

Monitoring of Cytomegalovirus Infection in Solid-Organ Transplant Recipients by an Ultrasensitive Plasma PCR Assay

Karine Hadaya,¹ Werner Wunderli,¹ Christelle Deffernez,¹ Pierre-Yves Martin,² Gilles Mentha,³ Isabelle Binet,² Luc Perrin,¹ and Laurent Kaiser^{1*}

Central Laboratory of Virology, Division of Infectious Diseases,¹ Division of Nephrology,² and Transplantation Unit, Department of Surgery,³ University Hospitals of Geneva, 1211 Geneva 14, Switzerland

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Early and accurate monitoring of cytomegalovirus (CMV) infection in solid-organ transplant recipients is of major importance. We assessed the potential benefit of an ultrasensitive plasma-based PCR assay for renal transplant recipients. The pp65 CMV antigen (pp65 Ag) assay using leukocytes was employed as a routine test for the monitoring of CMV in 23 transplant recipients. We compared the pp65 antigenemia with the CMV load quantified by an ultrasensitive PCR (US-PCR) with a limit of detection of 20 CMV DNA copies/ml of plasma. CMV infection was detected in 215 (67%) of 321 plasma samples by the US-PCR compared with 124 (39%) of 321 samples by the pp65 Ag assay. The US-PCR assay permitted the detection of CMV infection episodes following transplantation a median of 12 days earlier than the pp65 Ag assay. Moreover, during CMV infection episodes, DNA detection by the US-PCR was consistently positive, whereas false negative results were frequently observed with the pp65 Ag assay. We found a good correlation between the two assays, and the peak viral loads were significantly higher in patients with CMV-related complications (median, 5,000 DNA copies/ml) than in those without symptoms (1,160 DNA copies/ml) ($P = 0.048$). In addition, patients that did not require preemptive therapy based on the results of the pp65 assay had CMV loads significantly lower (median, 36 DNA copies/ml) than those that needed treatment (median, 4,703 DNA copies/ml) ($P < 0.001$). These observations provided cutoff levels that could be applied in clinical practice. The ultrasensitive plasma-based PCR detected CMV infection episodes earlier and provided more consistent results than the pp65 Ag assay. This test could improve the monitoring of CMV infection or reactivation in renal transplant recipients.

Cytomegalovirus (CMV) remains a leading cause of complication in solid-organ transplant recipients (SOTR) and has a significant impact on transplant outcome (3, 25, 26, 28). CMV disease is associated with high viral loads and is effectively prevented by a prophylactic antiviral strategy (7, 20, 29). Low CMV replication levels may also have deleterious effects on the long-term allograft function and on the incidence of opportunistic infections (11). The availability of a sensitive test capable of detecting CMV replication at an early stage is thus of importance. The pp65 antigen (pp65 Ag) assay is a reference test for the monitoring of CMV viremia and has been validated in clinical trials with immunosuppressed patients (10, 18). However, this assay has several limitations, including its dependence on the leukocyte count and its inability to quantify low viral loads. PCR assays have been developed to improve the detection and the monitoring of CMV infection. Qualitative PCR assays have not provided insight into the dynamics of viral replication and are of limited utility for the prevention of clinical complications (2, 15). In contrast, quantitative PCR assays, whether performed with whole blood, leukocytes, or plasma, are highly sensitive for CMV load determination and are effective tools for establishing the suitability of the initiation of preemptive therapy (14, 22, 24). However, most of these assays lack standardization and have not been validated with large cohorts of transplant patients (32). Recently, a standard-

ized and commercially available assay, the Cobas Amplicor CMV Monitor test, has been developed for plasma DNA quantification (4, 5, 21). However, previous studies have shown a limited sensitivity of PCR performed with plasma compared to that of assays performed with whole blood or with leukocytes (12, 23, 34). For this reason, we have improved the sensitivity of the Amplicor CMV Monitor test by lowering its detection limit from 400 to 20 DNA copies/ml (17). This ultrasensitive PCR (US-PCR) has been validated by a previous study performed with hematopoietic stem cell transplant recipients (17).

The aim of the present study was to evaluate the US-PCR as an alternative to the routinely used pp65 Ag assay for the monitoring of CMV infection in SOTR. Both methods were compared for detection sensitivity and the dynamics of CMV load on prospectively collected blood samples.

