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MAJOR ARTICLE

Correlation of Cytomegalovirus (CMV) Disease Severity and Mortality With CMV Viral Burden in CMV-Seropositive Donor and CMV-Seronegative Solid Organ Transplant Recipients

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Background. The rate of cytomegalovirus (CMV) viral load increase and peak viral loads are associated with CMV disease in kidney and liver transplant recipients, but relationships to disease severity or mortality have not been shown.

Methods. Using stored serial serum specimens from renal (n = 59) and liver (n = 35) transplant recipients (D+R-; CMV-seropositive donors, CMV-seronegative recipients) from 2 prospective, randomized, controlled, interventional prophylaxis trials of CMV immune globulin (CMVIG), CMV viral load was measured using the COBAS quantitative polymerase chain reaction assay and the World Health Organization CMV standard. Patients with severe CMV-associated disease were classified according to trial definitions. Pairwise comparisons of mean viral load among deceased, surviving diseased, and nondiseased patients were analyzed by 2-way analysis of variance. To determine if viral load could predict mortality, receiver operating characteristic (ROC) curves were constructed using area under the curve (AUC) of the viral load and peak viral concentration (V_{max}).

Results. Viral load (mean $\log_{10}$ [AUC], peak viral load [V_{max}]) for patients with severe CMV disease was significantly higher compared with nondiseased patients ($P < .001$). Similarly, higher viral burden was significantly associated with mortality ($P < .001$). Viral load AUC and V_{max} AUROCs for predicting mortality were 0.796 and 0.824, respectively, for renal patients, and 0.769 and 0.807, respectively, for liver patients.

Conclusions. Using specimens from studies preceding the antiviral prophylaxis era, CMV viral load was associated with severe CMV disease and death, supporting CMV viral load quantification as a proxy for CMV disease severity and disease-associated mortality end points in solid organ transplantation.

Keywords. cytomegalovirus; cytomegalovirus disease; liver transplantation; renal transplantation; viremia.

Cytomegalovirus (CMV) viral load is associated with CMV disease in several different populations [1–6]. In particular, both the extent and duration of CMV load in blood or plasma are associated with CMV disease in kidney, liver, and stem cell transplantation [2, 3, 7], and viral load kinetics reflects the likelihood of disease recurrence [8]. Unlike HIV, for which viral load can predict survival [9], few studies exist for CMV infection, and only in the context of hematopoietic stem cell transplantation (HSCT). In solid organ transplantation (SOT) [10–13], a recent meta-analysis has begun to address this issue [14]. For CMV, while currently under discussion, health authorities do not currently permit the use of viral load as a surrogate marker for a primary outcome in registrational studies as they do for HIV [9, 14, 15]. Because rates of CMV disease are greatly reduced in SOT due to the widespread use of prophylactic and preemptive antivirals, it is increasingly difficult to enroll sufficient patient sample sizes in clinical trials to demonstrate that a new agent can reduce the incidence or resolve CMV disease [16]. A more practical approach for determining the efficacy of a novel antiviral agent in SOT populations, HSCT populations, or in vaccine development could be the use of viremia as a primary end point [17] and to guide the initiation of preemptive antiviral therapy.

In this context, we analyzed sera from 2 cohorts of D+R- (CMV-seropositive organ donor and CMV-seronegative recipients) kidney and liver SOT recipients, who were part of 2

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separate, prospective, prophylaxis trials of cytomegalovirus immune globulin (CMVIG) to prevent CMV infection and disease [18, 19]. We chose samples from these patients because viremia correlates with CMV end organ disease, and D+R- patients have the highest incidence of viremia, the highest peak viral load relative to D+R+ and D-R+ patients, and the longest duration of both viremia and treatment [3, 20]. Both prophylaxis studies were largely conducted before the antiviral era, and therefore provide an advantage for analyzing the effect of viral load without the confounding effects of additional therapies on the occurrence of CMV disease.

METHODS

Patient Cohorts

Samples were selected from D+R- renal and liver transplant patients (Figure 1) [18, 19]. The liver transplant patient samples were from the D+R- patient subset in a larger prophylactic trial of CMVIG that enrolled patients regardless of serologic status.

