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DETERMINANTS OF PROTRACTED CYTOMEGALOVIRUS INFECTION IN SOLID-ORGAN TRANSPLANT PATIENTS

THESE

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Facteurs prédictifs d'une infection à CMV prolongée chez les transplantés d'organes solides

MISE EN PERSPECTIVE : On observe fréquemment chez les transplantés d' organe solide (greffés) des infections récurrentes à CMV après un premier traitement. Notre étude consiste à décrire le cours d'une infection à CMV chez les greffés et d'identifier les facteurs prédictifs d'une infection prolongée avec des récidives.

METHODES : A l'aide d'une PCR quantitative, nous avons analysé rétrospectivement dans les leucocytes et dans le plasma la variation longitudinale de la charge virale en DNA de CMV chez les greffés donbt l'infection à CMV avait été prise en charge d'une manière thérapeutique ou préemptive, sur la base de cultures sanguines quantitatives.

RESULTATS: Parmi les 101 patients greffés (cœur, foie, rein), 63 ont présenté au moins un épisode infectieux à CMV, dont 32 ont développé des récidives. Chez les patients ayant présentés des infections récurrentes, l'analyse du taux de DNA de CMV dans le sang périphérique et le plasma a montré que la majorité (27) de ces patients avait une charge virale élevée pendant une période prolongée ($\geq$ 1 mois), malgré un traitement préemptif ou thérapeutique. Les facteurs prédictifs d'une infection prolongée se sont avérés être l'âge, la séropositivité pour CMV du donneur, et toutes les mesures de la charge virale pendant l'épisode infectieux initial. La séropositivité pour CMV du receveur protègeait fortement contre une infection prolongée. Le taux de DNA à CMV dans le plasma à la fin d'un traitement permettait au mieux de distinguer les patients développant ou pas une infection prolongée.

CONCLUSIONS : Chez les patients transplantés d'un organe solide, l'infection prolongée à CMV est associée à l'âge, la séropositivité chez le donneur, la séronégativité chez le receveur, et une charge virale élevée pendant le premier épisode infectieux. Elle représente une infection prolongée plutôt que des épisodes séparés de virémie.

Le taux de DNA de CMV dans le plasma à la fin du traitement est le meilleur facteur pronostic d'une infection prolongée. Chez les greffés avec un risque élevé de développer une infection prolongée, un traitement prophylactique s'avère particulièrement bénéfique et intéressant du point de vue du coût, puisqu'il évite des traitements multiples.

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DETERMINANTS OF PROTRACTED CYTOMEGALOVIRUS INFECTION IN SOLID-ORGAN TRANSPLANT PATIENTS[1]

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Background. **Recurrent infection frequently follows the response to the initial treatment of cytomegalovirus (CMV) infection in solid-organ transplant (SOT) recipients. The objective of this study was to describe the course of CMV infection in SOT patients and to identify factors that would predict protracted CMV infection with recurrences.**

Methods. **Quantitative polymerase chain reaction (PCR) assay for CMV DNA in leukocytes and in plasma were used to assess viral load changes retrospectively in consecutive SOT patients, whose CMV infection episodes had been attended therapeutically or preemptively using quantitative blood culture.**

Results. **Among 101 SOT patients, CMV infection occurred in 63, of whom 32 developed recurrent infection after the initial episode. In patients with recurrent infection, PCR indicated that a majority (27) of recipients had high level of CMV DNA in peripheral blood leukocytes and plasma throughout a protracted (≥1 month) period including after preemptive or therapeutic ganciclovir courses. Predictors of protracted high-level infection were increasing age, CMV donor seropositivity, and all measures of viral load during the initial episode. CMV recipient seropositivity protected strongly against protracted infection. End of treatment plasma CMV DNA best discriminated between patients who did or did not develop protracted infection.**

Conclusions. **In SOT patients, protracted CMV infection is associated with increasing age, donor seropositivity, recipient seronegativity, and high viral load during the first episode. End of therapy plasma CMV DNA level best predicts the occurrence of protracted infection. In patients with a high risk of protracted infection, prophylaxis is likely to be particularly cost effective.**

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Cytomegalovirus (CMV) remains a leading cause for infectious morbidity in solid-organ transplant (SOT) patients (*1–6*). Numerous approaches, using vaccines, immunoglobulins, acyclovir, and ganciclovir, have been proposed to limit the morbidity caused by CMV in SOT recipients (*6–12*). Currently, the optimal preventive approach for the prevention of CMV disease in the various solid-organ transplant recipient remains to be determined (*13, 14*). The efficacy of prophylactic oral ganciclovir has been demonstrated for liver (*9*), kidney (*15*), and heart (*16*) transplant patients, whereas valacyclovir prophylaxis seemed a safe and effective way to prevent CMV disease after renal transplantation (*17*). This approach seems to offer the added bonus of reducing the

incidence of acute rejection in high-risk, seropositive donor–seronegative recipient (D+/R−) patients (*17*, *18*). In contrast, the rationale of preemptive approaches has been to concentrate the use of intravenous ganciclovir in patients at highest risk of developing CMV disease. It has proved efficacious in liver (*12*), kidney (*19*), and lung and heart transplants (*20*). To the best of our knowledge, the prophylactic and preemptive approaches have not been compared in a prospective randomized study. The potential for CMV infection to recur after (or persist despite) ganciclovir treatment of CMV disease has been recognized in as many as 12% to 59% of the patients, depending on the type of organ transplant (*21*). Identified predictors of recurrence have been primary CMV infection (*22–24*), antirejection therapy and type of transplanted organ (probably reflecting higher levels of immunosuppression) (*25*, *26*), the severity of the initial episode (*22*), and more recently, a higher viral load during the initial episode (*23*, *24*). Recurrent CMV infection has also been described in 4/14 (29%) kidney-transplant patients treated preemptively with ganciclovir for 3 weeks (*19*), raising the question as to what are the predictors of recurrent infection after preemptive treatment and whether recurrent infection should be treated preemptively (*27*).

For several years, we have been managing SOT patients with CMV infection according to a preemptive ganciclovir approach using a quantitative shell vial assay for the detection of CMV viremia as a monitoring tool (*28*). Recently, we experienced an increased rate of recurrent CMV viremia episodes after preemptive treatment of CMV infection.

This article describes the incidence of protracted CMV infection with recurrences in a population of 101 consecutive SOT recipients, including using peripheral blood leukocyte and plasma quantitative PCR for CMV DNA. We demonstrate that recurrent infection indeed reflects a protracted CMV infection that is not resolved at the end of preemptive treatment. We also analyze potential risk factors that may help identify patients at risk for protracted infection after the initial infection episode.