MATERIALS AND METHODS

Patients. In our institution we routinely monitor SOTR for CMV infection on a weekly basis for a 3-month period after transplantation. Follow-up is extended for high-risk patients and those in which antiviral therapy has been initiated. Twenty-three unselected consecutive SOTR were enrolled in the study (19 kidney recipients, 2 pancreas and kidney recipients, 1 islet and kidney recipient, and 1 liver recipient) irrespective of the time of transplantation. Mean age was 52 years (range, 33 to 73 years), and 14 were male. All patients were either CMV immunoglobulin G (IgG) positive before transplantation (R+; $n = 15$) or recipients of an organ from a CMV IgG-positive donor (D+; $n = 8$). Eleven patients were D+R+, eight were D+ but CMV IgG negative before transplantation (R-), and four were R+ but received an organ from a CMV IgG-negative donor (D-). Induction therapy consisted of anti-CD25 antibodies, and five patients received rabbit antithymocyte globulin for delayed graft function. Baseline immunosuppression was achieved by using either cyclosporine A or tacroli-

* Corresponding author. Mailing address: Central Laboratory of Virology, Division of Infectious Diseases, University Hospitals of Geneva, 24 Rue Micheli-du-Crest, 1211 Geneva 14, Switzerland. Phone: 41 22 37 24 994. Fax: 41 22 37 24 990. E-mail: laurent.kaiser@hcuge.ch.

TABLE 1. Comparison of results of the US-PCR and the pp65 Ag assay for detection of CMV in 321 blood samples

Result of pp65 Ag assay (no. of samples)	No. found by US-PCR to be:	
	Positive (<i>n</i> = 215)	Negative (<i>n</i> = 106)
Positive (124)	124	0
Negative (197)	91	106

mus in combination with mycophenolate mofetil and steroids in 21 of 23 patients. During the follow-up period, at a median of 142 days posttransplantation (range, 60 to 312 days), seven patients (30%) presented with a total of eight episodes of acute rejection, which were treated with methylprednisolone (*n* = 7) combined with rabbit antithymocyte globulin (*n* = 4). Graft success and patient survival rates were 100% during the follow-up period.

An isolated positive pp65 Ag assay result was interpreted as an indication of asymptomatic CMV infection. A positive pp65 Ag assay result associated with clinical symptoms (fever, muscle pain, and asthenia) and/or biological abnormalities (leukopenia, thrombocytopenia, and abnormal liver function tests) was interpreted as an indication of CMV syndrome. Diagnosis of CMV disease required a positive pp65 Ag assay result and proven organ involvement.

Clinical samples. Three hundred twenty-one whole blood samples were collected from 23 SOTR. Patients were enrolled irrespective of the time of transplantation. Thus, collection of serial blood samples started within the first month posttransplantation for 14 patients and between the second and seventh month posttransplantation for the remaining 9 patients. Semiquantification of CMV viremia levels by the pp65 Ag assay was performed immediately, whereas quantification of DNA levels in plasma was performed later with the same frozen samples. The results of the pp65 Ag assay and the PCR for each individual blood sample were subsequently compared. The median number of samples analyzed per patient was 14 (range, 6 to 27).

Antiviral therapy. A preemptive antiviral therapy approach based on the results of the pp65 Ag assay was the routinely used strategy for study patients, including those who were D+R- and those receiving high doses of methylprednisolone or rabbit antithymocyte globulins. However, three of eight recipients from the D+R- group received primary prophylaxis. Ganciclovir therapy was initiated and terminated as deemed necessary by the physicians in charge.

pp65 Ag and PCR assays. Five milliliters of whole blood was processed for extraction of peripheral blood leukocytes within 4 h by using a standardized procedure (13, 30, 31). Briefly, after leukocyte isolation by dextran sedimentation, 2.5×10^5 leukocytes were cytocentrifuged onto glass slides and then fixed and permeabilized to allow for the detection of the pp65 Ag by monoclonal antibodies (CINAKit; Argène, Biosoft, Varilhes, France). The mean number of antigen-positive cells was determined by counting cells on two slides, and the result was expressed as the number of pp65-positive cells per 2.5×10^5 leukocytes (range, 1 to 300 positive cells).

