

Comparison of Quantitative Cytomegalovirus Real-time PCR in Whole Blood and pp65 Antigenemia Assay: Clinical Utility of CMV Real-time PCR in Hematopoietic Stem Cell Transplant Recipients

Successful preemptive therapy for cytomegalovirus (CMV) infection in transplant patients depends on the availability of sensitive, specific, and timely diagnostic tests for CMV infection. Although the pp65 antigenemia assay has been widely used for this purpose, real-time quantification of CMV DNA has recently been recognized as an alternative diagnostic approach. However, the guidelines for antiviral therapy based on real-time quantitative polymerase chain reaction (RQ-PCR) have yet to be established. From November 2004 to March 2005, a total of 555 whole blood samples from 131 hematopoietic stem cell transplant (HSCT) recipients were prospectively collected. RQ-PCR was conducted using an Artus[®] CMV LC PCR kit (QIAGEN). Both qualitative and quantitative correlations were drawn between the two methods. Exposure to the antiviral agent influenced the results of the two assays. Additionally, the discrepancy was observed at low levels of antigenemia and CMV DNA load. Via ROC curve analysis, the tentative cutoff value for preemptive therapy was determined to be approximately 2×10^4 copies/mL (sensitivity, 80.0%; specificity, 50.0%) in the high risk patients, and approximately 3×10^4 copies/mL (sensitivity, 90.0%; specificity, 70.0%) in the patients at low risk for CMV disease. Further study to validate the optimal cutoff value for the initiation of preemptive therapy is currently underway.

Key Words : Cytomegalovirus; Hematopoietic Stem Cell Transplantation; Antigenemia; Real-time PCR; Cutoff

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INTRODUCTION

Cytomegalovirus (CMV) disease continues to be an important cause of morbidity and mortality in hematopoietic stem cell transplantation (HSCT) recipients. We have been conducting a preemptive strategy based on the CMV pp65 antigenemia assay for the prevention of CMV infection and disease in a risk-adapted manner (1). Although the antigenemia assay has been used extensively as a tool to monitor CMV infections, there are several limitations, including 1) the need for immediate sample processing, 2) the nonstandardization of sample processing between laboratories, 3) the time-consuming and laborious procedure, and 4) the lack of objectivity of quantification (2-4). Furthermore, as the detection of antigenemia is applicable only to blood specimens (only virus associated with cells), it requires an adequate number of leukocytes.

Recently, several studies have demonstrated that real-time quantitative polymerase chain reaction (RQ-PCR) for CMV

is useful for the rapid diagnosis of CMV infection and for the monitoring of clinical responses to antiviral therapy (5-11). However, there is, as yet, no consensus regarding the cutoff level and the appropriate blood compartment to be tested by RQ-PCR assay in HSCT recipients.

In this study, we compared RQ-PCR with the pp65 antigenemia assay for the monitoring of CMV infection in allogeneic HSCT recipients. Additionally, we have determined the proper cutoff level of CMV DNA load for preemptive therapy, in order to switch the monitoring method from the antigenemia assay to an RQ-PCR method.

MATERIALS AND METHODS

Patients and samples

From November 2004 to March 2005, whole blood spec-

imens were prospectively collected from allogeneic HSCT recipients who had been admitted to the Catholic HSCT Center. During the study period, the pp65 antigenemia assay and RQ-PCR for CMV were performed simultaneously twice a week after engraftment until day 100, after which they were conducted on a biweekly or monthly basis until the termination of immunosuppressant therapy. During this period, antigenemia assay was used to guide the preemptive therapy in a risk-adapted manner (1).

CMV pp65 antigenemia assay

The antigenemia assay was performed as previously described, with some modifications (12).

In brief, EDTA-treated whole blood samples were fractionated by dextran sedimentation and lysis of erythrocytes. The leukocytes were then centrifuged and placed on Cytospin (Thermo Electron Corporation, Waltham, MA, U.S.A.) slides (2.0×10^5 leukocytes per slide), fixed in paraformaldehyde, reacted with monoclonal antibodies C10/C11 (Clonab CMV; Biotest, Dreieich, Germany), and immunostained by the alkaline phosphatase/anti-alkaline phosphatase method, with the presence of dark brown nuclear staining considered as a positive reaction. The results were expressed as the number of positive cells per 200,000 leukocytes.

