Interrelationships among Quantity of Human Cytomegalovirus (HCMV) DNA in Blood, Donor-Recipient Serostatus, and Administration of Methylprednisolone as Risk Factors for HCMV Disease following Liver Transplantation

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Human cytomegalovirus (HCMV) is a recognized cause of both morbidity and mortality following solid organ transplantation [1, 2]. HCMV is the most common cause of infection following liver transplantation, with an incidence of 20%–60%, and ~50% of infected patients will proceed to develop HCMV disease. Risk factors for disease include receipt of large doses of immunosuppressive therapy, transplantation of an organ from an HCMV-seropositive donor into an HCMV-seronegative recipient, and the appearance of viremia [2–5]. Clinical manifestations appear typically within the first 3 months after transplant and range from persistent fever and mononucleosis-like syndrome to more severe invasive organ disease, such as HCMV hepatitis, gastrointestinal disease, and pneumonia [6].

With the development of effective antiviral agents against HCMV, the emphasis has focused more recently on earlier intervention strategies to reduce incidence of disease [7–12]. A randomized, controlled prophylactic trial of ganciclovir versus high-dose acyclovir showed ganciclovir to be more effective at preventing HCMV disease [7, 10]. Indeed, one randomized trial has shown short-course preemptive ganciclovir therapy to be more effective than high-dose acyclovir at preventing HCMV disease [9]. The results of many studies have confirmed that rapid detection of HCMV DNA-emia via polymerase chain reaction (PCR) of plasma, sera, and whole blood can provide prognostic information for liver transplant recipients [13–20], suggesting that preemptive therapy could be used in these patients, as has been described for HCMV infection following bone marrow transplantation [21].

Stagno et al. [22] in 1975 first reported the relationship between the quantity of HCMV and the development of disease. They demonstrated that HCMV virus titer was directly related to disease in congenitally infected infants [22]. Recent cross-sectional and longitudinal studies in human immunodeficiency virus–positive patients and renal transplant and bone marrow transplant patients have shown that quantity of virus (HCMV) is usually higher in patients with symptomatic infection [23–27]. In liver transplant recipients, one study using a semiquantitative PCR assay to follow 40 liver transplant patients showed a significant correlation between high levels of HCMV DNA in blood and development of disease [28]. However, no detailed longitudinal studies of liver transplant recipients have been done using fully quantitative PCR methods to determine the relative contributions of virus load in the context of other risk factors for disease; we now report such a study.

Materials and Methods

Study population. Between November 1992 and April 1996, 162 patients with a total of 1433 surveillance blood samples were followed prospectively for HCMV infection by a qualitative PCR assay, as previously described [29–31]. In this context, viremia was defined as qualitatively PCR-positive in blood. The median age of patients in this study was 50 years (range, 9–70).

An immunosuppressive regimen was initiated immediately after transplant, consisting of triple therapy of azathioprine (1.5 mg/kg daily), methylprednisolone (0.8 mg/kg daily), and cyclosporin A...
(4 mg/kg/24 hr intravenously [iv]) from days 1 to 3 after transplant, adjusted according to plasma levels and renal function of the patient. Moderate to severe rejection, diagnosed histologically, was treated with methylprednisolone (1 g daily iv for 3 days), which was repeated if a subsequent rejection episode occurred. Steroid-resistant rejection was treated with either OKT3 for 5 days or antithymocyte globulin for 10 days, and cyclosporin was replaced with FK506 (0.1 mg/kg daily) in the case of chronic rejection.

Ampicillin (1 g four times daily iv), netilmicin (3.5 mg/kg twice daily iv), and metronidazole (500 mg three times daily iv) were given for bacterial prophylaxis and amphotericin (5 mL four times daily) for fungal prophylaxis. No antiviral prophylaxis was given for treatment of HCMV infection. However, patients who were herpes simplex virus (HSV) antibody-positive received low-dose acyclovir (5 mg/kg three times daily iv initially, followed by 200 mg four times daily orally) for at least 1 month after transplant. In patients with diagnosed HCMV disease, immunosuppressive therapy was reduced, and iv ganciclovir was administered at 5 mg/kg twice daily (adjusted according to renal function). For those diagnosed with HCMV pneumonitis, human immunoglobulin was given iv, in addition to ganciclovir. If the patient did not respond, foscarin (60 mg/kg every 8 h or 90 mg/kg every 12 h) was given.

Pretransplant sera from donors and recipients were tested for HCMV by EIA for IgG antibodies (Biokit, Barcelona, Spain). The recipient was also tested for HSV IgG antibodies by EIA (Biokit) according to the manufacturer’s instructions.

