Time to Detection of Cytomegalovirus (CMV) DNA in Blood Leukocytes Is a Predictor for the Development of CMV Disease in CMV-Seronegative Recipients of Allografts from CMV-Seropositive Donors following Liver Transplantation

Rafael Manez, Shimon Kusne, Charles Rinaldo, Jose Maria Aguado, Kirsten St. George, Paolo Grossi, Bonnie Frye, John J. Fung, and Garth D. Ehrlich

Departments of Surgery, Medicine, and Pathology, University of Pittsburgh, Pennsylvania

In 35 cytomegalovirus (CMV)-seronegative recipients of livers from CMV-seropositive donors, 32 (91%) developed CMV infection and 24 of them (75%) experienced disease. Polymerase chain reaction for CMV DNA in leukocytes had the best positive and negative predictive values for the development of disease within 2 months from transplantation, and shell-vial or tube culture viremia was the best predictor thereafter. In patients who developed CMV disease, CMV DNA was first detected at 46 days (median; range, 13–128) after transplantation, significantly earlier than the 77 days (range, 46–174) for those who did not develop CMV disease (P = .02). By a semiquantitative method, the CMV DNA level in the first positive sample did not predict disease development. However, the maximum CMV DNA level during infection was significantly higher in patients who developed CMV disease. In CMV-seronegative recipients of livers from CMV-seropositive donors, the time to DNA positivity following transplantation may predict disease progression and be useful as a guide for the initiation of preemptive therapy.

Cytomegalovirus (CMV)-seronegative recipients of grafts from CMV-seropositive donors are at the highest risk for development of symptomatic CMV infection after organ transplantation. Use of high-dose oral acyclovir and CMV hyperimmune globulin in these patients can prevent the development of disease in kidney transplant recipients [1, 2]. The same regimens, however, have failed to provide protection in liver recipients [3, 4]. The prophylactic use of ganciclovir immediately after transplantation has only marginal benefit in decreasing the incidence of disease in CMV-seronegative recipients of liver allografts from CMV-seropositive donors [3].

Virus excretion or shedding precedes CMV disease in transplant recipients. These markers have recently been used to identify patients at the highest risk for CMV disease [5]. Moreover, a short course of ganciclovir given as preemptive therapy when shedding occurred prevented progression to disease [5]. However, almost all of the patients enrolled in that study were CMV-seropositive recipients. It is not clear whether shedding occurs, and for how long before disease develops, in CMV-seronegative recipients of grafts from CMV-seropositive donors. Since preemptive therapy could be very useful in these transplant recipients, we investigated which laboratory parameter detects CMV earlier and, therefore, would be useful to guide the initiation of antiviral therapy in this high-risk population.

Materials and Methods

Study population. From January 1992 to July 1993, 35 CMV-seronegative liver transplant recipients of grafts from CMV-seropositive donors were prospectively followed until CMV disease developed or for 6 months after transplantation if they did not develop CMV disease. Two patients received intestinal grafts along with the liver graft.

Immunosuppression included tacrolimus (FK 506) and steroids as previously described [6]. All of the patients received CMV prophylaxis with ganciclovir (Syntex, Palo Alto, CA) given intravenously at 5 mg/kg twice a day for the first 14 days after transplantation, followed by oral acyclovir at 3200 mg/day until 12 weeks of therapy [3]. The dosages of both ganciclovir and acyclovir were adjusted for renal function.

Virologic and serologic studies. Donor and recipient CMV IgG and IgM antibodies were measured in all patients by a semiautomated immunofluorescence test (FIAX Test System; Bio-Whittaker, Walkersville, MD). IgG CMV antibody titers >20 were considered positive. IgM CMV antibody tests were reported as negative, equivocal, or positive. Weekly samples of blood, urine, and throat swabs for CMV culture and serum for serology were obtained during the first 3 months after transplantation and twice a month thereafter. Additional cultures and serum samples were taken during any hospital admission or clinical illness.