Two milliliters of the same whole blood samples were processed for extraction of plasma and stored at -70°C for subsequent PCR analysis. The PCR assay was performed by using the Cobas Amplicor CMV Monitor test platform (Roche Diagnostic Systems Inc., Branchburg, N.J.). Kits for the PCR assays were kindly provided by Roche Molecular Systems, Inc. In this procedure, plasma DNA is extracted and the amplified product is detected by hybridization to a labeled oligonucleotide probe which comprises a sequence of 365 nucleotides located in the amino terminus of the CMV DNA polymerase gene. A CMV quantitation standard amplicon is added to each specimen, and the final PCR product is measured by a colorimetric reaction with a lower detection limit of 400 copies/ml of plasma. We recently modified this PCR assay to increase its sensitivity (17). Briefly, we increased the plasma input (600 μl) and added a centrifugation step ($50,000 \times g$ for 80 min at 4°C) using a refrigerated centrifuge (Biofuge 28 RS; Heraeus AG, Osterode, Germany). The supernatant was discarded, and the pellet was suspended in 600 μl of lysis buffer containing a proportion of quan-

titation standard similar to that introduced in the unmodified PCR assay. After precipitation by ethanol, the pellet was suspended and used for the PCR. Further steps were performed according to the manufacturer's recommendations, and determination of the number of copies per milliliter was adjusted for plasma input. These modifications allowed the limit of detection to be lowered from 400 to 20 DNA copies/ml and have been extensively validated in previous experiments with healthy blood donors and bone marrow transplant recipients (17). Although the US-PCR assay can detect less than 20 copies/ml, it has been formally validated to detect 20 copies or more/ml. In the present study, we have also reported the few positive results below the validated limit of detection in order to show that the assay provides consistent results when low levels of viremia are detected.

Statistical analysis. Comparisons between independent variables were assessed by the Mann-Whitney U test. The Wilcoxon signed rank test was applied for comparison of the different subgroups, and linear regression was used to compare the two assays. The difference was considered significant when *P* was <0.05 . Statistical analysis was performed with SPSS 9.0 (SPSS Inc., Chicago, Ill.) and with Epi Info 5.01b (Centers for Disease Control, Atlanta, Ga.).