Study Design for Sample Collection

The designs for both clinical studies have been reported previously [18, 19]. Briefly, patients were randomized to receive either CMVIG, no treatment (renal), or placebo (liver) at the time of transplantation. The dosing schedule for the renal study was 150 mg/kg within 72 hours of transplantation, then 100 mg/kg every other week for 4 doses, and then 50 mg/kg at months 3 and 4. For the liver transplant study, CMVIG dosing was 150 mg/kg within 72 hours of transplantation, then 150 mg/kg every other week for 4 doses, and then 100 mg/kg at months 3 and 4 post-transplant. Serum samples were taken at baseline and before transplant and were available weekly for 2 months (both studies), then every other week for 2 months (liver study), and monthly for the renal study for the following 6 months or at the time of any hospitalization. For this analysis, only samples up to day 100 post-transplant were assessed. If CMV disease was suspected, additional samples were obtained. Sera were generally stored at –20°C. Plasma or whole blood was not saved as part of the study design. In the original clinical trials, the presence of viral infection was determined via culture of buffy coat or body fluids using traditional culture methods or CMV shell vial assays. No attempt was made to quantify viral load during the time these studies were completed (circa 1984–1991), and the World Health Organization (WHO) CMV standard [21] was not available. Both study protocols were approved by the respective institutional review boards (IRBs) before they were conducted [18, 19], and an IRB approved the current analysis, which used specimens and data with patient identifiers removed.

CMV DNA Quantification

CMV load measurements were performed with serum samples using the COBAS AmpliPrep/COBAS TaqMan CMV Test [CAP/CTM CMV test]) system, a real-time polymerase chain reaction (PCR) test approved for the quantification of CMV DNA (Roche Molecular Systems, Pleasanton, CA) and qualified for use with ethylenediaminetetraacetic acid (EDTA)–plasma specimens. As only serum was collected, the assay was qualified using CMV-spiked serum samples to demonstrate that detection limit, linearity, and precision of CMV quantification in serum were comparable to the parameters measured in EDTA plasma (unpublished and data not shown). The first WHO International Standard for Human Cytomegalovirus (HCMV) National Institute for Biological Standards and Control (NIBSC) code, 09/162, was used for viral load quantification. CMV International Units (IU) and values were expressed as

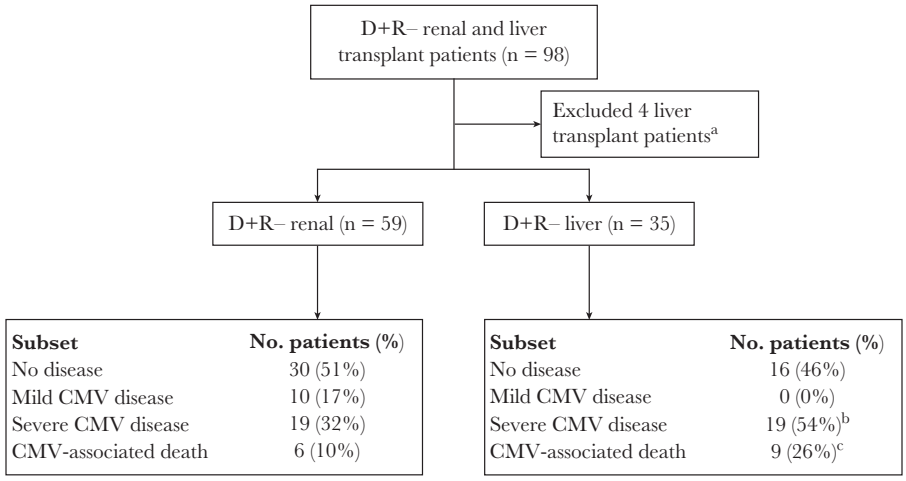

Figure 1. Patient disease characteristics. [a]Four liver transplant patients were excluded from analyses due to insufficient sampling. One patient had no disease; of the 3 patients with severe disease, 1 patient died, and 1 patient was followed to day 15 and then received a new transplant. [b]One liver transplant patient with mild disease was included in the severe disease category for analysis purposes. [c]The total number of deaths was 10 (Table 1). One patient was excluded from death comparison analyses because cause of death was unrelated to cytomegalovirus (CMV).

WHO Equivalent Units (0.91 IU = 1 copy; see test manufacturer's package insert) [21, 22]. Using this standard, we ascertained that the lower limit of quantification was 150 copies/mL (135 IU/mL) and that the assay accuracy and precision were comparable in both serum and plasma. All values were reported as IU/mL.

