganciclovir therapy, and rate of recurrence of positivity after treatment. The mean periods of time before occurrence of the first positive result for the different tests were compared using an analysis of variance model. The agreement between CMV detection assays was evaluated using the kappa coefficient. For most purposes, kappa values of >0.75 may be considered to represent excellent agreement beyond chance, values of <0.40 may be considered to represent poor agreement, and values between 0.40 and 0.75 represent fair-to-good agreement (9). Comparison of the numbers of CMV copies in PMNLs and plasma was performed using Wilcoxon's test. Finally, correlation between the quantitative assays for the CMV viral load was evaluated with Pearson's correlation coefficient. Statistically significant differences were set at the 5% level. All statistical analyses were done using the SAS System, version 6.12.

RESULTS

Incidence of positive AG and PCR-based assay results. Fifty consecutive allogeneic transplant (45 blood and 5 marrow) recipients were enrolled in this study. The positivity rates for the various CMV detection assays were calculated from a group of 40 allogeneic recipients who survived ≥77 days after transplantation. The numbers of patients with at least one positive result by the AG, PCR-Lquant, PCR-Pquant, and PCR-Pqual assays were 27 of 40 (67.5%), 25 of 40 (62.5%), 17 of 40 (42.5%), and 14 of 40 (35.0%), respectively. The number of subjects with a positive test was significantly higher for the AG assay compared with the PCR-Pqual ($P < 0.001$) and PCR-Pquant ($P = 0.002$) assays. Similarly, more persons had a positive PCR test result using leukocytes versus plasma ($P = 0.003$ and 0.008 for the qualitative and quantitative assays, respectively). There was no significant difference in the positivity rate between the AG and PCR-Lquant ($P = 0.50$) assays or between the PCR-Pquant and PCR-Pqual ($P = 0.38$) assays. Among the 10 persons who died before day 77 after transplantation from diverse regimen-related toxicities (mean period of follow-up, 25.2 days; range, 7 to 49 days), only 1 presented evidence of CMV reactivation in the blood by all of the PCR-based assays on day 35, which occurred 10 days before his death due to a myocardial infarct. This was the only person who developed CMV disease during the surveillance period, as confirmed by the presence of typical intranuclear inclusions in both lungs and the gastrointestinal tract on autopsy. Since serial AG tests were persistently negative, this patient never received preemptive therapy. Overall, when the entire cohort is considered, 27 of 50 (54.0%) and 26 of 49 (53.1%) enrolled persons had positive AG and PCR-Lquant test results, respectively. Two persons had positive AG (on days 28 and 42) but negative PCR-Lquant test results, whereas two others had positive PCR-Lquant (on days 35 and 84) but negative AG test results, including the previously discussed person with CMV disease.

The mean and median times before detection of the first positive results for each assay are reported in Table 1. Although leukocyte-based test (AG and PCR-Lquant) results were positive earlier than those of plasma-based PCR assays, the differences among the results of the four tests were not significant when data from patients with all four positive test results ($n = 13$, $P = 0.61$) or at least one positive test result ($n = 29$, $P = 0.74$) were analyzed. The observed concordance and kappa coefficient of agreement for all CMV assays are shown in Table 2. The concordance between all paired comparisons was above 90%. However, excellent agreement (kappa coefficient, >0.75) was found only for the two plasma PCR assays. Overall, no results were available for 12 of 683 (1.8%) AG tests and for 102 of 683 (14.9%) PCR-Lquant tests due to severe neutropenia at the time of specimen collection. It must be emphasized that AG tests had priority over PCR assays in our treatment protocol. The overwhelming majority of missing values were from specimens collected either before or on day 21 after transplantation (91.7 and 90.2% for the AG and PCR-Lquant tests, respectively).

Evaluation of CMV viral load. The maximum viral load in the three quantitative assays for persons receiving preemptive therapy based on a positive AG assay result is reported in Table 3. The number of CMV copies was significantly higher in leukocytes than in plasma ($P = 0.02$) for 18 patients who tested positive by both PCR assays. After logarithmic transformation of the test values, the correlation between the quantitative assays for the CMV viral load was as follows: AG assay versus PCR-Pquant assay, $r = 0.56$, $P = 0.004$; AG assay versus PCR-Lquant assay, $r = 0.71$, $P = 0.001$; PCR-Pquant versus PCR-Lquant assay, $r = 0.45$, $P = 0.004$.

TABLE 2. Concordance and kappa coefficient agreement among results of different CMV detection assays

Test comparison	Concordance (%)[a]	Agreement[b]
PCR-Pqual vs AG	612/670 (91.3)	0.37
PCR-Pquant vs AG	617/670 (92.1)	0.44
PCR-Lquant vs AG	523/581 (90.0)	0.47
PCR-Pqual vs PCR-Pquant	665/682 (97.5)	0.81
PCR-Pqual vs PCR-Lquant	536/582 (92.1)	0.56
PCR-Pquant vs PCR-Lquant	542/582 (93.1)	0.63

[a] Number of concordant results/number of concordant + number of discordant results.
[b] Kappa coefficient.