Urine and throat swab specimens were examined for CMV in both tube culture and shell-vial assay for early antigen detection, as previously described [7]. Blood buffy coat leukocytes were cultured for CMV by tube culture and shell-vial assay. CMV DNA from the leukocyte preparations was amplified by the polymerase chain reaction (PCR), and the levels were evaluated by a semiquantitative method.
Diagnosis of CMV infection and disease. CMV infection was defined by IgG seroconversion with or without development of IgM antibodies, detection of CMV DNA in the blood, or isolation of CMV from urine, throat, or blood. Asymptomatic infection was defined by CMV IgG seroconversion or detection of CMV by any laboratory method in the absence of symptoms. Symptomatic infection was defined according to the criteria of the Paris CMV workshop [8] and included proved CMV disease and presumptive CMV disease. Proved CMV disease required symptoms or signs (or both) from an affected organ plus CMV detected from that organ. Presumptive CMV disease required CMV viremia by either shell-vial assay or standard culture along with fever >38°C for ≥2 days in the absence of another clinical source, combined with one of the following findings: atypical lymphocytosis >3%; white blood cell count <4000/mm³, or platelets <100,000/mm³.

PCR-based amplification and detection of CMV DNA. All PCR-based analyses were done in the University of Pittsburgh’s Core PCR Facility, which was specifically designed and staffed to minimize the problems inherent in nucleic acid-based amplification systems [9].

Leukocyte specimens were prepared for PCR amplification by a robotic pipetting station (Biomek 1000; Beckman Instruments, Fullerton, CA) customized with a computer-controlled heating and cooling block for enzymatic digestions. The specimens were individually loaded into the thermal-control block where the robot pipetted 25 µL of each of lysis solutions A and B [9] (containing proteinase K [PK]) into each specimen before the temperature was ramped to 60°C for 1 h for proteinase digestion. This was followed by a denaturation step at 99°C for 15 min, cooling to 4°C, and addition of the PCR master mix cocktail to each specimen; the cocktail was prepared by the robot in a separate block during the denaturation step. The master mix was formulated to contain sufficient extra water to replace the loss due to evaporation that occurred during the specimen heating cycles. The tubes were then sealed and transported to the amplification laboratory for thermal cycling.

All specimens were tested in duplicate, on a blood volume basis, using a multiplex PCR-based assay that simultaneously supports amplification of three nonoverlapping CMV genomic target sequences [10]. Therefore, 6 data points were generated per specimen. In addition, all specimens were checked for their ability to support amplification of a human test gene, β-actin, in a parallel reaction. Multiple negative control specimens, included in each experimental run, were prepared by adding a mock lysate prepared without leukocytes to the PCR reagent master mix cocktail.

PCR products were detected by a multiplex liquid hybridization assay of the amplified DNAs with 32P end-labeled oligonucleotide probes located internally to the amplification primers. The assay was read by gel retardation analysis of the hybridization products, followed by autoradiography [10]. Patient specimens were analyzed semiquantitatively by comparing their signal intensity with that generated by a dilution series of CMV genomic controls using visual discrimination by an experienced clinical molecular diagnostician. For each set of specimens analyzed, a positive control panel consisting of 1 pg, 100 fg, and 10 fg of the control viral genomic DNA was amplified corresponding to ~3000, ~300, and ~30 genomic equivalents of CMV, respectively. A signal of 4+ was >3000 copies, a signal of 3+ was ~3000 copies, a signal of 2+ was ~300 copies, and a signal of 1+ was ~30 copies.

### Table 1. Time to infection and CMV DNA signals in infected patients with and without disease.

<table>
<thead>
<tr>
<th></th>
<th>Disease (n = 24)</th>
<th>No disease (n = 8)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median days to infection (range)</td>
<td>46 (13-128)</td>
<td>77 (46-174)</td>
<td>.02</td>
</tr>
<tr>
<td>First positive blood PCR signal</td>
<td>0 1</td>
<td>2 7</td>
<td>NS*</td>
</tr>
<tr>
<td>Maximum blood PCR signal</td>
<td>0 1</td>
<td>2 3</td>
<td>.005*</td>
</tr>
</tbody>
</table>

* Comparison between ≥3+ signals to <3+ signals. PCR, polymerase chain reaction; NS, not significant.