Clinical Outcomes

CMV disease or severe CMV-associated disease was classified according to the definitions of the clinical trials [18, 19]. Briefly, CMV disease was defined as the presence of organ dysfunction along with biopsy-proven CMV in the affected organ, and CMV-associated disease was defined as biopsy-proven CMV disease in 2 or more organs along with opportunistic infections. Laboratory measures were blinded to treatment or prevention strategy. In both studies, the definition of severe CMV or CMV-associated disease included the following: CMV pneumonia, opportunistic infections (fungal or parasitic) with CMV infection, retinitis, or central nervous system involvement. In the renal study, white blood cell count (<3000/μL) was also included as a measure of severe CMV disease. In the liver transplant study, patients with involvement of >2 organs also qualified as having severe CMV disease. Viral quantification and clinical outcomes were assessed from day 0 through day 100 to ensure assessment of primary CMV infection and disease.

Statistical Analysis

This study was a post hoc analysis of patient samples from 2 randomized controlled prospective multicenter trials using molecular methods not available at the time of the trials. Sample size was determined by the number of available, usable serum samples.

Analyses of the viral load data were performed without any prior knowledge of patient outcomes. For each patient, area under the time-vs-viral concentration curve (AUC) and peak viral concentration (V_{max}) were calculated on a fixed interval from day 0 to day 100. AUC was calculated using the composite trapezoid rule [23]. Values for samples with undetectable viral loads were set to half of the lower limit of quantification of the assay before AUC and V_{max} calculation, and all individual patient AUC and V_{max} values were log-transformed. Log_{10} (AUC) and log_{10} (V_{max}) were analyzed separately in the liver and renal transplant studies. Pairwise comparisons of mean viral load for different disease states were analyzed using 2-way analysis of variance (ANOVA), controlling for CMVIG treatment status by including it as a fixed categorical variable. Pairwise comparisons of mean viral load among deceased, surviving diseased, and surviving nondiseased patients were analyzed by 2-way ANOVA, again controlling for CMVIG treatment status by including it as a fixed categorical variable. Statistical significance of pairwise comparisons was assessed at $P < .05$ using the 2-sided Tukey-Kramer test. The utility of viral load as a predictor of mortality and morbidity was assessed by computing the area under the receiver operating characteristic (AUROC) curves for predicting CMV-associated death (death vs survival) and severe CMV disease (severe CMV disease vs absent to mild CMV disease). Assessment of viremia by CMVIG treatment status was done by calculating the proportion of patients with detectable viremia within 100 days of transplant according to qPCR viral load above the lower limit of quantification and qPCR viral load above 2000 IU/mL. Time to viremia by CMVIG treatment status was assessed by Kaplan-Meier analysis, with times censored at the earliest of last follow-up visit and day 100 post-transplant. Statistically significant differences in time to viremia between the CMVIG-treated and -untreated groups were assessed by log-rank test at $\alpha = 0.05$. For the Kaplan-Meier analysis, viremia was defined as qPCR viral load above the lower limit of quantification. All statistical analyses were performed using R, version 3.1.1 [24]. Receiver operating characteristic (ROC) curves and AUROCs were calculated using the R package "AUC" [25]. Kaplan-Meier analysis was performed using the R package "survival" [26].

Role of the Funding Source

The original renal transplant trial [18] was supported in part by National Institutes of Health (NIH) grants AM31389 and RR-00054 from the General Clinical Research Centers Program of the National Center for Research Resources, NIH, to the New England Medical Center. The liver transplant trial [19] was supported by NIH grant R10 DK31389. Genentech, Inc. funded the current analysis and participated in the analysis and interpretation of results.

RESULTS

Patient Demographics

As previously described, patients included in this study were D+R- renal transplant recipients and the D+R- patient subset from the liver transplant study [18, 19]. Specimens were available from all 59 renal transplant patients and 35 high-risk liver transplant recipients (Figure 1; Table 1). Thirty-two percent of renal patients and 51% of liver patients had severe CMV disease; 10% of renal patients and 29% of liver patients died as result of CMV disease (Table 1). During the renal trial, ganciclovir was only available on a compassionate basis; the 4 patients treated have been previously reported [27]. During the liver transplant trial, ganciclovir became licensed for use, which is reflected in the increased number of patients who received ganciclovir, as well as the timing of the ganciclovir treatment post-transplant after CMV disease was diagnosed (Table 1). The median time of the first dose of ganciclovir after transplant was 45.5 days in the renal transplant study (n = 4) and 35.5 days in the liver transplant study (n = 17) (Table 1).