MATERIAL AND METHODS

Patient Population

All adult patients who underwent a heart, liver, or kidney transplantation at the Centre Hospitalier Universitaire Vaudois between January 97 and December 98 were considered for inclusion in this retrospective study.

Transplant recipients who died perioperatively or did not have a minimal follow-up of at least 3 months for reasons other than death, namely loss of follow-up or primary graft failure with discontinuation of immunosuppressive drugs, were excluded from this study.

Immunosuppressive Regimens

Recipients of renal transplants received various induction regimens. Five patients did not receive any antilymphocyte antibody induction. Fifty-two patients received antilymphocyte antibody induction (ATGAM, Pharmacia & Upjohn, Peapack, NJ, n=22; ATG-Fresenius, Fresenius Medical Care, Homburg, Germany, n=17; Orthoclone OKT3, Janssen-Cilag (Baar, Switzerland), n=2; Thymoglobuline, Imtix-Sangstat, (Lyon, France), n=5; or Simulect, Novartis Pharma, Basel, Switzerland, n=6). Induction included intravenously (IV) administered methylprednisolone, 500 mg, on the first day, which was tapered gradually within the first 4 days postoperatively. Since 1997, cyclosporine (CsA, Sandimmune, Novartis, Basel, Switzerland) has been administered IV at 3 mg/kg four times per day for the first 2 days and then orally at 5 mg/kg four times per day. After 1997, only Sandimmune Neoral was given at the same dose. CsA oral doses were adjusted to a predefined trough level in whole blood. CsA blood levels were measured by enzyme-multiplied immunoassay technique (EMIT, Behring, Marburg, Germany) using a Cobas Mira+ machine (Roche, Basel, Switzerland). In high-risk patients, azathioprine was added (2 mg/kg/day). Patients receiving a second renal transplant or having acute rejection episodes were given mycophenolate mofetil (16 patients).

Cardiac transplant recipients underwent an antilymphocyte antibody induction regimen (ATGAM or ATG of 10 mg/kg/day) during 5 days and received 1 g IV of methylprednisolone perioperatively followed by 375 mg four times per day for 4 days. Subsequently, 1 mg/kg of prednisone was administered orally four times per day and tapered gradually; and 4 mg/kg of CsA, administered orally four times per day, and 2 mg/kg of azathioprine, administered orally four times per day, were given as soon as the patient was able to take them. One patient had azathioprine replaced by mycophenolate mofetil. Doses of oral CsA were adjusted according to trough levels in whole blood (approximately 150–250 ng/mL).

Hepatic transplant recipients received 300 mg of methylprednisolone IV on the operative day. On the ensuing days, they received methylprednisolone (or prednisone orally as soon as they were able to take it) in doses diminishing by 40-mg steps, until they reached a daily dose of 20 mg. One to two mg/kg of CsA were administered IV four times per day, then orally at three times the daily IV dose, with a target blood level between 150 and 300 ng/mL. Azathioprine was administered IV at 1 to 2 mg/kg four times per day, and then orally.

Acute rejection episodes were operationally defined for the purpose of this study by the administration of treatment against rejection episodes (i.e., >3 daily pulses of methylprednisolone or antilymphocyte therapy administered independently from the induction regimen), which were diagnosed by the usual clinical or histological criteria.

Definitions

Cytomegalovirus infection and disease were defined according to established criteria (*29*). A laboratory diagnosis of CMV infection was defined by positive shell vial culture of any biological sample. Viremia was defined as positive culture from blood leukocytes. In the absence of signs and symptoms (see below), this was considered asymptomatic infection. CMV infection with symptoms or signs attributable to CMV (symptomatic infection) was considered CMV disease.

Presumptive CMV disease was defined as one or more of the following symptoms associated with positive CMV shell vial culture (usually viremia): presumptive CMV syndrome: fever higher than 38.3°C and lasting at least 2 days, or leukopenia (<4000/μL), or thrombocytopenia (<100 G/L), or all three symptoms. Presumptive CMV organ disease was diagnosed as follows: (a) pneumonia: clinical symptoms with radiographic changes or hypoxemia; (b) hepatitis: serum transaminase disturbances (more than twice the previous value) or jaundice without other causes of hepatitis, or both; (c) gastrointestinal disease: gastrointestinal symptoms with diarrhea or bleeding, or both; and (d) neurologic disease: neurologic symptoms with encephalitis, transverse myelitis, or other central nervous system symptoms, together with CMV detected in the cerebrospinal fluid.

Proven CMV disease was defined as above but required a histological confirmation for hepatitis, gastrointestinal disease, and neurological disease. Pneumonia in transplant patients could be based on evidence of pneumonia with CMV detected in the bronchoalveolar lavage or lung biopsy, whereas retinitis with typical ophthalmologic lesions was accepted without virological proof.

Initial CMV infection was defined as the first CMV infection episode documented after transplantation. Recurrent CMV infection, symptomatic or not, was defined as the reappearance of a positive blood leukocyte shell vial culture after an initial CMV infection episode, separated by at least one negative blood culture, whether induced by anti-CMV treatment or not. Intermittently positive viremia with less than 3 IU/10^6 peripheral blood leukocytes (PBL) were not considered separate, recurrent episodes. Protracted CMV infection was defined as infection with a high viral load (>10 IU by quantitative culture or >10^5 copies of CMV DNA by real-time PCR per million PBL) observed in at least two separate samples at least 1 month apart.

Treatment administered to a patient was referred to as *preemptive* if it was administered because of recognized CMV disease risk factors in the absence of symptoms, and *therapeutic* if it was administered because of symptomatic disease.

Virological Monitoring

The CMV antibody status of donors and recipients was determined by enzyme-linked fluorescent assay for anti-CMV IgG (Vidas, bioMérieux, Marcy l'Etoile, France). For the monitoring of CMV infection, according to guidelines for the management of CMV infection that were proposed during the time of the study, clinicians were asked to submit blood specimens for CMV shell vial cultures weekly for the first 6 weeks after transplant and once every two weeks for the next 6 weeks. Weekly cultures for 4 weeks and biweekly cultures for an extra 4 weeks were proposed after the treatment of an acute rejection episode. Obtaining cultures for 1 month was also proposed after a CMV viremia episode, independent of treatment and time after transplantation. Samples taken at the time of peak viral load of an untreated episode or within 48 hr of initiation of antiviral treatment were called *peak samples*. Samples taken within 48 hr of the termination of a treatment were called *end-of-treatment* samples.