The control DNA used for comparative analysis was obtained by phenol-chloroform extraction of density-banded CMV virus purchased from Advanced Biotechnologies (Columbia, MD). This DNA was quantitated by averaging multiple UV absorption spectrophotometric results at A260nm. Viral genomic copy number was calculated on the basis of a 240-kb genome. The analytic sensitivity of the assay was retrospectively determined to be one input target molecule (unpublished data) using limiting dilution of a plasmid clone containing the late antigen gene, pp64 (gift of M. Stinski, University of Iowa, Ames).

Statistical analysis. The Mann Whitney U test, a nonparametric test equivalent to the standard two-sample t test, was used to compare medians. Association between the maximum level of CMV DNA and development of disease was assessed by Fisher’s exact test.

Results

Of 35 patients, 32 (91%) developed CMV infection and 24 of them (75%) experienced disease. Definitive CMV disease was present in 18 patients (75%), whereas the other 6 patients developed presumptive CMV disease. Median times from transplantation were 56 days (range, 13–174) to the development of infection and 68 days (range, 31–128) to the appearance of disease. In patients who experienced disease, the infection occurred at a median of 46 days (range, 13–128) after transplantation, which was significantly earlier than the median interval of 77 days (range, 46–174) for patients who did not develop disease (P = .02; table 1). In 16 patients, CMV disease occurred within 2 months from transplantation; in the other 8, it occurred after 2 months.

PCR for CMV DNA in leukocytes was the first positive test in 29 (91%) of the 32 patients who developed CMV infection, preceding positive results by all other methods a median of 7 days (range, 0–43). In 2 asymptomatic patients, leukocytes...
remained negative for CMV DNA during the entire study period, despite positive urine and throat swab cultures plus IgM seropositivity and IgG seroconversion. On the other hand, 1 of the 2 patients, who received a combined liver-intestine transplant, experienced enteritis diagnosed by histopathology and isolation of CMV by shell-vial and tube culture in the intestinal biopsy with repeatedly negative viremia, as determined by shell-vial, tube culture, and PCR.

CMV was detected by PCR in 96% of patients who developed CMV disease and by shell-vial or tube culture (or both) in 83%. As shown in figure 1, CMV DNA preceded the development of disease by a median of 11 days (range, 0–35). In contrast, CMV shedding in the urine or throat, as detected by shell-vial or tube culture, did not precede the development of disease. Of the 16 patients who developed disease within 2 months after transplantation, 14 (88%) were positive for CMV DNA prior to the disease. In contrast, only 2 of the 8 patients who developed disease >2 months after transplantation were positive for CMV DNA before the development of disease.

Table 2 shows the positive and negative predictive value of qualitative CMV DNA in leukocytes and shell-vial or tube culture in blood buffy coats and urine for the development of disease. Whereas CMV DNA had the best positive predictive value within 2 months of transplantation (88%), shell-vial or tube culture in blood buffy coats was the best predictor for the development of disease thereafter (100%). CMV DNA had the best negative predictive value at any time after transplantation. This was 100% in patients who developed disease >2 months after transplantation and 75% in those who developed disease within 2 months after transplantation, as a consequence of the false-negative result from the patient who received a combined liver-intestine transplant.

The level of CMV DNA in the first positive sample did not predict the development of disease (table 1). However, the overall maximum level of CMV DNA in the blood during the infection correlated with the appearance of disease: High levels (≥3+) were found in 79% of symptomatic and in only 25% of asymptomatic patients (odds ratio, 11.4; 95% confidence interval, 1.3%–135.4%; P = .005).

Discussion

The most significant factor associated with development of CMV disease in this cohort of patients was the time from

Table 2. Positive and negative predictive value of qualitative CMV DNA in blood leukocytes and shell-vial or tube culture in blood and urine for the diagnosis of CMV disease ≤2 and >2 months after transplantation.