Quantitative real-time PCR for CMV DNA

DNA was extracted from 200 μ L of whole blood using a QIAamp DNA blood mini kit (QIAGEN) in accordance with the manufacturer's instructions. PCR was performed on the LightCycler[®] 1.2 instrument (Roche Diagnostics, Mannheim, Germany) using Artus[®] CMV LC PCR kit (QIAGEN). Each reaction tube contained 15 μ L of master mix and a 5 μ L aliquot of the extracted sample DNA or sterile water (as a negative control). The thermocycling protocol consisted of an initial 10 min incubation at 95°C to activate the DNA polymerase. Then a touch-down procedure followed that consisted of 5 sec at 95°C, annealing for 20 sec at temperatures decreasing from 65°C to 55°C during the first 10 cycles (with 1°C decreasing steps for each cycle) and an extension step at 72°C for 15 sec. The annealing temperature for the remaining 40 cycles was 55°C for 20 sec. The products were analyzed by melting-curve analysis by applying 95°C for 1 sec, 50°C for 15 sec, and 80°C for 0 sec. All acquired fluorescence data were analyzed using LightCycler software. The detection limit of the assay is 64.9 copies/mL clinical specimens. It detected CMV DNA in a linear range from 500 to 1×10^7 copies/mL. RQ-PCR values of <500 copies/mL were assigned an arbitrary value of 500 copies/mL.

Statistical analysis

Analysis was conducted using the SPSS software, version

13.0 (SPSS Korea, Seoul, Korea). The proportion of positive and negative results were compared by the chi-square test. Pearson's correlation was used to compare antigenemia with RQ-PCR values. Samples negative for either of the two tests were excluded from the correlation analysis. *P* values of less than 0.05 were considered significant.

Receiver-operating characteristic (ROC) curve analysis was conducted in order to determine a cutoff value of the CMV DNA load in whole blood for the initiation of preemptive therapy. Clinical practice had been based on the pp65 antigenemia assay, and therefore this assay was used as a standard to determine the optimal cutoff value for CMV DNA load by RQ-PCR.

RESULTS

Demographic characteristics of the patients

One hundred and thirty-one patients (87 adult and 44 pediatric) were consecutively enrolled during the study period, and a total of 555 whole blood samples were collected from these patients (average 4.2 samples per patient). There were 68 men and 63 women, with an average of 27.0 yr in a range of 1 to 58 yr. The most common underlying disease was acute leukemia. And more detailed demographic characteristics of the patients are summarized in Table 1. Seventy-two (55.0%) patients received transplants from HLA-identical siblings and 59 (45.0%) patients from unrelated HLA-matched donors (Table 1). Neutropenia (absolute neutrophil count <1,000/ μ L) at the time of blood sampling was detected in 16.9% of total specimens (94/555).

Table 1. Demographic characteristics of the patients

	No. (%) of patients
Age, mean (range), yr	27 (1-58)
Adult (>18 yr)	87 (66.4)
Pediatric	44 (33.6)
Sex, men:women	68 (51.9):63 (48.1)
Underlying diseases	
AML	42 (32.1)
ALL	35 (26.7)
SAA	22 (16.8)
MDS	15 (11.5)
CML	12 (9.2)
MM	3 (2.3)
Other diseases*	2 (1.6)
Donor type	
Matched sibling	72 (55.0)
Matched unrelated	59 (45.0)

*Included Fanconi anemia and chronic lymphocytic leukemia.

AML, acute myelogenous leukemia; ALL, acute lymphocytic leukemia; SAA, severe aplastic anemia; MDS, myelodysplastic syndrome; CML, chronic myelogenous leukemia; MM, multiple myeloma.

Incidence of CMV infection and qualitative results of antigenemia and real-time PCR

CMV infection was detected in 84/555 (15.1%) whole blood samples by RQ-PCR and 143/555 (25.8%) samples by pp65 antigenemia. Per patient analysis, CMV infection was observed in 34 (30.0%) patients by RQ-PCR and 45 (34.4%) patients by pp65 antigenemia. Both methods detected CMV reactivation more frequently in the unrelated transplant recipients than in the sibling recipients (37.3% vs. 16.7%, $P=0.007$ by RQ-PCR, 47.5% vs. 23.6%, $P=0.004$ by antigenemia).