Blood was harvested in 8-mL EDTA-Monovettes tubes (Sarstedt, Sevelen, Switzerland) and processed within 2 hr in the Diagnostic Virology Laboratory for CMV shell vial assay. PBL prepared by hypotonic lysis of red blood cells were used in a quantitative shell vial assay using in house human embryonic lung fibroblasts as recently described (*28*, *30*). Aliquots of plasma and PBL were frozen at −80°C until extracted for PCR.

Preemptive Treatment Guidelines

Treatment with ganciclovir (2×5 mg/kg/day or adapted to renal function) for 15 days was proposed for all symptomatic patients and for patients with asymptomatic viremia and the following risk factors for CMV disease: (1) D+/R− serostatus; (2) treatment rejection within the previous 2 months; and (3) viremia higher than 10 positive nuclei/10^6 leukocytes. This cut-off value was used because it was shown recently that levels of viremia above it are predictive of impending CMV disease (*28*). The treatment had to be initiated no longer than 24 hr after diagnosis of symptomatic CMV disease or 72 hr after the indication to treatment of asymptomatic CMV infection was recognized. In case of ganciclovir toxicity, foscarnet (60 mg/kg IV, three times per day) was proposed. No antiviral prophylaxis was used in these patients.

Compliance to Performing Surveillance Cultures

Compliance was defined as the percentage of shell vial cultures made in accordance with the prescribed microbiological monitoring guidelines (e.g., 9/9 cultures=100%). For a culture to be counted as performed, it had to be performed within 2 days of the prescribed date. If more than 1 culture per week was performed, only one per week was counted to determine compliance.

Retrospective Analysis of CMV DNA Load in PBL and in Plasma Samples

Quantitative real-time PCR. PBL and plasma specimens from transplant patients during and after viremic episodes were retrospectively analyzed for CMV DNA. The laboratory personnel performing the PCRs was blinded to the clinical and quantitative culture data.

DNA was extracted from PBL samples using the QiaAmp blood kit (Qiagen, Basel, CH) and amplified by real-time PCR using primers and probes for the target sequence of the HCMV UL 86 gene coding for the major capsid protein (*31*). To control for the efficiency of the extraction procedure, a real-time PCR system was set up for human β-globin. TaqMan Universal Master Mix was purchased from Perkin-Elmer (Rotkreuz, Switzerland). Primers and probes were from Microsynth (Balgach, Switzerland).

Amplification and detection were performed with an ABI 5700 system (Perkin-Elmer). Beta-globin and CMV PCR were performed in separate reaction mixtures in duplicates. For each specimen, inhibition controls were run in duplicate by spiking 1000 copies of a linearized plasmid carrying CMV UL 86 ORF, encoding for the major capsid protein (CMV *Eco*RI D fragment in pACYC184, kindly provided by Dr. Deborah Spector, UCSD, San Diego) in the sample extract. Calculation of cell equivalent and CMV DNA copy numbers in extracts was based on standard lines of 10-fold dilution series of human DNA extracted from HeLa cells and of pACYC containing the *Eco*RI D fragment (that encompasses UL86) in carrier DNA in duplicates. Results were expressed as CMV DNA copy number per 10^6 PBL. Details concerning the primers, probes, and amplification procedures, as well as their analytical performance can be obtained from the corresponding author upon request.

Quantitative competitive PCR: Amplicor CMV monitor assay. This commercial method was used to measure CMV DNA load in plasma samples. The manufacturer's instructions were followed for all stages of the process, including sample preparation, amplification, and detection. Contamination was prevented by the inclusion of Amperase (uracyl-N-glycosydase) in the master mix and by substituting dUTP for dTTP.

Statistical Analysis

Demographic, clinical, and microbiological data were collected. For the comparison between patients with and without CMV recurrent episodes, only data within the first 3 months (i.e., the period of the planned virological surveillance) were included. For this comparison, the patient who died during an initial CMV episode was also excluded, because he did not have the opportunity to develop recurrent infection.

Data of patients with and without recurrent or protracted infection (see these definitions) were tabulated and compared by Fisher's exact test for categorical variables and by Wilcoxon rank sum test for continuously distributed variables. All tests were two-sided. The following possible risk factors for recurrence were analyzed: demographic data, type of organ transplant, donor and recipient CMV serostatus, immunosuppressive agents used (both for induction and treatment of acute rejection), type of treatment of initial episode (therapeutic or preemptive), acute rejection, and viral load determinations.

These data were also analyzed by logistic regression analysis to examine the odds ratios associated with these variables. After identifying variables influencing significantly the risk of protracted infection by univariate analysis, age, donor, and recipient CMV serostatus, viral load were entered into a stepwise multivariate logistic analysis. Computations were performed using the Stata software (Stata Corporation, College Station, TE).

RESULTS

Demographic Characteristics of the Patients

From 1997 to 1998, 111 patients underwent solid-organ transplant in our hospital. Four patients died during the perioperative period and are excluded from this study. Two patients returned to dialysis and had immunosuppression discontinued 22 and 25 days after transplantation because of acute vascular rejection requiring graft explantation and cyclosporine-induced microangiopathy respectively. Four pa-

tients were lost to follow-up during the 3-month period of surveillance according to the preemptive approach guidelines. The remaining 101 patients are the subject of this study. One patient had a simultaneous heart and kidney transplant and has been included arbitrarily in the kidney transplant category. Table 1 demonstrates the demographic characteristics, including the risk factors for developing CMV infection and disease. A majority of the patients (56%) underwent kidney transplantation. Patients had a variable proportion (from 2/9 to 9/9) of their surveillance cultures performed. Overall, however, a large proportion of surveillance cultures were indeed performed (85±22%). Only one kidney-transplant patient had preemptive IV ganciclovir administered while he was receiving OKT3 antibody treatment for acute rejection.

Incidence of CMV Infection and Disease

Table 2 demonstrates the incidence and characteristics of CMV infection. Overall, 63 (62%) patients developed CMV infection, for a total of 140 episodes. CMV infection caused symptoms in 39% of the 63 initial episodes. In one instance, CMV arguably was a contributing cause of death: a kidney-transplant patient experienced acute vascular rejection 3 weeks after transplantation and was treated with a course of OKT3 antibody. One week later, he developed CMV viremia, which was treated with ganciclovir. He nevertheless developed multiorgan failure and died 11 days after ganciclovir initiation. The postmortem revealed a disseminated B-cell lymphoma and CMV pneumonitis.