TABLE 3. Maximum CMV viral loads per person determined by quantitative detection assays

Test	No. of persons with positive assays	Maximum CMV viral load			SD
		Mean	Median	Range	
pp65 AG assay[a]	27	2.1	1	0.5–9	2.6
PCR-Pquant[b]	18	8,111.4	1,460	289–65,600	18,220.2
PCR-Lquant[c]	26	10,920.4	2,455	492–88,900	20,788.6

[a] Number of positive cells per 10^5 leukocytes.
[b] Number of CMV copies per milliliter of plasma.
[c] Number of CMV copies per 4×10^6 leukocytes.

Effect of preemptive ganciclovir therapy on CMV detection assay results. The negativity rates of the different assays were calculated for the patients who had a positive test result at the onset of ganciclovir therapy. After the first week of i.v. ganciclovir therapy (5 mg/kg twice a day), the negativity rate were 68.8, 76.9, 44.4, and 30.8% for the AG, PCR-Lquant, PCR-Pquant, and PCR-Pqual tests, respectively. The percentages of negative specimens reached 93.8, 88.5, 61.1, and 76.9% at the end of the second week of i.v. ganciclovir therapy. Pairwise analyses revealed no significant differences between the negativity rates of the tests after 1 week of ganciclovir, but this finding may be explained by the small number of treatment episodes analyzed (ranging from 12 to 26 episodes, depending on the comparison). Lastly, the percentage of recurrence of positivity (second viremic episode) after successful preemptive therapy with ganciclovir was calculated for each of the assays. The recurrence rates were 22.2% for AG (6 new episodes after 27 treated episodes), 30.4% (7 of 23) for PCR-Lquant, 20.0% (3 of 15) for PCR-Pquant, and 27.3% (3 of 11) for PCR-Pqual (all pairwise comparisons revealed no significant differences).

DISCUSSION

In this study, we compared different commercially available detection assays for the monitoring of CMV infections in allogeneic blood or marrow transplant recipients receiving a brief course of preemptive ganciclovir therapy initiated because of a positive pp65 AG assay result. The major finding of our work is the superiority of leukocyte-based tests (pp65 AG and quantitative PCR assays) over plasma-based tests (qualitative and quantitative PCR assays) with higher sensitivity, earlier positivity, and more rapid negativity following ganciclovir administration. To our knowledge, this study provides the first evaluation of the standardized and automated COBAS AMPLICOR CMV MONITOR Test with samples from a population composed exclusively of blood and marrow transplant recipients.

Despite major advances in treatment and prevention, CMV infection remains an important cause of morbidity and mortality in allogeneic transplant recipients. Prolonged prophylaxis of high-risk persons with ganciclovir started from engraftment and continued until posttransplantation day 100 is highly effective in preventing CMV disease but associated with significant myelotoxicity, an increased incidence of invasive fungal infections, and late CMV disease due to delayed CMV-specific T-cell response recovery (4, 11, 14). CMV viremia is highly predictive of CMV disease, with a positive predictive value of approximately 70% (17); however, early CMV detection has been problematic with insensitive methods such as conventional cell culture and the shell vial assay, which may become positive when disease is already present (7, 12).

Recently, there has been a renewed interest in the targeting of high-risk subjects by using more-sensitive assays based on the detection of CMV antigens or nucleic acids in the blood (the so-called preemptive or early-treatment strategy). In that regard, Boeckh et al. have shown that the use of the CMV pp65 AG test using a cutoff value of three positive cells per two slides (1.5×10^5 PMNLs/slide) for enacting short ganciclovir treatment resulted in an unacceptably high rate of CMV disease (14%) compared to the universal ganciclovir prophylactic approach (rate of 2.7%) (4). More recently, the same authors reported that the use of a modified preemptive strategy based on a lower threshold for the pp65 AG assay (any positive result) combined with a longer course of ganciclovir (until day 100) was significantly more successful than their original strategy in reducing the incidence of early CMV disease (rate of 3.8%) (2). Use of a high AG assay threshold for initiating early ganciclovir treatment has also resulted in a high rate of CMV disease in another study (21). PCR testing of blood samples has also been studied in the context of preemptive therapy for BMT patients. For instance, early ganciclovir treatment based on two consecutive positive PCR tests of whole blood has been associated with a 5.4% incidence of CMV disease, compared to 23.5% using cell culture (7).

Results of the previous studies have clearly established the necessity of using a sensitive detection method and, in the case of quantitative assays, a low cutoff value for enacting preemptive antiviral therapy in allogeneic blood or marrow transplant recipients. Such conclusion is based mainly on the following three observations. First, a significant proportion of allogeneic BMT patients develop CMV disease despite a low systemic viral load (6, 19). Second, rapid progression (sometimes in less than a week) of the CMV viral load may occur, particularly in persons with severe graft-versus-host disease (1, 4). Lastly, development of CMV pneumonitis is associated with a high mortality rate in the BMT population despite the use of antiviral therapy and gamma globulins (18).