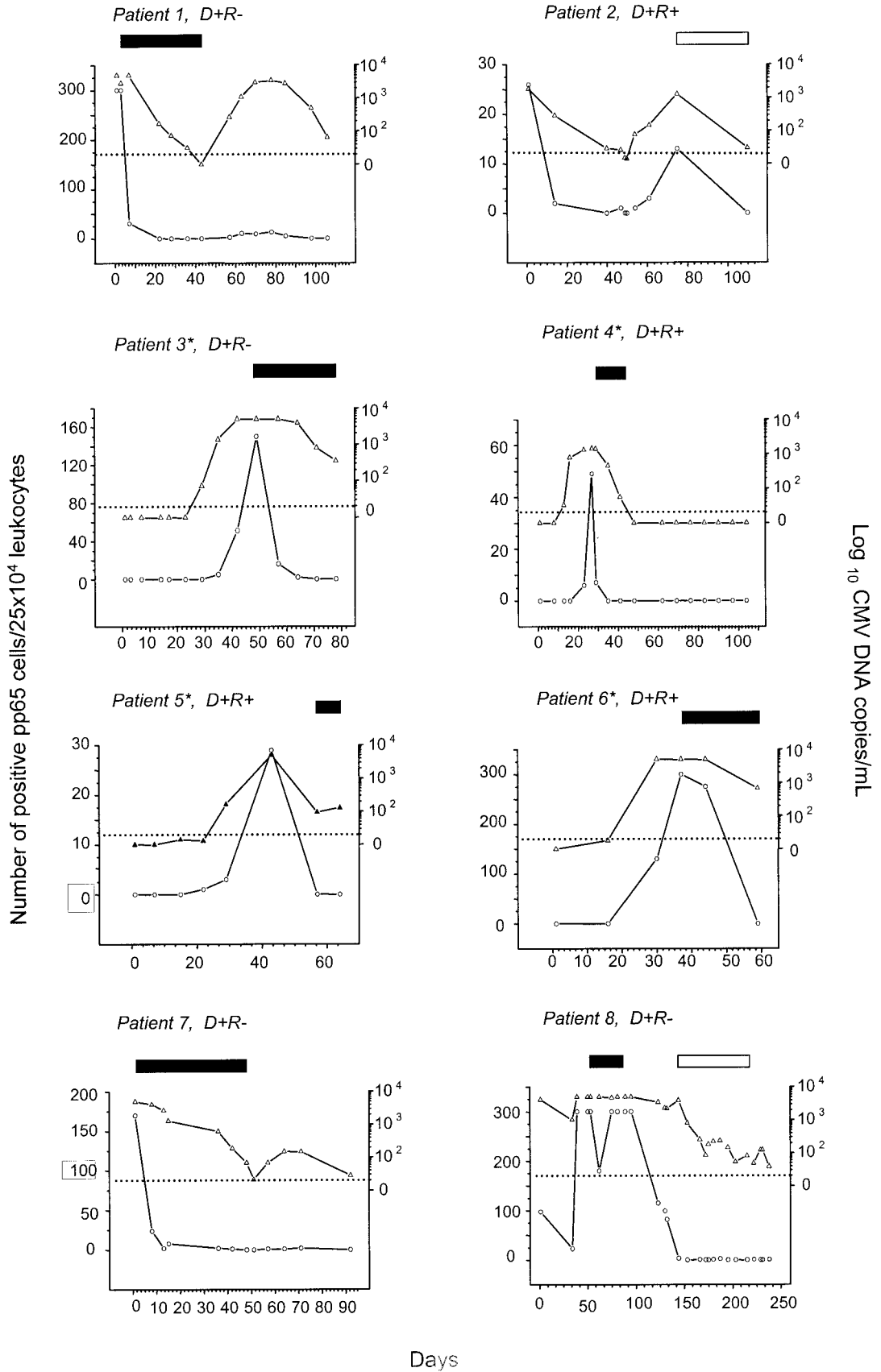
RESULTS

Assay correlation. A total of 321 blood samples were collected from 23 SOTR. The pp65 Ag assay and the US-PCR detected CMV loads in 124 (39%) and 215 (67%) of 321 samples, respectively (*P* < 0.001). All pp65 antigenemia-positive samples were also positive by the US-PCR. However, 91 (42%) of the samples positive by the US-PCR were negative by the pp65 Ag assay (Table 1). Of these 91 samples, 11 gave positive results by the US-PCR a median of 12 days (range, 6 to 21 days) before positive results were obtained with the pp65 Ag assay; 47 remained positive after results of the pp65 Ag assay were negative for a median of 16 days (range, 7 to 42 days); 33 were consistently positive by the US-PCR during episodes of CMV viremia, whereas results of the pp65 Ag assay were intermittently negative (Fig. 1). The samples positive by the US-PCR and negative by the pp65 Ag assay were associated with low levels of CMV DNA in plasma, i.e., a mean of 87 copies/ml (range, 5 to 1,655 copies/ml).

Monitoring of CMV infection and disease. CMV DNA was detected at least once during the follow-up period in all patients. Concomitantly or subsequently, all patients were also positive by the pp65 Ag assay. Among patients monitored routinely from the first month of transplantation (Fig. 1), CMV DNA in plasma was detected by US-PCR before the pp65 Ag assay in 8 of 14 cases. In these patients, the first positive results were observed a median of 32 days (range, 18 to 137 days) after transplantation with the US-PCR compared with 43 days (range, 25 to 150 days) after transplantation with the pp65 Ag assay (*P* < 0.01). In 6 of 14 cases, both assays were positive simultaneously (Fig. 1).

All 8 D+R- patients developed primary infections, and all 15 D+R+ and D-R+ patients developed reactivations. Asymptomatic infection, CMV syndrome, and CMV disease were diagnosed in 18 (78%), 4 (17%), and 1 (5%) patient, respectively. CMV disease occurred in a D+R- patient (pa-

FIG. 1. Results of monitoring of CMV loads in individual patients by using the pp65 assay and the US-PCR. Numbers of days after the collection of the first blood samples processed for detection of pp65 antigenemia and CMV DNA-emia are indicated along the abscissas. The left y axes report the numbers of pp65 antigen-positive cells per 2.5×10^5 leukocytes (circles). The right y axes report the numbers of CMV DNA copies expressed per milliliter of plasma (triangles). The dotted lines represent the validated limit of quantification by the US-PCR, which is 20 CMV DNA copies/ml. The patients monitored from the first month after transplantation are those identified with an asterisk. The horizontal black bars represent the duration of ganciclovir treatment, and the white bars represent the duration of valacyclovir treatment.