More than half (n=76, 54%) of the episodes represented instances of recurrent infection. In fact, 32 patients experienced recurrent infection with 20 patients experiencing more than 1 recurrence (up to 8 recurrent episodes), for a median number of 2 recurrences and a mean of 2.24 episode per patient. The proportion of symptomatic infection was lower in recurrent infection, but nevertheless about one-fifth (21%) of these episodes were symptomatic. In addition, another 21% of these episodes were of such a viral load level that preemptive treatment was prescribed according to the guidelines in the same manner as for initial episodes. Among clinically significant recurrent CMV episodes, we observed for instance a life-threatening CMV pneumonia during a first recurrence (second CMV episode) 2 months after heart transplant, a thrombotic thrombocytopenic purpura during a high-level first recurrence (second CMV episode) 2 months after liver transplant, and an accelerated course of recurrent hepatitis C toward cirrhosis in a liver transplant experiencing multiple episodes of CMV viremia with proven CMV hepatitis.

The proportion of recurrent episodes during this 1997 to 1998 period was dramatically increased (54%) over the previous 2-yr period (1995–1996) when it was 25% (*28*). When risk factors for CMV disease were compared between these two study periods, the only significant CMV risk factor change to explain this fact was an increased proportion of D+/R− high risk patients from 15% to 26%.

Performance of Real-time Quantitative PCR

During this study period, the surveillance for CMV infection was based on a quantitative culture assay. To test whether the quantitative detection of CMV DNA could provide more information than culture, samples of peripheral blood leukocytes were saved and retrospectively analyzed by a newly developed real-time quantitative PCR. Experiments using plasmidic target DNA diluted in human DNA, or DNA extracted from CMV-infected fibroblasts (*30*), indicated that the assay has a 1 to 10^7 copies dynamic range with cycle thresholds varying from 38 to 15, proportionally to the Log of the input CMV plasmid copy number with a r^2=0.998. Repeated measures on samples containing from 236 to 28,500 copies of CMV DNA revealed that the coefficient of variation of the assay averaged 55±18% (SD).

The CMV DNA content in 395 clinical specimens (1–20 specimen per patient) of peripheral blood leukocytes of the present patient population was therefore determined. In addition, plasma samples were analyzed for CMV DNA by Cobas Amplicor CMV Monitor assay. In fact, of 63 patients with CMV infection, 34 had PBL and 32 had plasma samples available for this analysis.

Course of CMV Viral Load in Selected Patients with CMV Infection

Figure 1 shows examples of the evolution of the viral load, as measured by quantitative rapid culture and by quantitative CMV DNA assay in the leukocytes (real-time PCR) and in plasma (Cobas Amplicor CMV Monitor) in several patients who presented different patterns of CMV infection. Figure 1a depicts the evolution of a patient with primary infection (D+/R− serostatus) presenting with recurrent viremia despite three treatment courses. The last viremia episode, characterized by an especially high level of CMV load lasting for several weeks, starting more than 3 months after transplantation, was accordingly not treated preemptively but attended with watchful waiting. The only symptom was a marked fatigue accompanied by thrombocytopenia, which both resolved simultaneously with a precipitous viral load drop in the absence of antiviral treatment. Of note, a similar pattern of spontaneous eventual resolution of CMV infection, as defined by no recurrent CMV disease or viremia after a last episode of high-level viremia with a leukocyte CMV DNA load in the 10^6 to 10^7 range, followed by a spontaneous precipitous decline of CMV load in the blood (Fig. 1A, from day 105–135 after transplantation) was observed in nine patients (seven with the D+/R− serostatus pattern and two with the D+/R+ pattern).

In fact, CMV DNA was continuously detectable in the leukocytes and plasma of this patient, ranging from 10^5 to 10^6 copies/10^6 leukocytes and from 10^4 to 10^5 copies/mL plasma throughout the three initial viremic episodes that were treated and reaching close to 10^8 copies and $>10^5$ copies, respectively, in PBL and plasma during the last high-level viremia, in the absence of treatment.

Figure 1B depicts the course of D+/R− heart transplant patient with a CMV infection course similar to the previous one. Figure 1C depicts the course of a D+/R+ kidney-transplant patient who presented a clinically nonsignificant CMV recurrent infection after preemptive treatment of an initial episode. Figure 1D depicts the course of a D+/R+ kidney-transplant patient who presented with spontaneous recurrent episodes of viremia that did not require preemptive treatment according to our guidelines. Figure 1E and F present the infection course of a D−/R+ heart-transplant patient and a D+/R+ kidney-transplant patient who presented without any recurrence after an initial infection episode treated preemptively or not.

Together, these detailed longitudinal descriptions of the course of CMV infection in the wake of transplantation indicate a wide variability not only in the peak viral load at the time of initial infection, but also in the profile of viral load changes over time after initial infection. Because some of the patients presented with low viral load recurrences of little clinical significance, we attempted to replace the culture-derived concept of "recurrent infection," i.e., viremia episodes separated by a negative culture. Acknowledging the fact that CMV infection was not resolved in these patients as indicated by PCR data at the end of preemptive treatment (Fig. 1A and B), we incorporated both a viral load and a duration criteria in the new definition of "protracted high-level infection," (i.e., the observation, at least 1 month apart, of a viral load >10 CMV IU or $>10^5$ copies of CMV DNA/10^6 PBL). As indicated by Table 2, the somewhat more stringent definition of protracted infection led to the reclassification of five patients with recurrent infection as not having protracted high-level infection (sample patients depicted in Fig. 1C and D). The duration of protracted high-level infection varied substantially with a median time from first to last high virus load sample of 73 days (range: 30–227).

Risk Factors for Protracted Infection

It would be of major interest to identify early predictors of protracted high-level infection to adjust the management of CMV infection to this risk. To identify risk factors for protracted CMV infection, patients with CMV infection were classified according to whether they developed (n=27) or not (n=36) protracted infection and compared with respect with a host of clinical and microbiological potential risk factors. For this analysis, only events occurring within the 3-month period of planned surveillance were taken into account. Table 3 demonstrates that patients with protracted infection were significantly older than patients with nonprotracted infection. The risk of protracted infection did not significantly differ across the different types of organ transplant. The donor/recipient serostatus pattern was also a very strong predictor for protracted infection, which rose from 0% to 95%

TABLE 1. Baseline characteristics of patients and incidence of CMV and other opportunistic infections

Characteristic	Organ			Total
Organ transplanted	Heart	Kidney	Liver	
Number of patients (n)	19	57	25	101
Age (yr, mean±SD)	56.2±5.7	48.3±11.4	48.9±9.8	49.9±10.5
Gender (M/F, no)	18/1	40/17	19/6	77/24
CMV serostatus pattern (n, %)				
D−/R−	4 (21%)	9 (16%)	6 (24%)	19 (19%)
D−/R+	4 (21%)	14 (25%)	4 (16%)	22 (22%)
D+/R−	7 (37%)	12 (21%)	7 (28%)	26 (26%)
D+/R+	4 (21%)	22 (39%)	8 (32%)	34 (34%)
Acute rejection (n, %)	12 (63%)	31 (54%)	6 (24%)	49 (49%)
% of planned surveillance cultures performed	83±19%	94±11%	66±28%	85±22%