Table 1. Demographic and Clinical Outcomes of Renal and Liver Transplant Patients at High Risk for CMV Infection

Parameter	Renal Patients (n = 59)	Liver Patients (n = 35)
Age, mean (SD), y	30.9 (14)	36.8 (18.5)
Gender, male/female, No. (%)	35 (59)/24 (41)	24 (69)/11 (31)
CMVIG prophylaxis, No. (%)	24 (41)	17 (49)
Deceased donor, No. (%)	30 (51)	35 (100)
Living related donor, No. (%)	29 (49)	0 (0)
CMV disease, No. (%)	29 (49)	19 (54)
Severe CMV disease, No. (%)	19 (32)	18 (51)
Deaths, No. (%)	6 (10)	10[a] (29)
Ganciclovir treatment, No. (%)	4 (6)	17 (49)
Days from transplant to start of ganciclovir treatment, median (q25–q75[b])	45.5 (43.4–54.5)	35.5[c] (28.3–38.8)

Abbreviations: CMV, cytomegalovirus; CMVIG, CMV immune globulin.

[a]One liver transplant patient whose death was unrelated to CMV disease and who had no evidence of CMV infection was included in the disease analyses.

[b]q25–q75, median interquartile range.

[c]Excludes 1 liver transplant patient who was treated with ganciclovir until death at day 39.

Association of Viral Burden in D+R- Patients With Severe CMV Disease

In the renal cohort, patients were categorized using both symptoms and evidence of CMV infection as having no disease, mild CMV disease, and severe CMV disease (including severe CMV-associated disease) (Figure 2A; Supplementary Table 1). Among liver patients, those with CMV-associated disease were considered severe and compared with those with no disease (Figure 2B; Supplementary Table 1). In both renal and liver D+R- patients, the diseased subsets had significantly higher mean $\log_{10}$ (AUC) viral loads (renal, severe, 5.73; liver, 6.34) (Supplementary Table 1), a measure of the extent and duration of infection, than patients without disease (4.34 for both renal and liver; $P < .001$) (Supplementary Table 1). Mean $\log_{10}$ (V_{max}) values for both renal and liver transplant patients were also significantly higher in those with disease compared with those without (renal, severe, 4.62 vs 2.66; liver, 5.29 vs 2.63) (Supplementary Table 1). When comparing mild relative with severe disease subsets of renal transplant patients, there were no significant differences in mean $\log_{10}$ (AUC) viral load (5.78 vs 5.73) (Figure 2A; Supplementary Table 1) or mean $\log_{10}$ (V_{max}) (4.62 vs 4.62) (Figure 2A; Supplementary Table 1). For both renal and liver transplant patients, CMVIG status was included in the statistical model to reduce confounding of the association between viral load and disease status.

Association of Viral Load Measures With Mortality Outcomes

Within the group of D+R- renal transplant patients, 29 patients had mild or severe CMV-associated disease, and 6 of these patients died as a result of clinical sequelae associated with CMV infection (Figure 3A; Supplementary Table 2). We saw significant differences ($P < .001$) in mean viral burden between patients who died relative to surviving patients without disease (mean $\log_{10}$ [AUC], 6.13 vs 4.34; mean $\log_{10}$ [V_{max}], 5.14 vs 2.66), but not relative to surviving patients with mild or severe disease (mean $\log_{10}$ [AUC], 5.65; mean $\log_{10}$ [V_{max}], 4.49) (Supplementary Table 2). Among D+R- liver transplant patients, 19 patients had severe CMV-associated disease, and 9 of these patients died (Figure 3B; Supplementary Table 2). Similar to what we observed with renal transplant patients, the mean viral burden in liver transplant patients who died (mean $\log_{10}$ [AUC], 6.39; mean $\log_{10}$ [V_{max}], 5.48) was significantly different ($P < .001$) from that of surviving patients with no disease (mean $\log_{10}$ [AUC], 4.36; mean $\log_{10}$ [V_{max}], 2.65), but not from that of surviving patients with severe disease (mean $\log_{10}$ [AUC], 6.3; mean $\log_{10}$ [V_{max}], 5.11) (Figure 3B; Supplementary Table 2). Although not statistically significant, we did see numerical differences between transplant patients who died vs those who survived with CMV disease; these differences were more pronounced in the renal subsets (Figure 3A; Supplementary Table 2) relative to the liver subsets (Figure 3B; Supplementary Table 2). As in the analysis of association between viral load and morbidity, CMVIG status was included in the statistical model to reduce bias.