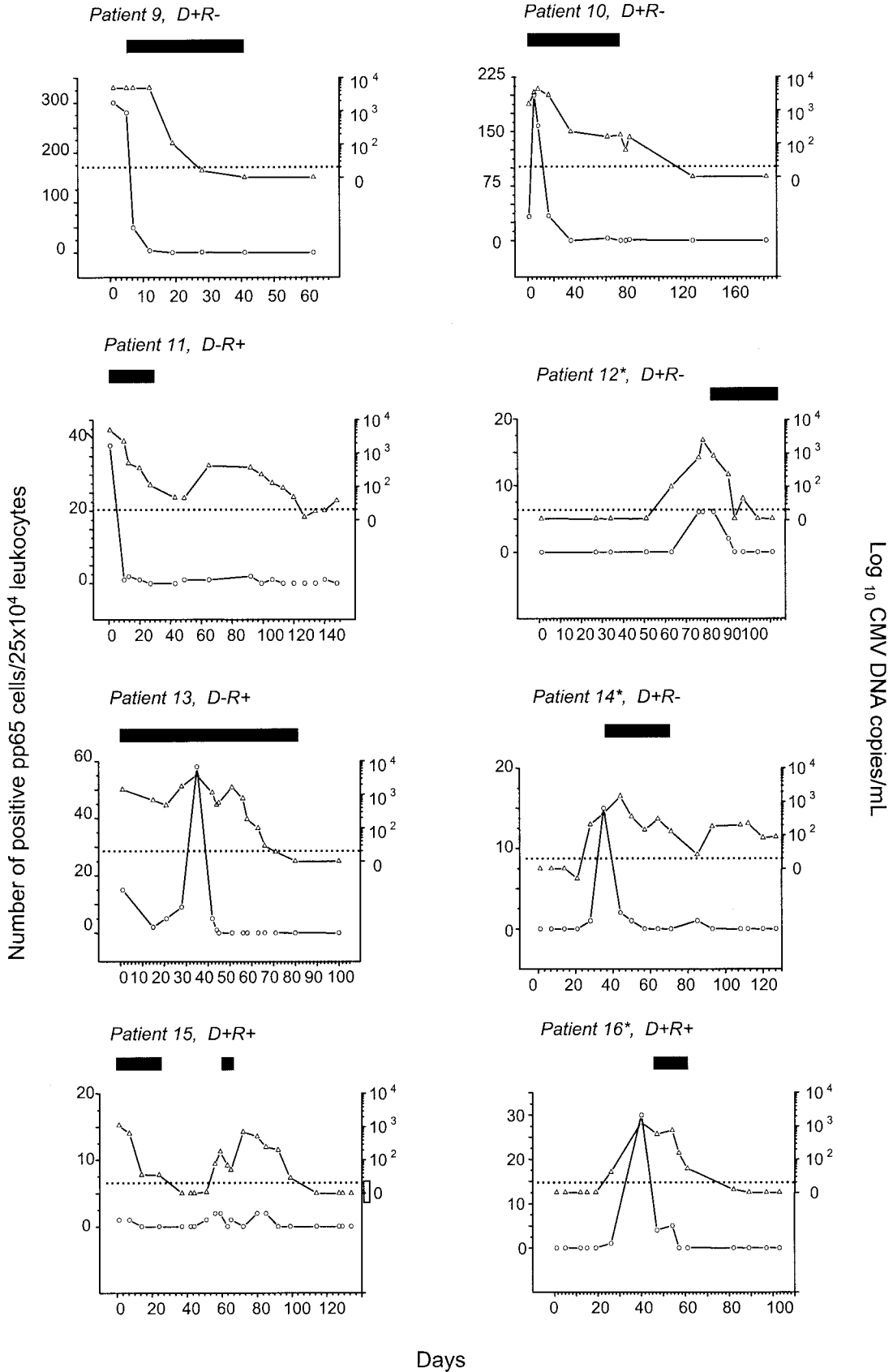


FIG. 1—Continued.