TABLE 2. Incidence and characteristics of CMV infection

	Heart	Kidney	Liver	Total
CMV infection (n, %)	10/19 (53%)	40/57 (70%)	13/25 (52%)	63/101 (62%)
Recurrent infection (n, % of CMV-infected)	4/10 (40%)	20/40 (50%)	8/13 (62%)	32/63 (51%)
Protracted infection (n, % of CMV-infected)	3/10 (30%)	16/40 (40%)	8/13 (62%)	27/63 (43%)
Total infection episodes (n)	18/140	93/140	29/140	140
Type of initial episode				
Total (n, %)[a]	10/18 (56%)	40/93 (43%)	13/29 (45%)	63 (46%)
Asymptomatic, untreated (n, %)[b]	3 (30%)	14 (35%)	4 (31%)	21 (33%)
Asymptomatic, treated preemptively (n, %)[b]	4 (40%)	9 (23%)	3 (23%)	15 (23%)
Symptomatic, despite preemptive treatment (n, %)[b]	0 (0%)	1 (2%)	1 (8%)	2 (3%)
Symptomatic, treated (n, %)[b]	3 (30%)	16 (40%)	5 (38%)	25 (39%)
Type of recurrent episodes				
Total (n, %)[a]	8/18 (44%)	52/93 (56%)	16/29 (55%)	76 (54%)
Asymptomatic, untreated (n, %)[c]	3 (38%)	33 (63%)	8 (50%)	44 (58%)
Asymptomatic, treated preemptively (n, %)[c]	3 (38%)	12 (23%)	1 (6%)	16 (21%)
Symptomatic, despite preemptive treatment (n, %)[c]	0	0	0	0
Symptomatic, treated (n, %)[c]	2 (25%)	7 (13%)	7 (44%)	16 (21%)
Mortality due to CMV	1	1	0	0
Opportunistic infections[d]	5	1	2	8

[a] % of all, initial and recurrent, episodes.
[b] % of initial episodes for each transplant category.
[c] % of recurrent episodes for each transplant category.
[d] Among heart transplant patients: toxoplasmosis (2), tuberculosis (1), *P. carinii* pneumonia (1), and *Nocardia* pneumonia (1); kidney transplant patients: *P. carinii* pneumonia (1); liver transplant patients: *Legionnella* pneumonia (1), visceral Leishmaniosis (1).

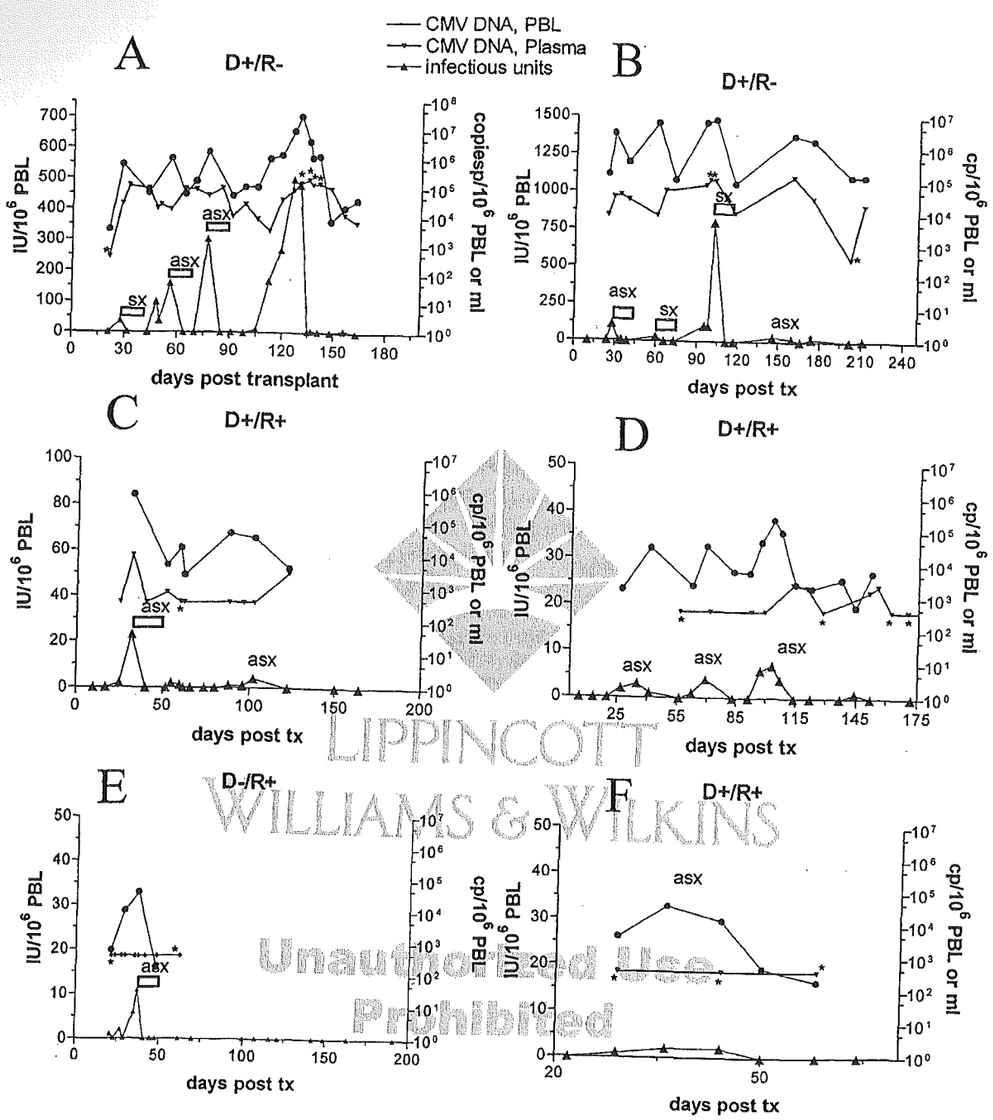

FIGURE 1. **Course of CMV infection in six patients (A–F) after SOT. D/R, donor–recipient CMV serostatus. PBL infectivity as measured by quantitative shell vial assay (blue triangles, arithmetic scale on left y axis) and CMV DNA (PBL by real-time PCR, red circles, and plasma by Cobas Amplicor CMV Monitor, black inverted triangles, logarithmic scale on right y axis) are shown throughout the period of active follow-up of the patients. Open rectangles, ganciclovir treatment; asx, asymptomatic episodes, sx, symptomatic episodes. *Out of range values (<400 or >10^5 copies/mL).**

across the different patterns ($P<0.001$, Fisher's exact test). When serostatus pattern groups were compared pairwise, the risk for protracted infection was significantly higher in D+/R− patients compared with D+/R+ patients ($P<0.001$) but not in D+/R+ compared with D−/R+ patients ($P=0.12$). Protracted infection was not significantly associated with any peculiar immunosuppressive drug used in the induction or maintenance of immunosuppression, or with acute rejection or its treatment.