Concordant results of the two tests were observed in 460 (82.9%) out of 555 specimens ($\kappa=0.483$, $P<0.0001$, Table 2). Out of 95 discrepant samples, 77 (81.1%) were determined to be positive on antigenemia assay but negative on RQ-PCR and 18 were negative on antigenemia assay but positive on RQ-PCR. Significant factor influencing the discrepant qualitative results was the exposure to the anti-CMV drugs ($P<0.001$). Upon subgroup analysis, specimens collected during anti-CMV therapy showed concordance, with a kappa value of 0.303 ($P=0.001$). However, the antiviral naïve samples evidenced concordance with a higher kappa value of 0.539 ($P<0.0001$). Out of discrepant samples, antigen-positive but RQ-PCR negative samples were more frequently exposed to the anti-CMV agents than antigen-negative but RQ-PCR positive samples. However, this was not statistically significant (48.1% vs. 33.3%, $P=0.101$). The discrepant data revealed a significantly lower value of antigenemia (mean \pm SD, cells/200,000 leukocytes; 8.1 ± 17.6 vs. 23.0 ± 111.4 , $P=0.007$) and CMV DNA load (mean \pm SD, copies/mL; $5,690 \pm 18,453$ vs. $35,539 \pm 267,087$, $P=0.018$) than those was observed with the concordant data. The presence of neutropenia at the sampling time did not affect the concordance of the two methods.

Correlation between pp65 antigenemia and quantitative real-time CMV PCR

A statistically significant correlation was observed between the CMV DNA load by RQ-PCR and the number of pp65 antigen-positive cells ($r=0.569$, $P<0.0001$, Fig. 1). Adjust-

Table 2. Comparison of the qualitative results of CMV real-time PCR and pp65 antigenemia

Antigenemia	RQ-PCR for CMV DNA		No. of samples
	Positive	Negative	
Positive	66	77	143
Negative	18	394	412
Total no. of samples tested	84	471	555

Kappa=0.483 ($P<0.001$)

CMV, cytomegalovirus; RQ-PCR, real-time quantitative polymerase chain reaction.

ed analysis with possible confounding factors, such as the presence of neutropenia at the sampling time, also evidenced correlation ($r=0.590$, $P<0.0001$). Subgroup analysis revealed no correlation between the two methods with the specimens collected during anti-CMV therapy. However, the samples which were naïve to the anti-CMV agents were significantly correlated ($r=0.743$, $P<0.0001$).

Upon longitudinal analysis for the individual patients, the follow-up curves of the CMV DNA load by RQ-PCR and pp65 antigenemia were uniform and no discrepancies were noted between the two methods in response to anti-CMV therapy (Fig. 2). The duration of follow up of enrolled patients was a median of 1,126 days with a range of 22-3,099 days. There were 2 confirmed cases of CMV diseases (Fig. 3). Patient A was a 7-yr old girl who had undergone cord blood transplantation and experienced acute GVHD (grade IV). CMV infection was detected by both methods at the 20th day after transplantation and preemptive ganciclovir (GCV) therapy was instituted and both viral copies and antigenemia were decreased to zero. After the cessation of GCV, the viral DNA loads increased earlier than antigenemia and both increased continuously despite the GCV therapy. GCV was switched to foscarnet (FOS) but the patient developed CMV pneumonia and died as a result of it. Patient B was a 57-yr old woman who underwent peripheral blood stem cell transplantation from a matched sibling donor. She received alemtuzumab as a conditioning regimen. CMV infection was detected by both RQ-PCR and pp65 antigenemia on the 21st day after transplantation. Viral load and the number of pp65 antigen-positive cells were somewhat discrepant during the early period of antiviral therapy. She received GCV and FOS with CMV immunoglobulin alone or in combination, guided by pp65

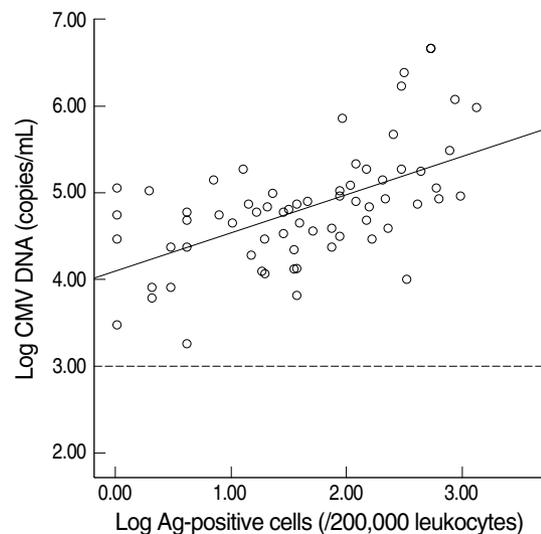


Fig.1. Correlation between the pp65 antigenemia and CMV RQ-PCR in whole blood sample (Pearson correlation coefficient=0.569).