Evaluation of Viral Load as a Marker of Mortality and Morbidity in Renal and Liver Transplant Patients

We investigated the potential utility of viral load AUC or V_{max} as a predictor of CMV-related mortality in renal and liver transplant patients by constructing ROC curves for surviving vs nonsurviving transplant patients (Figure 4). The AUROC can be interpreted as the average probability of correctly predicting binary disease status using a threshold based on a given viral load measure (ie, AUC or V_{max}). The AUROCs for predicting mortality in renal transplant patients using viral load AUC and V_{max}, respectively, were 0.796 and 0.824 (Figure 4A) and 0.769 and 0.807 in liver transplant patients (Figure 4B). Using similar analyses, we found that viral load could also predict severe CMV disease (Supplementary Figure 1).

Viral Load in Relation to Use of CMVIG

We also analyzed viral load in relation to CMVIG. There was a modest effect among those who received CMVIG

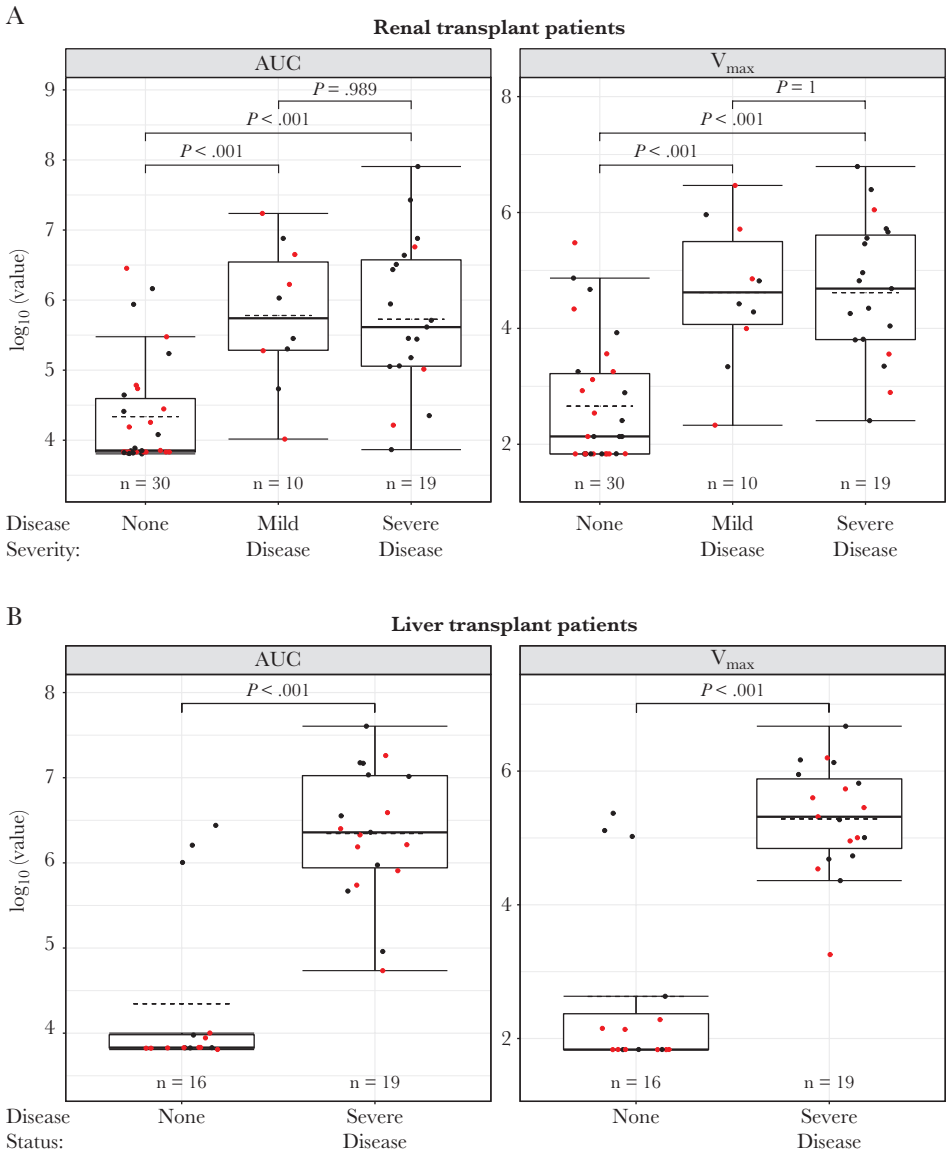

Figure 2. Cytomegalovirus (CMV) viral burden in (A) renal transplant patients and (B) liver transplant patients with or without CMV disease. Viral load areas under the curve (AUCs) and peak viral loads (V_{max}) were determined in subsets of patients with no disease, mild disease, or severe CMV disease and included CMV-associated disease. *P* values were calculated using the Tukey-Kramer test. Circles represent individual patients within each subset given CMV immune globulin (red) or placebo (black); boxes represent interquartile range; whiskers represent the upper and lower 25% of values; bold lines represent group median values; dashed lines represent group mean values.

prophylactically, both in the delayed timing of the first onset of viremia and in the decreased proportion of those with viremia and those with a viral load over 2000 IU/mL (Supplementary Figure 2; Supplementary Table 3). As mentioned previously, we accounted for the use of CMVIG in our modeling of viral load on morbidity and mortality.

DISCUSSION

Historically within fields of infectious diseases such as HIV, hepatitis B, and hepatitis C, viral burden has been directly related to outcome with respect to disease [9, 28, 29]. Although CMV viral load has been shown to be related to the development of disease with CMV infection [1, 14, 30] and mortality in a study of hematopoietic stem cell transplantation [13], this is the first study to demonstrate an association with mortality as well as severity of disease in SOT recipients. This study has several advantages: the availability to use serial samples, the lack of any specific antiviral therapy for CMV disease in the vast majority of patients, the blinded application of predetermined definitions of severe disease that were not related to viral load measurements, and the performance of laboratory analyses by investigators without knowledge of end points. In addition, the assay to quantify viral burden was performed using the WHO