Viral Load During the Initial Episode as a Risk Factor for Subsequent Protracted Infection

The relationship between viral load measures during the initial viremia episode and the development of protracted

TABLE 3. Distribution of potential risk factors among patients with and without protracted CMV infection

Type of CMV infection risk factor	Nonprotracted (n=36)	Protracted (n=27)	P value[b]
Age[a]	48.5 (25–68)	56 (35–67)	0.0185
Organ[c]			
Heart (n=10)	7/10 (70%)	3/10 (30%)	0.309
Kidney (n=40)	24/40 (60%)	16/40 (40%)	
Liver (n=13)	5/13 (38%)	8/13 (62%)	
CMV serostatus pattern[c]			
D−/R− (n=1)	1/1	0/1	$P<0.001$
D−/R+ (n=16)	15/16 (94%)	1/16 (6%)	
D+/R+ (n=27)	19/27 (70%)	8/27 (30%)	
D+/R− (n=19)	1/19 (5%)	18/19 (95%)	
Tacrolimus	5/36 (14%)	4/27 (15%)	1.000
Anti-IL2R antibody	2/36 (6%)	1/27 (4%)	1.000
Azathioprine	13/36 (36%)	8/27 (30%)	0.788
Mycophenolate mofetil	8/36 (22%)	6/27 (22%)	1.000
Acute rejection	23/36 (64%)	15/27 (46%)	0.136
Pulse methylprednisolone (total grams)[a]	22/36 (61%)	12/27 (44%)	0.212
	2.5 (0–6)	3 (1.5–6)	0.351
OKT3	6/36 (17%)	4/27 (15%)	0.944

[a] Median (range).

[b] Statistical comparisons were performed by Wilcoxon's rank sum test for continuous variables and by Fisher's exact test for categorical variables.

[c] For organ transplant category, serostatus pattern, % indicate the proportion of patients with or without protracted infection within each category or pattern.

infection was also analyzed. Figure 2 demonstrates that all measures of viral load during the initial viremia episode were significantly higher among patients who developed a protracted infection compared with those who did not. However, there was a substantial overlap between the viral load data of the two groups regarding peak PBL infectivity, peak and end of treatment PBL CMV DNA, and peak plasma CMV DNA, so that none of these measures could be used alone to predict whether the patient would experience a protracted infection. In contrast, end of treatment plasma CMV DNA best distinguished between patients who developed or not a protracted infection (Fig. 2, lower right panel). In fact, by using a cut-off value of 2000 CMV DNA copies/mL plasma, this test had a sensitivity of 100% and a specificity of 89% for predicting protracted infection.

Logistic Analysis of Risk Factors for Protracted Infection

Finally, logistic regression was used to assess the quantitative relationship between risk factors (including age, CMV donor and recipient serostatus, occurrence of acute rejection, immunosuppressive drugs used for induction, baseline immunosuppression and treatment of acute rejection, virus load during the first episode using the various assays, and mode of treatment of initial episode [preemptive or therapeutic]). Table 4 demonstrates patient characteristics that significantly predicted protracted infection by univariate analysis: per decade of age, the odd of developing protracted infection increased nearly twofold. A CMV-seropositive donor conferred an approximately 20-fold odds increase of developing protracted infection, whereas being a CMV-seropositive recipient diminished the odds close to 100-fold. Among the various measures of CMV viral load during the initial episode, all predicted an increased risk, with an odds ratio per Log increase varying from 2.79 for end of treatment CMV PBL DNA to 33.15 for end of treatment CMV plasma DNA, which again appeared as the strongest predictor of the risk to develop protracted infection. Significant univariate predictors were examined in a stepwise multivariate regression model. The only viral load measure entered in this model was the peak PBL infectivity, because data for this measure were available for all patients, in contrast to PCR data. As can be seen from Table 4, in the multivariate model, recipient seropositivity and peak PBL infectivity remained significant independent predictors of protracted infection. Age remained of borderline significance, whereas donor seropositivity was dropped from the model for lack of significance.

DISCUSSION

Recurrent CMV infection and disease are common problems in solid-organ transplant patients (*21*, *22*, *24*, *25*). Various risk factors for recurrent CMV disease and infection have been recognized in these studies (see *Introduction*). However, to date, our study is the largest examining both clinical determinants and molecular measures of viral load during the first episode as predictors for recurrent infection. In addition, our patient population was characterized by a high rate of recurrent infection, in part because of the fortuitously high proportion of high-risk (D+/R−) patients among our patients during the study period. Thus, this study offers an unprecedented opportunity to analyze the determinants of recurrent CMV infection in solid-organ transplant patients.

During the study period, the patients were monitored virologically using a quantitative shell vial culture (*28*), and stored blood samples were retrospectively analyzed for CMV DNA in PBL and plasma fractions. Although blood culture turned quickly negative during ganciclovir treatment (Fig. 1A and B), CMV DNA was frequently detected in the PBL and plasma fractions during, at the end, and after ganciclovir treatment, in particular in patients who