CMV, cytomegalovirus; RQ-PCR, real-time quantitative polymerase chain reaction.

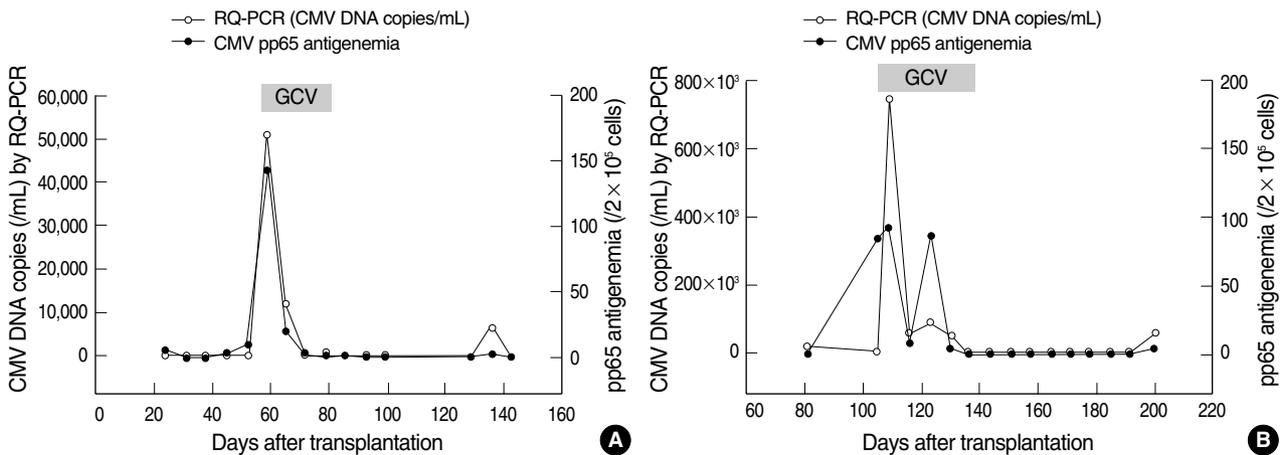


Fig. 2. Representative serial changes in CMV DNA load by real-time PCR and antigenemia in two patients (A, B) who received antiviral therapy. CMV, cytomegalovirus; GCV, ganciclovir; RQ-PCR, real-time quantitative polymerase chain reaction.