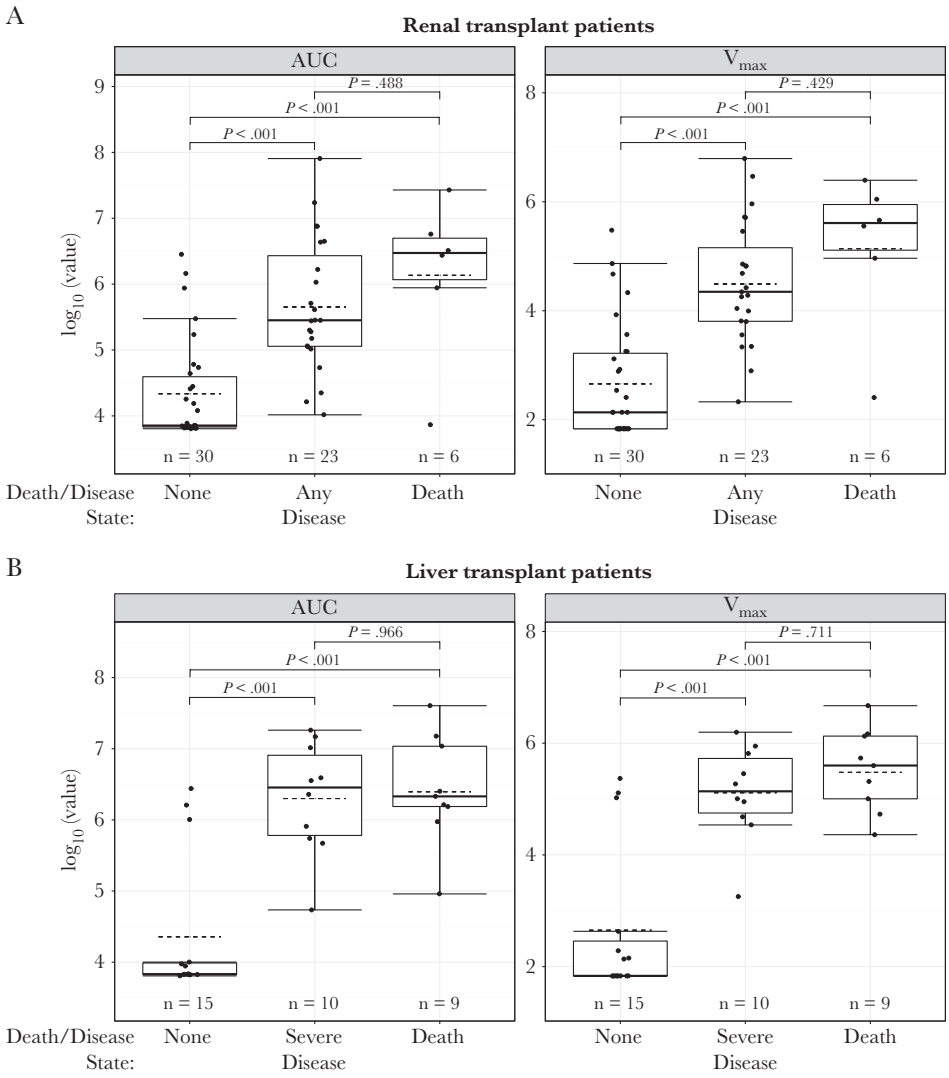

Figure 3. Association of cytomegalovirus (CMV) viral burden with disease or mortality in (A) renal transplant patients and (B) liver transplant patients. Areas under the curve (AUCs) and peak viral loads (V_{max}) were determined in subsets of patients who died as a result of severe CMV or CMV-associated disease (renal, n = 6; liver, n = 9) compared with survivors (renal, n = 53; liver, n = 25). Pairwise comparisons of AUC and V_{max} were performed using the Tukey-Kramer test. Black circles represent individual patients within each subset; boxes represent interquartile range; whiskers represent the upper and lower 25% of values; bold lines represent group median values; dashed lines represent group mean values.

International Standard for CMV [21] that was not available at the time these studies were conducted. Our data demonstrate the relationship of viral load to serious clinical outcomes with respect to CMV infection and disease, including associated mortality.

This analysis has some limitations. First, we measured viral load in serum, not plasma; however, in serum, the assay performance was similar to results generated in EDTA plasma (data not shown). In addition, the specimens were used for multiple other analyses [30–33], and therefore subjected to several freeze/thaw cycles. We cannot, therefore, directly compare the exact values to values generated in current studies, nor can we consider these specific values to be predictors of outcomes. Another possible limitation derives from using data from D+R- patients, who are at highest risk of developing CMV infection and disease. Whether we can generalize the results to other serologic patient categories (D+R+, D-R-, D-R+) remains to be determined.

This study is unique because ganciclovir was rarely administered to the patients enrolled in the renal study [27]; although ganciclovir became available during the liver study, its use was still infrequent. Ganciclovir treatment was used in some patients with severe disease, and its use would have biased the results toward a lower viral load in the AUC, reducing the association with mortality. However, despite such use in 4 patients in the renal transplant study and 17 patients in the liver transplant study (Table 1), the association remained quite robust.