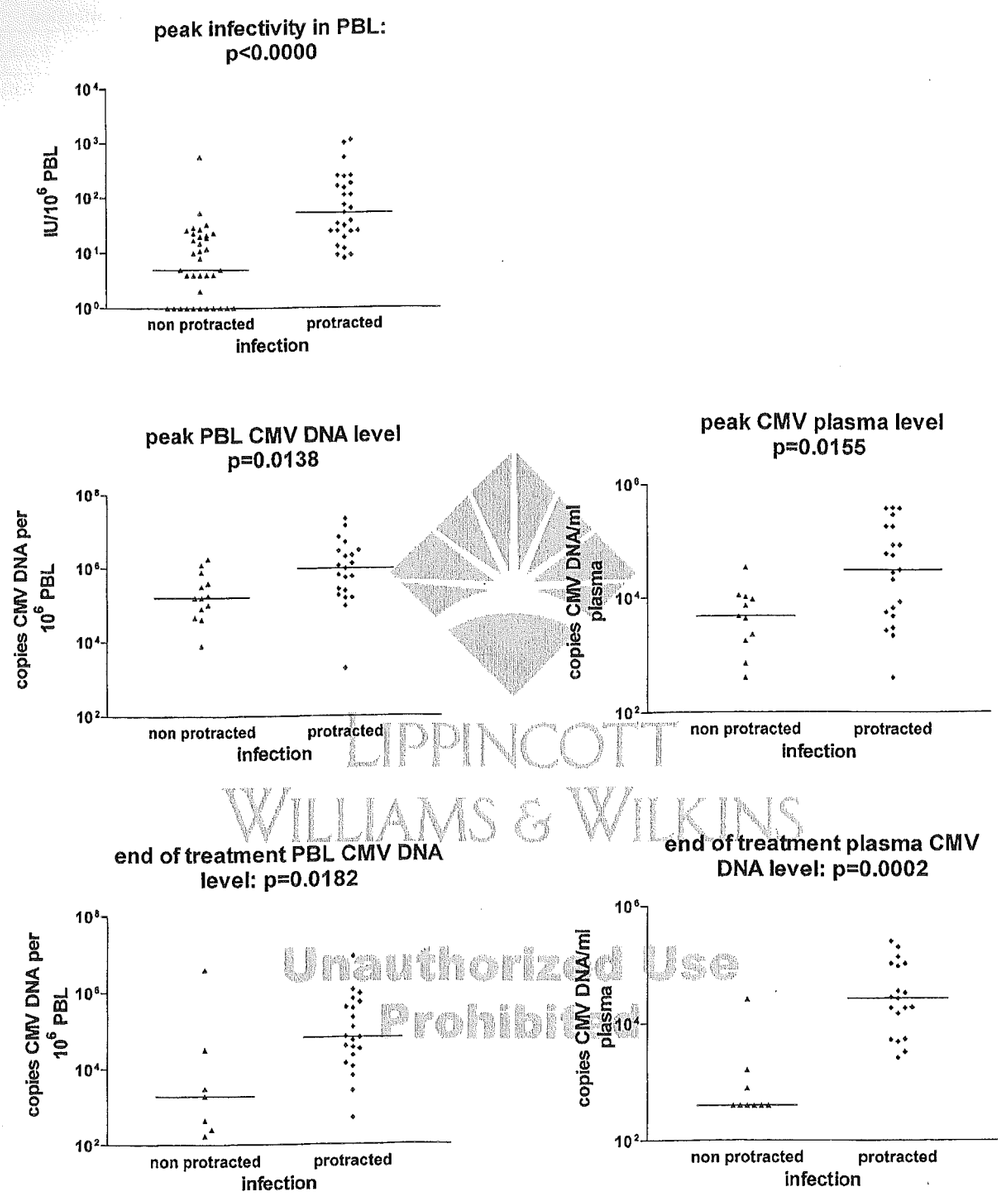

FIGURE 2. **Viral load during initial CMV infection episodes, analyzed by infection outcome. Peak value was defined as the value from the samples with the highest value during the infection episode, or when antiviral treatment was administered, the value of a sample taken within 48 hr of treatment. End of treatment value was defined as the value of a sample taken within 48 hr of treatment end. Individual values are shown, with the bar representing the median value. Values of patients with or without protracted infection were compared using the Wilcoxon rank sum test.**

later developed recurrent infections. This led us to interpret recurrent infection in these instances as the expression of a protracted, unresolved infection, with antiviral treatment suppressing transiently CMV replication, which resumed after treatment was stopped. This pattern apparently continued until a spontaneous control of CMV viremia occurred, which most probably reflected the development of effective immune responses.

TABLE 4. Univariate and multivariate logistic analysis relating risk factors to the development of protracted infection

Predictor	OR (95% CI)	P
Univariate		
Age (per decade increase)	1.90 (1.06–3.73)	0.03
D+ serostatus	19.5 (2.37–160.3)	0.006
R+ serostatus	0.014 (0.0017–0.125)	<0.001
Peak PBL infectivity (per Log increase)	12.48 (3.34–46.59)	<0.001
Peak PBL DNA (per Log increase)	2.90 (1.02–8.20)	0.045
End PBL DNA (per Log increase)	2.79 (1.11–7.02)	0.029
Peak plasma DNA (per Log increase)	4.18 (1.25–13.99)	0.02
End plasma DNA (per Log increase)	33.15 (2.64–415.8)	0.007
Multivariate (entering age, D[a] and R serostatus (excluding D−/R−) and start PBL infectivity)		
Age (per decade increase)	2.28 (0.91–5.69)	0.076
R+ serostatus	0.031 (0.003–0.329)	0.007
Start PBL infectivity (per Log increase)	7.79 (1.75–34.57)	0.004

[a] D+ serostatus dropped for $P=0.28$, >0.1.

We also observed in several instances low-level recurrent viremia, after or not ganciclovir treatment (Fig. 1, c and d). Because these remained asymptomatic in the absence of treatment, we coined a more stringent definition of recurrent infection, which would incorporate both a viral load criterion (>10 infectious units or 10^5 CMV DNA copies per 10^6 PBL) and a duration criterion (two high viral load measurements at least 1 month apart). We coined the term of protracted (high viral load) infection to stress this duration component of the definition. Our preemptive approach has the potential to prevent the appearance of symptomatic CMV disease in the majority of cases (*28*), thus leading to an underestimate of the pathogenic potential of CMV during initial and recurrent infection. As a consequence, we did not request symptoms to be present in our stringent definition of protracted infection.

Using the stringent definition, we had in mind to analyze predictors of clinically significant recurrence with a high probability of disease or of requiring preemptive treatment. In fact, among the 63 patients with CMV infection, only 27 (of the 32 patients with recurrent CMV viremia) had protracted high-level infection according to this stringent definition. Categorical statistical analyses for risk factors for recurrent infection were performed using either definition: correlations with risk factors were uniformly more significant using the stringent (protracted infection) compared with the plain (recurrent infection) definition. Other analyses (logistic regression) were performed using the stringent definition.

When analyzing the effect of clinical predictors, we did not find the type of organ transplant to affect significantly the probability of protracted infection. All three types were therefore analyzed together. However, because of the relatively small number of heart or liver patients, one should be cautious before extrapolating our analysis to different transplant categories.