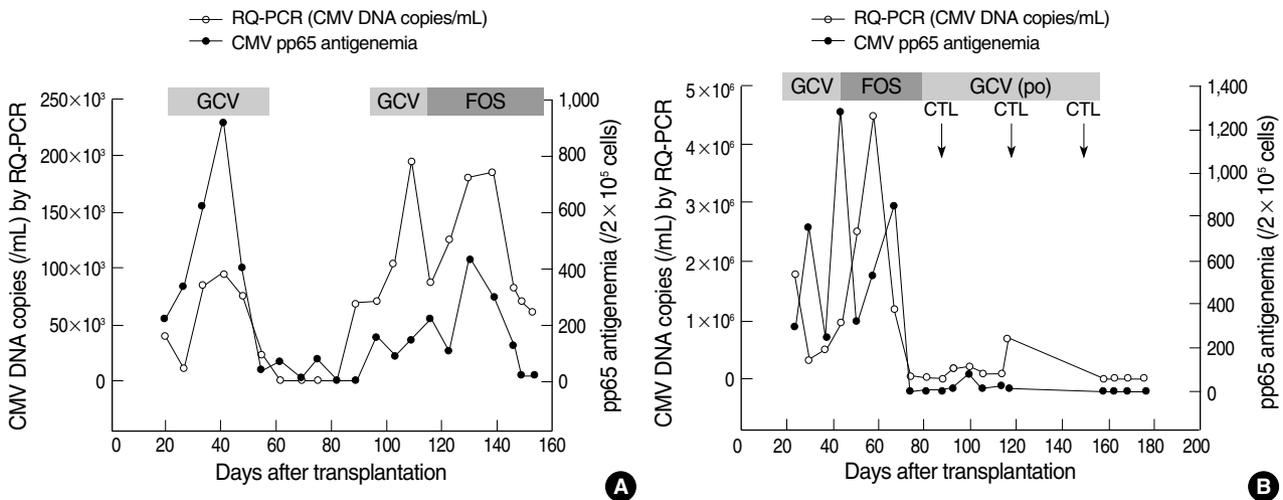


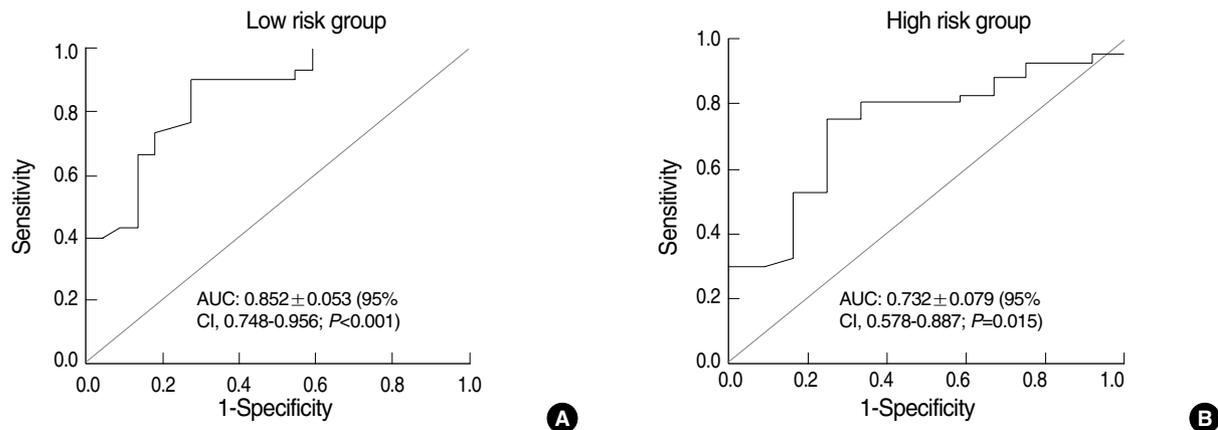
Fig. 3. Longitudinal trend of antigenemia values and CMV DNA load in two patients (A, B) who developed CMV diseases. The details are provided in the text. CMV, cytomegalovirus; CTL, cytotoxic T lymphocyte; FOS, foscarnet; GCV, ganciclovir; RQ-PCR, real-time quantitative polymerase chain reaction.

antigenemia. However, CMV retinitis and pneumonia developed. After 2 months of intravenous antiviral therapy, she was treated with maintenance oral GCV and CMV specific cytotoxic T lymphocyte (CTL) immunotherapy once per month. She survived, and the viral load was reduced below detectable levels by both methods.

Determination of CMV DNA cutoff values

Samples naïve to the anti-CMV agents were included only for ROC curve analysis. At present, the cutoff value of pp65 antigenemia for the beginning of preemptive GCV therapy was more than 5 antigen-positive cells in the low risk patients, and more than 1 antigen-positive cell in the high risk patients (1). If an antigenemia value of ≥ 5 was used as a standard, the optimal inflection point was located between

a CMV DNA load of 29,308 copies/mL (sensitivity, 90.0%; specificity, 68.2%) and 36,593 copies/mL (sensitivity, 90.0%; specificity, 72.7%), as shown in Fig. 4A. If more than 1 antigen-positive cell was utilized as a standard, the trade-off inflection point for CMV DNA load was located between 19,226 copies/mL (sensitivity, 80.0%; specificity, 41.7%) and 21,419 copies/mL (sensitivity, 80.0%; specificity, 58.3%) (Fig. 4B). Thus, tentative CMV DNA cutoff values were suggested approximately 3×10^4 copies/mL (sensitivity, 90.0%; specificity, 70.0%) in the low risk patients and approximately 2×10^4 copies/mL (sensitivity, 80.0%; specificity, 50.0%) in the high risk patients. A cutoff level of 3×10^4 CMV DNA copies/mL showed a positive predictive value of 79.4% and a negative predictive value of 96.5% in the low risk patients. A cutoff level of 2×10^4 CMV DNA copies/mL revealed a positive predictive value of 86.5% and a negative predictive