Samples of urine and blood were taken weekly until discharge, at subsequent regular outpatient clinic visits, and if clinically indicated and were tested by conventional cell culture and PCR for HCMV. Liver biopsies were taken, as indicated clinically, and processed for histology and cell culture by detection of early antigen fluorescent foci (DEAFF).

**PCR and quantitative-competitive PCR assays.** The PCR assays to detect HCMV qualitatively and quantitatively have been described [26, 29–31]. DNA was extracted from 200 μL of whole blood using commercially available extraction columns (Qiagen, Crawley, UK) according to the manufacturer’s instructions. An extract (5 μL) was used for the PCR analysis (equivalent to ~40 ng of cellular DNA).

**Clinical diagnosis.** HCMV infection was diagnosed by PCR, culture, DEAFF, or histology. HCMV disease was based on the criteria of the International CMV Workshop [32] as typical features of HCMV infection resulting in symptoms associated within 2 weeks of detection of virus as follows: (1) pyrexia with viremia (fever of ≥38°C for ≥48 h in the absence of rejection or bacterial or fungal infection associated with viremia), (2) hepatitis (alteration in liver function tests [alanine aminotransferase, ALT] at least 2 times the upper limit of normal in the absence of bacterial or fungal infection or in association with HCMV detection in liver biopsy by virologic and/or histologic techniques), or (3) pneumonitis (symptoms of hypoxia and/or characteristic interstitial chest radiograph pattern, unresponsive to antibiotics and with HCMV infection detected in bronchoalveolar lavage fluid).

**Statistical methods.** The significance of differences between maximum virus loads in each group was assessed by the Mann-Whitney U test. Univariate analysis was used to examine the development of disease and the factors of interest using Fisher’s exact test, with continuity adjustment as required (viremia, donor serostatus, and augmented prednisolone), or by the Mann-Whitney test (differences in virus load). Significant relationships were then quantified using logistic regression analysis, and the results were used to generate an equation to determine the probability of being symptomatic at any given virus load in the presence or absence of methylprednisolone in an independent manor irrespective of the timing of HCMV infection. For these analyses, odds ratios quoted refer to each 0.25 log_{10} increase in virus load and each 1-g increase in methylprednisolone.

**Results**

Prospective follow-up of 162 liver transplant patients by PCR for HCMV using 1433 blood samples identified 51 patients with HCMV viremia. Forty-seven patients had detailed analysis performed on all qualitatively PCR-positive blood samples using quantitative-competitive PCR [30]. The remaining 4 patients were asymptomatic and had single PCR-positive samples, which contained insufficient sample for quantification. Of the 51 patients, 20 (40.4%) experienced HCMV disease compared with none of the remaining 111 patients (Mann-Whitney U test, P < .0001). In the 20 symptomatic patients, the median time between date of transplant and onset of disease was 43.5 days (range, 25–126). The maximum virus load detected during the posttransplant surveillance period ranged from 10^{3.59} to 10^{5.77} genomes/mL of blood (median, 10^{4.92}).

Representative longitudinal profiles of virus load and corresponding ALT levels are shown in figure 1. Patients with persistent viremia and liver biopsy–proven HCMV hepatitis showed a temporal association between modulations in ALT levels and virus load (figure 1, patients 1 and 2). Patients with intermittent episodes of viremia who had no evidence of HCMV hepatitis did not have any substantial increases in ALT levels in conjunction with high virus loads (figure 1, patients 3 and 4). Patients who remained asymptomatic had relatively low virus loads and showed no significant associations between ALT levels and virus load during the period of HCMV infection (figure 1, patients 5 and 6).