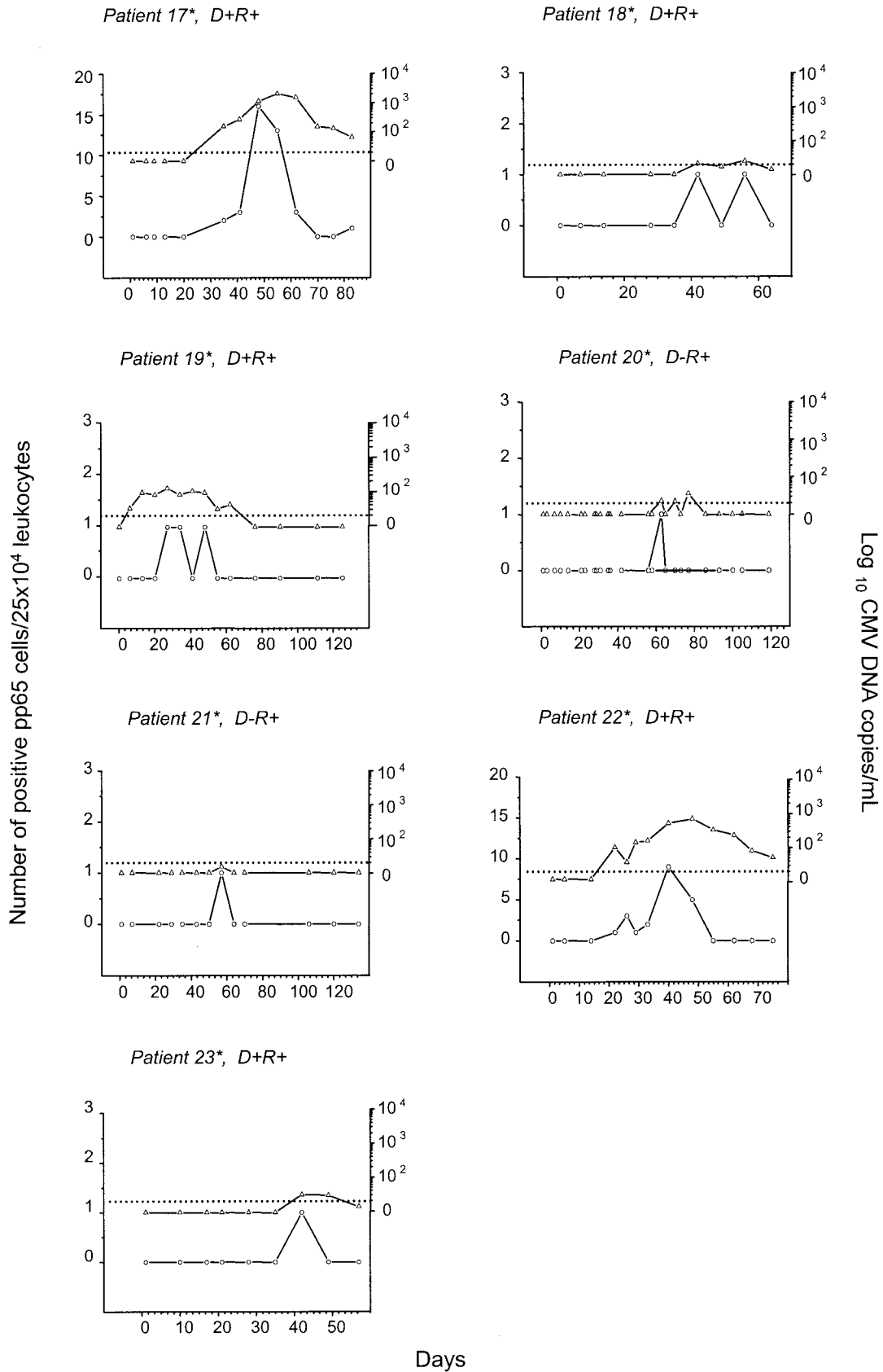


FIG. 1—Continued.

TABLE 2. Median peak CMV loads in asymptomatic and symptomatic patients as measured by the US-PCR and the pp65 Ag assay

Diagnosis	Median peak no. of DNA copies/ml (range)	Median peak no. of antigen-positive cells/ 2.5×10^5 leukocytes (range)
Asymptomatic CMV infection	1,660 ^a (15–5,000)	16 ^b (1–300)
CMV syndrome or disease	5,000 ^a (1,285–5,000)	170 ^b (30–300)

^a $P = 0.048$.