CMV DNA (copies/mL)	Sensitivity	Specificity
11,386	1.000	0.409
13,791	0.933	0.409
19,226	0.900	0.455
19,597	0.900	0.500
21,419	0.900	0.545
23,345	0.900	0.591
29,308	0.900	0.682
36,593	0.900	0.727
38,797	0.867	0.727

CMV DNA (copies/mL)	Sensitivity	Specificity
4,218	0.950	0.083
6,896	0.925	0.083
8,061	0.925	0.250
8,623	0.900	0.250
11,386	0.875	0.333
16,542	0.825	0.417
19,226	0.800	0.417
21,419	0.800	0.583
23,345	0.800	0.667

Fig. 4. (A) Receiver-operator characteristic (ROC) curve and table of statistics for some selected thresholds for pp65 antigenemia cutoff value (≥ 5) for the lower risk patients as a standard. (B) ROC curve and table of statistics for some selected thresholds for pp65 antigenemia cutoff value (≥ 1) for the high risk patients as a standard. AUC, area under the ROC curve. CMV, cytomegalovirus; CI, confidence interval.

value of 87.9% in the high risk patients.

DISCUSSION

Quantitative detection of CMV DNA using real-time PCR has become an increasingly popular method for the monitoring of CMV reactivation and for the initiation of antiviral therapy in allogeneic stem cell transplant recipients (4-11, 13-19). It has been suggested that RQ-PCR has several advantages over the antigenemia assay, including an increased sensitivity for the detection of CMV reactivation, the reliable detection of CMV reactivation during severe neutropenia in the early post-transplant period, the shorter time required for the procedure, and the convenient processing of large numbers of specimens (2, 3). For all of these reasons, we attempted to replace the antigenemia assay with RQ-PCR for the monitoring of CMV reactivation.

However, the replacement of the antigenemia assay with RQ-PCR is not a simple matter. The first task was the determination of the optimal specimen for RQ-PCR. There have been several reports addressing the proper specimen for RQ-PCR (3, 9, 15, 16, 20). Most of these studies have utilized plasma, whole blood, and leukocytes, and reported that these specimens correlated well with one another. Although CMV

is closely associated with leukocytes, CMV DNA can also be detected in the plasma while the virus is undergoing active viral replication. However, inconsistencies in the storage procedures for blood samples and the separation of plasma from whole blood can result in artificially increased CMV viral loads secondary to cell lysis. Inhomogeneity of leukocyte lysates can lead to variability and inaccuracies in results. Additionally, blood is simpler to process, as it requires one less centrifugation step than does plasma preparation. Due to its higher sensitivity and higher CMV DNA yield, whole blood has been accepted more recently as an optimal sample for CMV DNA quantification (3, 15, 16, 18, 20). Thus, we elected to test whole blood rather than plasma or leukocyte lysates for CMV viral load detection.

The next step was the establishment of precise relationship between the two methods. Most of all, we observed a qualitative and quantitative correlation between the pp65 antigenemia value and the viral load estimated by RQ-PCR. Additionally, in the longitudinal analysis of individual patients, the follow-up curves of RQ-PCR and pp65 antigenemia were relatively uniform and well correlated in response to antiviral therapy. The majority of the discordant samples were either in patients receiving anti-CMV therapy, in whom the DNA versus protein levels appeared to decline at different rates, or in the patients with very low viral burdens, in whom we sus-