The relationships among HCMV disease, maximal posttransplant virus load, and donor-recipient serostatus for HCMV are shown in figure 2. The median peak virus load in blood of the symptomatic patients was 10^{5.65} genomes/mL (range, 10^{4.94}–10^{5.37}), which was significantly higher than that observed in the asymptomatic group (median, 10^{4.48}–10^{5.3}; P < .0001, Mann-Whitney test). In the D+/R− group of patients with primary infection, the median virus load (10^{4.48} genomes/mL; range, 10^{3.99}–10^{5.10}) was significantly higher than in the D−/R+ group experiencing reactivation of latent HCMV (median, 10^{3.40} genomes/mL; range 10^{3.66}–10^{4.92}; P < .01, figure 2B). There was also a significant difference in virus load between patients at risk of either reactivation or reinfection (D+/R+; median virus load, 10^{4.93} genomes/mL; range, 10^{3.64}–10^{5.30}; n = 19) and patients reactivating their own latent virus (D−/R+; P < .05, Mann-Whitney test).
Figure 1. Longitudinal profiles of relationship between virus load (○) and alanine aminotransferase (ALT: □) levels in symptomatic patients with biopsy-proven HCMV (patients 1 and 2), those with intermittent viremia and symptoms (patients 3 and 4), and asymptomatic patients (patients 5 and 6). GCV, ganciclovir.
In the D+/R+ group, the 9 patients who had HCMV virus loads above the median \((10^{4.94}\text{ genomes/mL})\) were significantly more at risk of disease than the 10 patients with levels below the median virus load \((P = .02, \text{ Fisher’s exact test})\). Virus load was also significantly higher in the symptomatic patients (median, \(10^{5.57}\text{ genomes/mL}\); range, \(10^{4.94} - 10^{7.53}\); \(n = 7\)) versus the asymptomatic patients (median, \(10^{4.58}\text{ genomes/mL}\); range, \(10^{3.64} - 10^{5.3}\); \(n = 12\), \(P < .01, \text{ Mann-Whitney U test}\)). The symptomatic patients in the D+/R+ group had a median virus load comparable to that of symptomatic patients within the primary infection group.

Univariate analysis revealed that virus load, donor seropositivity, and total methylprednisolone administered for rejection episodes were all risk factors for HCMV disease, with odds ratios (ORs) of 2.22, 4.11, and 1.30, respectively (table 1). The relative contributions of each of these risk factors was then quantified by multivariate logistic analyses; the data are shown in table 2. In bivariate logistic models, the risk associated with donor seropositivity was negated once virus load had been controlled for (OR, 3.24; \(P = .25\)) but was unaffected by controlling for administration of methylprednisolone. The multivariate analyses showed that both elevated virus load and total dose of methylprednisolone were independent risk factors for HCMV disease, with ORs of 2.70 (per 0.25-log\(10\) increase) and 1.61 (per 1-g increase), respectively.

On the basis of the logistic regression analysis, we constructed disease probability curves for increasing virus loads in the univariate setting (figure 3A) and similar curves to investigate the influence of total methylprednisolone on the virus load–disease curve in the multivariate setting (figure 3B). The graph illustrates a rapid increase in the probability of disease

**Table 1.** Identification of virus load, donor-recipient serostatus at baseline, and receipt of methylprednisolone immunosuppression as risk factors for HCMV disease, by univariate analysis.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Odds ratio</th>
<th>95% confidence interval</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus load (per 0.25 log(10))</td>
<td>2.22</td>
<td>1.37–3.59</td>
<td>.001</td>
</tr>
<tr>
<td>Donor-recipient serostatus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D+/R−</td>
<td>4.11</td>
<td>1.02–16.67</td>
<td>.05</td>
</tr>
<tr>
<td>D−/R+</td>
<td>0.17</td>
<td>0.02–1.64</td>
<td>.13</td>
</tr>
<tr>
<td>D+/R+</td>
<td>1.0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Methylprednisolone (per 1 g)</td>
<td>1.30</td>
<td>1.05–1.60</td>
<td>.01</td>
</tr>
</tbody>
</table>
Table 2. Use of multivariate logistic regression models to examine pairs of the risk factors identified by previous univariate analyses.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Odds ratio</th>
<th>95% confidence interval</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>Virus load and D/R serostatus</td>
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</tr>
<tr>
<td>Virus load (per 0.25 log10)</td>
<td>2.15</td>
<td>1.26–3.66</td>
<td>.005</td>
</tr>
<tr>
<td>D+/R–</td>
<td>3.24</td>
<td>0.43–24.33</td>
<td>.25</td>
</tr>
<tr>
<td>D–/R+</td>
<td>1.36</td>
<td>0.09–21.58</td>
<td>.83</td>
</tr>
<tr>
<td>D+/R+</td>
<td>1.0</td>
<td></td>
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<tr>
<td>Methylprednisolone and D/R serostatus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D+/R–</td>
<td>4.80</td>
<td>0.98–23.51</td>
<td>.05</td>
</tr>
<tr>
<td>D–/R+</td>
<td>0.07</td>
<td>0.004–1.29</td>
<td>.07</td>
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<tr>
<td>D+/R+</td>
<td>1.0</td>
<td></td>
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</tr>
<tr>
<td>Virus load and methylprednisolone</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Virus load (per 0.25 log10)</td>
<td>2.70</td>
<td>1.41–5.17</td>
<td>.003</td>
</tr>
<tr>
<td>Prednisolone (per 1 g)</td>
<td>1.61</td>
<td>1.04–2.51</td>
<td>.03</td>
</tr>
</tbody>
</table>

NOTE. D/R = donor-recipient.