Although the occurrence of CMV infection was not significantly influenced by the patient age, patients with protracted infection were older than patients without: by logistic regression, each decade nearly doubled the odds of protracted CMV infection, a significant increase by univariate analysis that remained nearly significant by multivariate analysis. It has been widely observed that, with aging, substantial changes occur in both the functional and phenotypic profiles of T lymphocytes (*32*). This is usually considered as the explanation for the increased incidence of tuberculosis that is observed in elder patients in low tuberculosis-prevalence populations (*33*) and for the increasing incidence of zoster with age (*34*). To the best of our knowledge, our data for the first time quantitatively document the age-dependent decreasing capacity of the immune system in SOT patients over middle age decades to mount an anti-CMV immune response capable of containing viral replication.

The major predictor of protracted CMV infection was the donor–recipient serostatus pattern with a significantly escalating risk from the low-risk D−/R+, to the intermediate D+/R+, and to the high-risk D+/R− pattern (primary infection) group. This shows that the risk for protracted CMV infection parallels the risk for CMV disease in transplant patients (*4*, *13*). This finding is consistent with observations by others who found that CMV primary infection (the D+/R− pattern) and the severity of CMV disease during the initial episode are predictor for recurrent CMV infection and disease (*22–24*). Logistic regression allowed to analyze separately the influence of donor and recipient CMV serostatus on the risk of developing protracted infection: whereas an organ from a latently infected donor significantly increased the odds of protracted infection by univariate analysis, being a latently infected recipient diminished strikingly this odd by about 100-fold! In fact, in the multivariate model, donor seropositivity lost its significance, whereas recipient seropositivity remained strongly protective. We interpret these data as showing that the most important protective factor against protracted infection is the existence of an anti-CMV immune response before the transplantation procedure. CMV-specific cytotoxic T lymphocytes have been shown to control CMV viral load and to prevent CMV disease in the first months after renal transplantation (*35*). It is likely that the existence of a pool of CMV-specific memory T cells in seropositive recipients enables them to generate a protective immunity much faster than in seronegative, CMV-naïve recipients. This may in fact also be consistent with the observation that a low level of activated CD8+ T cells in the blood of kidney and liver transplant patients at the end of the treatment of the first CMV episode is associated with a high risk of recurrent CMV disease (*23*).

CMV viral load, particularly in the blood, carries prognostic information regarding the risk of impending CMV disease (*36*, *37*). By analogy, we wondered whether the viral load

during the first CMV infection episode would carry information as to the risk of protracted infection. By all measures, the viral load was significantly greater in patients who went on to develop protracted infection compared with those who did not. This is similar to the data reported by Sia et al. (*24*), who determined pre- and posttreatment PBL and plasma CMV DNA load by competitive PCR in 24 SOT recipients, 8 of whom went on to develop recurrent infection. However, there was a substantial overlap between the viral load data in patients with and without protracted infection in particular with respect to peak (pretreatment) viral load. In contrast, plasma CMV DNA at the end of treatment seemed the only test with a diagnostic performance sufficient for use as a predictor for protracted infection.

All our patients had peak viremia data, whereas only a subset had samples available for retrospective PCR analysis. Therefore, peak quantitative viremia was the only viral load measure introduced in the multivariate logistic regression model. This analysis showed that this viral load measure predicted the risk of protracted infection independent of age and D/R serostatus. The significance of the viral load as a risk factor for developing protracted infection is not surprising: it might reflect a higher level of immunosuppression (the weaker the immune response, the higher the CMV load) likely to persist beyond the initial infection episode; conversely, after an infection episode with a high level of replication, a higher residual load and a larger number of latently infected cells are available to cause a recurrence. This is indeed consistent with the observation that patients with severe multiorgan initial CMV episodes were more likely to develop recurrent CMV infection (*22*).

Van den Berg et al. (*23*) also analyzed 36 SOT patients, 11 who developed recurrent disease after treatment for CMV disease. These authors did not find quantitative antigenemia, or qualitative culture or PCR during the initial episode, to be helpful in identifying patients with subsequent relapse. Our finding that viral load predicts protracted infection may reflect either the larger size of our study or differences in the methods used to assay viral load.

It is unclear whether a prolonged preemptive treatment would prevent recurrent infection. In fact, in seronegative recipients, it could only further reduce the viral load, while the lack of protective immune responses would persist. Falagas et al. (*22*) did not find duration of treatment, over a 2- to 3-week range, to affect the likelihood of recurrent CMV disease in liver transplant recipients. Alternatively, patients with a high risk of recurrent infection may benefit from secondary CMV prophylaxis. However, a study provided disappointing results regarding the efficacy of oral ganciclovir in this indication (*38*). Recent studies have indicated the ability of oral ganciclovir and valacyclovir primary prophylaxis to prevent CMV infection and disease in both CMV-seropositive and -seronegative recipients (*9*, *17*, *18*).

CONCLUSIONS

In the absence of studies directly comparing the prophylactic and the preemptive approach in the management of CMV infection in SOT patients, the relative worth of each of these approaches is still debated (*14*). In this respect, our data help identify patients not only with an increased probability of CMV disease but also with a higher probability of protracted significant CMV infection despite preemptive treatment. On average, primary prophylaxis may save up to 2 to 3 preemptive and therapeutic courses in seronegative recipients, making it particularly cost effective compared with the preemptive approach in this subset of patients, with the added proven bonus of preventing acute rejection (*17*, *18*). In contrast, in seropositive recipients, of whom only a minority will require usually a single preemptive course, the preemptive approach is likely to be drug saving and cost effective.

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COMBINATION OF TACROLIMUS, METHOTREXATE, AND METHYLPREDNISOLONE PREVENTS ACUTE BUT NOT CHRONIC GRAFT-VERSUS-HOST DISEASE IN UNRELATED BONE MARROW TRANSPLANTATION

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Background. **Graft-versus-host disease (GVHD) is still a major problem in allogeneic bone marrow transplantation (BMT). Prophylactic regimens used against GVHD in unrelated BMT, including cyclosporine (CsA)-plus-methotrexate (MTX), CsA-plus-MTX-plus-prednisone, and tacrolimus (FK506)-plus-MTX, are still unsatisfactory (34–70% occurrence of grades II–IV GVHD). To address this problem, we examined the efficacy of FK506-plus-MTX-plus-methylprednisolone (mPSL) in 20 patients who underwent BMT from unrelated donors.**

Methods. **All patients received FK506 beginning the day before transplantation at a dose of 0.03 mg/kg per day by continuous intravenous (IV) infusion. MTX was administered at a dose of 10 mg/m^2 IV on day 1, and 7 mg/m^2 on days 3, 6, and 11. Intravenous administration of mPSL was started at a dose of 2 mg/kg per day on**

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