pect that sampling error or variable lower limits of detection may have contributed to the discrepancy. There have been some reports asserting that increasing or persistently high levels of antigenemia during treatment with GCV were associated with decreasing or persistently low levels of DNA load and viremia, thus revealing an unexplained dissociation (21, 22). Additionally, the pp65 antigen tends to disappear at a later time than CMV DNA after anti-CMV treatment. Thus, the determination of cutoff values for preemptive treatment was conducted only with the results of samples naïve to the anti-CMV agents. Unusually, sensitivity of the antigenemia assay was found to be higher than that of RQ-PCR for the detection of CMV reactivation in this study. We thought that anti-CMV therapy might have affected these results. About half of 77 specimens determined to be positive on antigenemia assay but negative on RQ-PCR, were under anti-CMV therapy. Additionally, false positivity of antigenemia caused by cytoplasmic background staining might also affect the results (23, 24). On the other hand, there was a report that purer nuclear acid extracts and a better depletion of PCR inhibitors by the automated nucleic acid isolation could additionally improve PCR performance and increase the sensitivity of RQ-PCR (13, 15). Thus, we began to use automated nucleic acid isolation after this study. As our study was conducted during a limited designated period, specimens were not fully collected from the initial reactivation of CMV after engraftment in every patient. In some patients, only samples from the middle of antiviral therapy or after the discontinuation of anti-CMV drugs were included. We surmised that the characteristics of this uneven sample also influenced the results. And because of this reason, we could not compare the point that which method could detect reactivation of CMV earlier than the other. We could analyze the data of 18 selected patients, whose serial samples were collected from the initial increase of CMV viral load to the termination of anti-CMV therapy. Subgroup analysis revealed that the first detection time of reactivation did not differ between the two methods and also the cutoff value did not differ from that of the overall group (data not shown). As the kinetics of the CMV DNA viral load differ from that of antigenemia, we think that the consistency in serial values between the two methods is more important than the concordance at a point.

Although RQ-PCR assays have become quite popular, guidelines for antiviral therapy based on these methods have yet to be established. Like the antigenemia assay, RQ-PCR assays also lack standardization. Different institutions have developed their own assays and local guidelines, and these vary depending on the type of specimen and the assay design. In our HSCT center, pp65 antigenemia guided preemptive therapy has been conducted in a risk adapted manner (1). Patients were classified into low and high risk groups in accordance with HSCT type and the grade of acute GVHD. High risk patients were defined as those who had unrelated donors and any grade of acute GVHD, or who had matched related

donors and grade II to IV acute GVHD. The patients with matched related donors and grade 0 to I acute GVHD were classified as low risk patients. GCV treatment was initiated when 5 or more CMV antigen-positive cells were detected in the low risk patients and when any antigen-positive cells were detected in the high risk patients. Thus, considering an antigenemia value of more than 5 antigen-positive cells or more than 1 antigen-positive cell as a standard, respectively, the optimal cutoff value was provided in Fig. 4. We suggested that the tentative cutoff value was 20,000 copies/mL for the high risk patients and 30,000 copies/mL for the low risk group. Usually, RQ-PCR was introduced in order to allow for the more sensitive, rapid, and accurate diagnosis of CMV reactivation in HSCT recipients, which allowed for preemptive therapy to be administered as early as possible. However, in this study, RQ-PCR showed lower sensitivity than antigenemia. So, we defined present cutoff value as the "tentative" one, because of the reasons described above. At the same time, we were concerned that these cutoff values were somewhat higher than those suggested previously (9, 17). Lilleri *et al.* suggested a cutoff value of 10,000 copies/mL for the initiation of preemptive therapy in the HSCT recipients, and that group conducted randomized clinical trials comparing RQ-PCR and antigenemia guided preemptive therapy (17, 25, 26). However, they did not conduct preemptive therapy in a risk adapted fashion. Additionally, Kalpoe *et al.* also suggested that preemptive therapy could be initiated at CMV DNA load of 10,000 copies/mL or 1,000 copies/mL, and then increased by 10-fold per week at the first episode of CMV reactivation (9). We believe that, in addition to the viral DNA load, the kinetics of the DNA load and the pattern of changes in the viral load are crucial and should be taken into consideration when making treatment decisions. Further study to correct present cutoff value and to validate the optimal cutoff value for the initiation of preemptive therapy is currently underway at our HSCT center. On the other hand, we thought that high negative predictive value (87.9% in the high risk group and 96.5% in the low risk group) could allow us to avoid unnecessary preemptive therapy with a fair aliquot of patients. Additionally, we suggested that the duration of preemptive therapy might be shortened by RQ-PCR guided manner, because more rapid negative conversion of viral reactivation was detected using RQ-PCR as previously reported (27).

In summary, we observed a correlation between the pp65 antigenemia assay and the viral load estimated by RQ-PCR in this study. Additionally, we realized that anti-CMV therapy might influence the results of the two assays. Based on a comparison with the established antigenemia assay, the tentative threshold at which preemptive anti-CMV therapy initiated was determined. The quantification of CMV DNA load from whole blood using RQ-PCR appears to be applicable to the clinical practice. An optimal cutoff value for guiding timely preemptive therapy should be clinically validated in

future studies.

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