In our study, CMV detection assays based on leukocytes, i.e., pp65 AG assay and quantitative PCR, were the most sensitive tests for detecting CMV viremia. Their sensitivities were comparable (67.5 and 62.5% for the AG and PCR assays, respectively) and significantly higher than that of either the quantitative or qualitative plasma PCR assay. Due to a low number of early CMV disease episodes in our population (only one patient with CMV pneumonitis and colitis), it is difficult to evaluate the specificity of those different CMV detection tests. However, as mentioned previously, even a low CMV viral load is highly significant in the context of BMT and thus the primary focus should be on improving sensitivity. Because the quantitative COBAS AMPLICOR CMV MONITOR Test has the same lower limit of detection with either leukocytes or plasma (that is, approximately 10 copies/PCR or 400 copies/ml of plasma or 4×10^6 PMNLs) (Williams et al., 98th Gen. Meet. Am. Soc. Microbiol.), it is reasonable to assume that the number of CMV copies was higher in PMNLs than in plasma. Indeed, we found a statistically significantly higher viral DNA load per patient in PMNLs compared to plasma (mean of 10,920 copies/4×10^6 PMNLs versus 8,111 copies/ml of plasma; $P = 0.02$). Extrapolation of these results per milliliter of whole blood in nonneutropenic patients would represent a mean viral load of 12,967 copies in PMNLs versus 5,353 copies in plasma, a 2.4-fold difference. These results are in agreement with those of our previous study using in-house quantitative PCR tests of samples from human immunodeficiency virus-infected persons (5) and those of Sia et al., who used the COBAS AMPLICOR CMV MONITOR Test for samples from solid organ transplant recipients (22, 23).

Leukocyte-based assays were found to be positive at an

earlier time after transplantation; they also became negative more rapidly after initiation of ganciclovir therapy than the tests based on plasma, although these differences did not reach statistical significance. Boeckh and colleagues have also found an earlier positivity time for PCR tests performed on leukocytes compared to assays based on plasma (3). Our finding of a faster clearance of CMV DNA from leukocytes compared to plasma after treatment (88 versus 61% negativity after 2 weeks of i.v. ganciclovir) was more unexpected. However, these data are also in agreement with the short half-life of CMV DNA in cells previously observed (8) and suggest different replication and/or clearance kinetics for CMV between the two compartments. Other factors could also influence the choice of a CMV detection assay for preemptive therapy in allogeneic transplant recipients. For instance, the COBAS AMPLICOR CMV MONITOR Test based on leukocytes has the following advantages compared to the pp65 AG assay: the possibility of a longer period of time before blood processing (up to 72 h versus 6 h), automation, and greater objectivity. On the other hand, the AG assay requires fewer PMNLs (2×10^5 versus 8×10^5) and is more suitable for laboratories with a low volume of samples. Surprisingly, we found only fair-to-good agreement (kappa = 0.47) between the positivity rates of the two leukocyte-based assays. This discrepancy could be explained, in part, by the choice of the AG assay for guiding antiviral therapy, which may have altered CMV replication kinetics, and the small viral load found in viremic persons. In theory, PCR tests performed on plasma could be more useful in assessing CMV viremia during neutropenic episodes, although this was not a major problem in our study. In fact, 90% of missing AG and leukocyte PCR test results due to insufficient PMNL counts occurred during the first 3 weeks after transplantation, at which time the risk of CMV reactivation is low.

In summary, our results show that either the pp65 AG or the COBAS AMPLICOR CMV MONITOR Test using leukocytes could be used to safely monitor CMV viremia in related allogeneic blood or marrow transplant recipients. Use of either test will result in administration of preemptive antiviral therapy in approximately two-thirds of the patients who survive the first 3 months after transplantation with a minimal risk of CMV disease (1 of 40 or 2.5% of persons with disease missed by the AG assay but retrospectively positive by PCR). The proportion of treated patients in our study is similar to the one recently reported by Gerna et al., who used either a low-level pp65 AG assay or an in-house quantitative leukocyte PCR assay to guide preemptive therapy in their BMT population (both approaches would have resulted in the treatment of 61% of the subjects) (10). A low threshold should be adopted for quantitative assays in that context, since even small amounts of CMV require immediate therapy. Determination of the viral load could be potentially more useful in assessing the response to antiviral therapy and in predicting relapses (23). Future studies should be aimed at evaluating the use of a more convenient source of viral DNA, such as whole blood, for the COBAS AMPLICOR CMV MONITOR Test and at studying the safety of shorter courses of preemptive therapy when CMV antigens or DNA are no longer detectable in leukocytes.

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