In this study, high HCMV virus load in blood was clearly a major risk factor for disease in liver transplant patients. Thus, patients experiencing symptomatic disease had a consistently higher median virus load than did patients who remained asymptomatic (P < .0001). The risk factors for disease of administration of augmented methylprednisolone and donor seropositivity identified in this study are consistent with previous data from our group and others [3–5, 33]. By using multivariate logistic regression analysis, the association of donor-recipient serostatus as a risk factor was explained by elevated virus load.

Discussion

In this study, high HCMV virus load in blood was clearly a major risk factor for disease in liver transplant patients. Thus, patients experiencing symptomatic disease had a consistently higher median virus load than did patients who remained asymptomatic (P < .0001). The risk factors for disease of administration of augmented methylprednisolone and donor seropositivity identified in this study are consistent with previous data from our group and others [3–5, 33]. By using multivariate logistic regression analysis, the association of donor-recipient serostatus as a risk factor was explained by elevated virus load.
Therefore, we suggest that virus load is the mechanism through which this risk factor is associated with disease (i.e., patients with primary infection have HCMV disease because of a high virus load).

In contrast, both methylprednisolone and elevated virus load were identified in the multivariate analysis as independent risk factors for disease. This observation implies that receipt of large quantities of methylprednisolone for rejection episodes predisposes the individual to HCMV disease at lower virus loads. In contrast, high virus loads are necessary to produce pathologic consequences in patients who experience relatively few rejection episodes and therefore require lower levels of augmented methylprednisolone.

The disease probability–virus load curves computed from the logistic regression analysis support the interpretation that HCMV disease is imminent once specific virus loads are reached. Thus, a 50% probability of disease was reached at a virus load of $10^{11}$ genomes/mL of blood, and a 90% probability of disease was reached at $10^{5.3}$ genomes/mL without consideration of methylprednisolone dose. The influence of increasing amounts of methylprednisolone on the virus load–disease probability curve was to shift this curve to lower virus loads. Hence, at a methylprednisolone level of 12 g, the 50% disease probability level was reached at a virus load of $10^{10.1}$ genomes/mL of blood (i.e., a 10-fold lower peak virus load than in patients receiving no methylprednisolone). This result supports the clinical practice of reducing steroid therapy, whenever possible, in the case of HCMV infection after transplant. Further analysis with more patients will elucidate whether the temporal increase in virus load can be used to direct preemptive therapy in patients most at risk of HCMV disease and suggests that controlled trials of such interventions should stratify patients according to dose of augmented methylprednisolone received.

The longitudinal virus load analysis undertaken in this study also provides insight into the pathogenesis of HCMV. The D+/R+ group had an intermediate risk of HCMV disease compared with patients suffering primary infection (D+/R−) and patients reactivating latent HCMV (D−/R+). High virus loads within the D+/R+ group were associated with increased risk of disease. Since high virus loads are associated with the D+/R− group, it is likely that D+/R+ patients with high virus loads were more likely to be experiencing primary infection than reactivation. This differential requires formal confirmation by restriction fragment length polymorphism studies of larger numbers of patients in the D+/R+ category.

Various strategies have been used in attempts to identify and reduce disease incidence in patients at risk of HCMV disease by applying different treatment regimens [5, 7–12]. Prophylaxis, although effective in reducing HCMV disease, also exposes patients not destined to develop disease to potentially toxic antiviral drugs. Thus, the need to find a reliable marker for disease is of paramount importance. In this study, it was possible to identify individuals most at risk for disease by using a combination of quantitative PCR, donor-recipient serostatus, and augmented methylprednisolone administration. The use of virus load increases above critical levels to identify patients at risk of future disease is an attractive option, but further studies are required to ascertain whether such an approach would be applicable in the clinical setting.

In conclusion, the detailed analysis of patients by measurement of virus load in surveillance samples may offer the possibility of designing algorithms that will predict whether a patient will go on to develop HCMV disease. Such monitoring, together with the knowledge of other risk factors, such as donor seropositivity and methylprednisolone usage, not only may identify patients at risk of disease but also will help prevent patients receiving unnecessary treatment with antiviral therapy.

References