^b $P = 0.036$.

tient 7) at day 88, despite a primary prophylaxis of 20 days. Three D+R– patients (patients 1, 9, and 12) developed late-onset CMV infections at days 146, 222, and 150 posttransplantation. Two of them (patients 1 and 12) had received primary prophylaxis for 65 and 31 days, respectively. The median peak DNA level in asymptomatic SOTR was 1,660 copies/ml (range, 15 to 5,000 copies/ml) compared with 5,000 copies/ml (range, 1,285 to 5,000 copies/ml) in patients who presented with CMV syndrome or disease ($P = 0.048$), as shown in Table 2. The median peak viremia level in asymptomatic SOTR was 16 pp65-positive cells/ 2.5×10^5 leukocytes (range, 1 to 300 positive cells) compared with 170 pp65-positive cells/ 2.5×10^5 leukocytes (range, 30 to 300 positive cells) in patients who presented with CMV syndrome or disease ($P = 0.036$).

Sixteen patients (70%) received ganciclovir therapy based on the results of the pp65 Ag assay. Ganciclovir was administered as a preemptive therapy in 11 of these patients and as a treatment for CMV syndrome or disease in 5. The reasons for the administration of deferred therapy were as follows: one patient was lost from follow-up (patient 7) and reappeared later with CMV disease, two patients developed late-onset CMV syndrome during a period of infrequent outpatient visits (patients 1 and 9), and the remaining two (patients 11 and 16) developed clinical symptoms during the period of observation after the first pp65 Ag assay results. The median peak viral load assessed by the US-PCR was significantly higher in the 16 SOTR who received antiviral therapy than in the 7 patients who did not: 4,703 copies/ml (range, 1,140 to 5,000 copies/ml) versus 36 copies/ml (range, 15 to 2,050 copies/ml) ($P < 0.001$). Nine (56%) of the treated patients had persistent detectable CMV DNA (median, 149 copies/ml; range, 12 to 5,000 copies/ml) for an average of 53 days (range, 8 to 154 days) after termination of ganciclovir treatment.

DISCUSSION

This study assessed whether a quantitative and ultrasensitive plasma-based PCR assay with a detection limit of 20 CMV DNA copies/ml could improve the monitoring of posttrans-