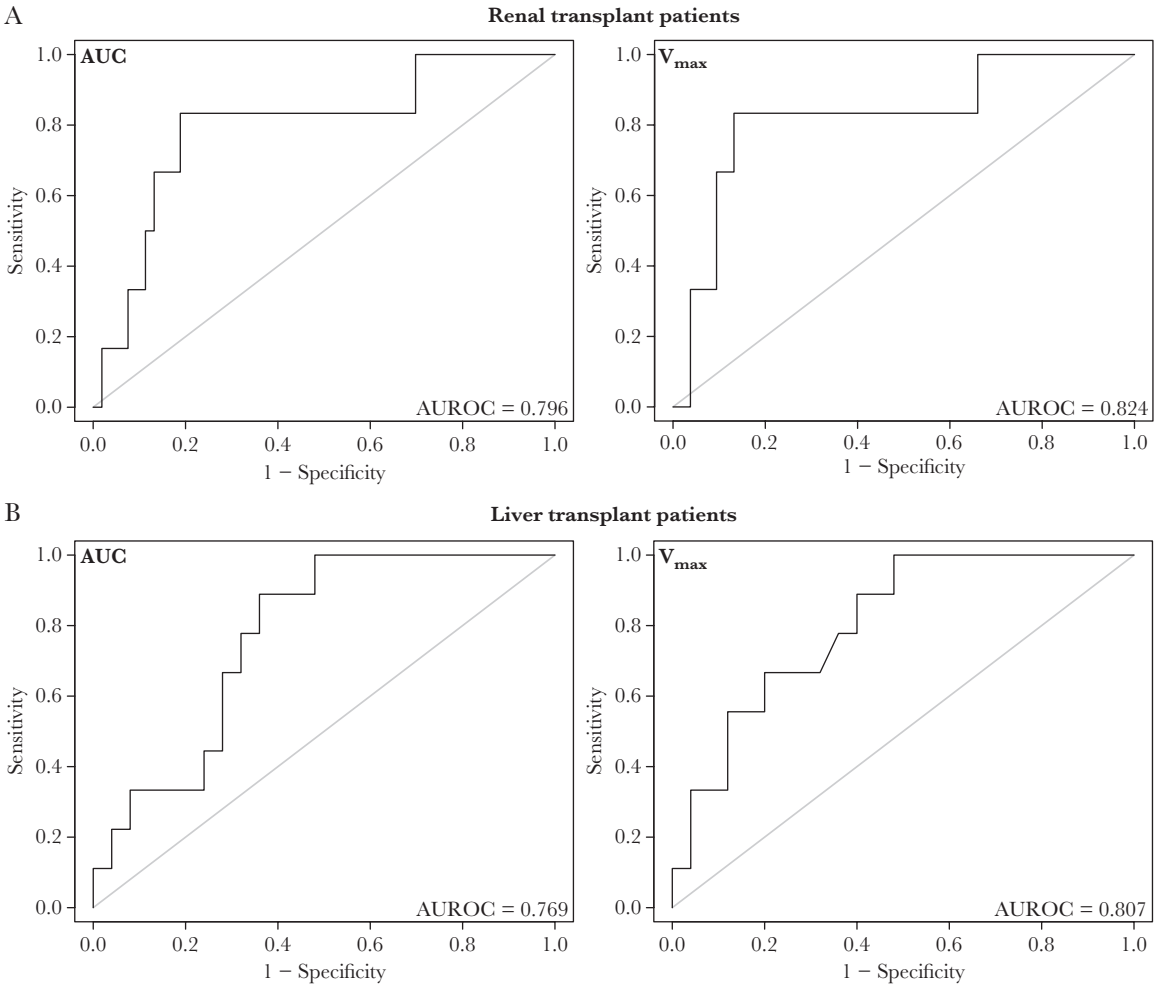

Figure 4. Receiver operating characteristic (ROC) curves for predicting mortality in renal and liver transplant patients using CMV viral load area under the curve (AUC) and peak viral load (V_{max}). Abbreviation: AUROC, area under the ROC curve.

In summary, we demonstrated a very strong association between the degree of CMV viral load and the occurrence of severe CMV disease and mortality in both renal and liver transplant recipients. Although these clinical end points are meaningful and relevant, assessing how new treatments affect them has become challenging due to the use of antivirals. Ideally, these findings would need to be confirmed in other replicative cohorts, but the typical current-day use of prophylactic and preemptive treatment of CMV infection is such that sample sets from patients with CMV disease, like those used in this study, are difficult to obtain. Nevertheless, these data strongly suggest that CMV viral load can be used as a surrogate marker for severe CMV disease and CMV-associated mortality. We therefore recommend the adoption of viral load as a primary end point in future trials to better evaluate the efficacy of potential anti-CMV therapies or vaccines.

Supplementary Data

Supplementary materials are available at Open Forum Infectious Diseases online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Acknowledgments

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Potential conflicts of interest. J.M. McBride, D. Sheinson, J. Jiang, N. Lewin-Koh, and J.A. Tavel are employees of Genentech, Inc., a member of the Roche group, and own Roche stock. X. Wu, who was an employee of Roche Molecular Systems at the time of the study, is currently an employee of Genentech, Inc. and owns Roche stock. D.R. Snydman has received a grant for this work from Genentech, Inc., a grant from Merck, and funds for consulting from Chimerix, Merck, Shire, and Moderna. B. Werner and J.K. Chow have no conflicts of interest to declare. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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