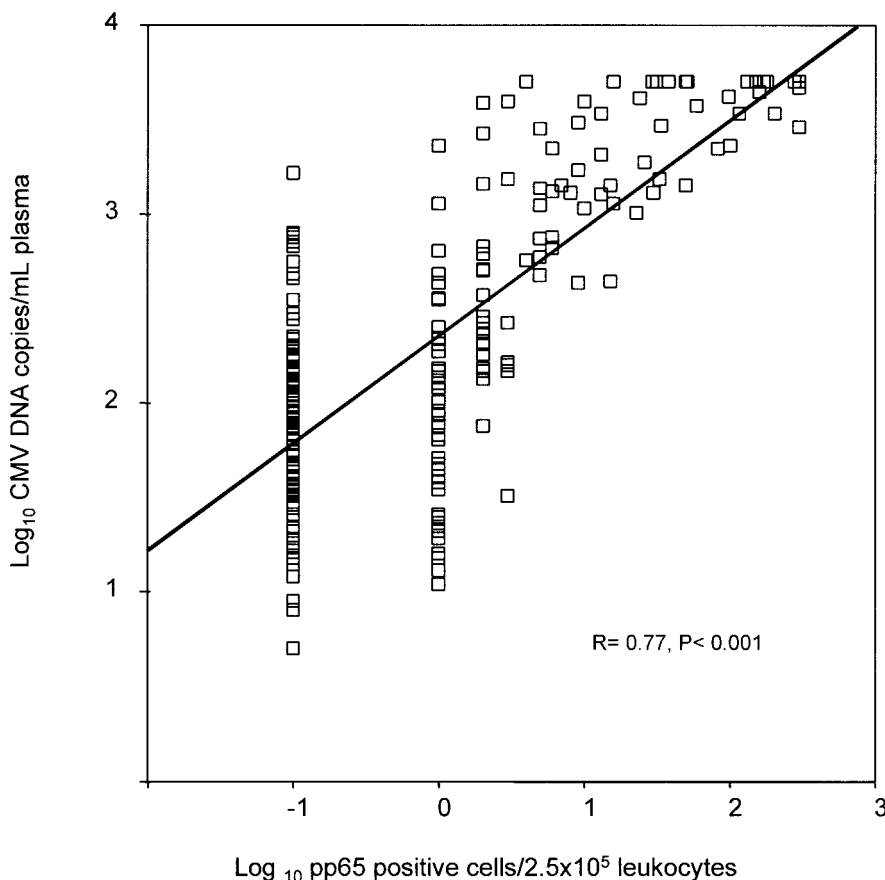


FIG. 2. Correlation between the CMV loads measured by the US-PCR (DNA-emia) and the pp65 Ag assay (antigenemia) in all samples except those negative by both assays.

plant CMV infection. Results demonstrated an increased sensitivity of the US-PCR for the detection of CMV load compared with that of the pp65 Ag assay (67% versus 39% positive samples). This led to an earlier identification of CMV infection episodes in the majority of cases, and CMV DNA was detectable for a longer period of time than pp65 antigenemia in either treated or untreated patients. These results are consistent with those of a previous study with hematopoietic stem cell transplant recipients showing that the US-PCR was much more sensitive than the standard Cobas Amplicor Monitor test and the pp65 assay (17). The US-PCR also provided consistent positive results during each CMV infection episode, whereas the pp65 Ag assay gave frequent false negatives (Fig. 1). This supports the need for a test that can quantify low viral loads and accurately describe the dynamics of CMV load.

Ganciclovir was given to more than 70% of our patients based on the results of the pp65 Ag assay only. Since we observed an excellent correlation between the two assays (Fig. 2) and since episodes of significant DNA-emia without subsequent antigenemia were not observed (Fig. 1), it is unlikely that the remaining cases would have been treated based on the US-PCR results. In addition, the patients with CMV-related symptoms or disease had higher peak viral loads (median, 5,000 copies/ml) than those without symptoms (median, 1,660 copies/ml). Moreover, patients that were treated (based on the pp65 assay) had much higher viral loads (median, 4,703 copies/ml; >1,140 copies/ml in all patients) than those that did not require treatment (median, 36 copies/ml; <2,050 copies/ml in all patients). This suggests that in SOTR viral loads higher than 5,000 copies/ml are associated with a high risk of CMV disease and that viral loads below 2,000 copies/ml are unlikely to be associated with CMV-related complications. If used appropriately, these cutoff levels could prevent unnecessary ganciclovir treatment.

Treatment initiation is based not only on the viral load itself but also on a combination of different criteria, including the kinetics of the viral load and the patient's immune condition. Early detection of low viral loads in kidney recipients at high risk for complications (e.g., D+R- patients and those receiving anti-T-cell antibody or high doses of methylprednisolone) is certainly of interest (9) and may lead to a timely initiation of therapy. The ability to detect persistent low-level CMV replication after termination of antiviral therapy is also of interest. The viral load at the completion of antiviral therapy has been shown to predict the risk of relapsing (8, 16, 19, 27, 33), and an ultrasensitive assay may secure treatment termination for high-risk renal transplant subjects. But most importantly, a negative result from an ultrasensitive assay can rule out CMV replication and may prevent unnecessary treatment. Of note, new drugs such as oral valganciclovir with improved, but still limited, bioavailability compared with that of their intravenous formulations will be increasingly prescribed. The detection of low CMV loads during prophylactic or preemptive therapy may lead to treatment adjustment and prevent the risk of emergence of resistant strains (1, 6).

In conclusion, our observations showed that the implementation of an US-PCR assay that can reproducibly quantify low levels of CMV DNA in plasma would improve the monitoring of CMV infection or reactivation in SOTR. In particular, this assay could improve the suitability of the initiation and

termination of antiviral therapy during CMV infection. To our knowledge, this is the first validation of a plasma-based PCR assay's showing superiority over the pp65 assay for SOTR. Our observations suggest also that it is unlikely that the implementation of a test with higher sensitivity for the detection of low CMV loads will lead to unnecessary antiviral therapies.

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