<table>
<thead>
<tr>
<th></th>
<th>≤2 months after transplantation (n = 16)</th>
<th>&gt;2 months after transplantation (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive predictive value, % (true positive/false positive)</td>
<td>Negative predictive value, % (true negative*/false negative)</td>
</tr>
<tr>
<td>CMV DNA</td>
<td>88 (15/2)</td>
<td>75 (3/1)</td>
</tr>
<tr>
<td>Blood</td>
<td>83 (5/1)</td>
<td>27 (4/11)</td>
</tr>
<tr>
<td>Urine</td>
<td>86 (6/1)</td>
<td>29 (4/10)</td>
</tr>
</tbody>
</table>

* 3 patients who did not develop CMV infection are also included as true negatives.
transplantation to infection. Earlier infections appeared to have higher virus load, most likely because the immunosuppression was more intense and was usually associated with disease. CMV DNA amplification by PCR in leukocytes and detection of CMV by shell-vial or tube culture in blood buffy coats were the methods that better predicted CMV disease, depending on the time after transplantation. When infection occurred within 2 months after transplantation, CMV DNA amplification by PCR in leukocytes showed the best positive and negative predictive value for the development of CMV disease. However, when the infection developed >2 months after transplantation, viremia detected by shell-vial or tube culture (or both) was the best predictor of disease.

The low negative predictive value of viremia detected by shell-vial or tube culture early after transplantation may be secondary to antiviral prophylaxis. As we previously showed, both high-dose acyclovir and ganciclovir influence the frequency of detection of CMV by shell-vial and tube culture [7]. CMV shedding in the urine or throat, as detected by shell-vial or tube culture, did not precede the development of disease in this population.

It has been suggested that a quantitative method for CMV DNA PCR could be more reliable for the diagnosis of CMV disease than the semiquantitative method used here [11, 12]. Although the DNA level in the first positive sample was not useful for predicting the development of disease, a correlation was observed between intensity of maximal signal and development of CMV disease. In CMV-seropositive recipients, CMV DNA detection by PCR often fails to correlate with disease and, therefore, specific quantitative or qualitative methods are needed [13, 14]. On the other hand, in this group of seronegative patients, any CMV DNA detection was significant because it indicated primary infection. The semiquantitative method used here is technically much easier and requires significantly less time than quantitative PCR methods. This method is useful in identifying patients at high risk for CMV disease when primary infection occurs within 2 months after transplantation.

In 20% of patients who developed CMV disease, a high level of CMV DNA was not detected at any time. These infections occurred late, when only twice-a-month sampling was done, suggesting that higher CMV DNA levels were present earlier but were missed. The lack of samples was also the most likely explanation for results from the 2 patients who developed asymptomatic CMV infection diagnosed by positive urine and throat cultures but who had leukocytes repeatedly negative for CMV DNA. However, clinically insignificant viral shedding by an organ locally without viremia may also be considered.

There was also a well-documented episode of CMV enteritis by histopathology and virology in a patient who received a combined liver-intestine transplant, with negative viremia and negative PCR for CMV DNA. Although we cannot rule out false-negative results due to technical problems, this was unlikely because of the number and different types of analyses. Further studies are required to evaluate the correlation of CMV viremia or DNAemia with gastrointestinal disease and the particular characteristics of CMV infection after intestinal transplantation [15]. This is a complex process involving mucosal infection with inflammation and tissue necrosis, along with vascular endothelial involvement and subsequent ischemic mucosal injury [16]. It is possible that the vascular occlusion that may occur in these circumstances isolates the area of infection, thereby preventing detection of CMV in the blood.

In summary, detection of CMV DNA by PCR in leukocytes allows diagnosis of CMV infection before the development of disease in CMV-seronegative liver recipients of grafts from CMV-seropositive donors and should be considered as a marker to institute preemptive therapy in this population. During the first 2 months after transplantation, therapy could be initiated when any level of CMV DNA is detected. In cases in which infection occurs >2 months after transplantation and the patient is asymptomatic, close follow-up is warranted, and preemptive therapy should be initiated when high levels of CMV DNA on PCR or viremia on shell-vial or tube culture are detected. CMV antigenemia has also been demonstrated as an earlier indication of infection than results from other serologic or virologic methods, and the level of antigenemia also correlates with disease [13, 17]. Further studies are needed to compare the cost and benefit of PCR of CMV in leukocytes and detection of CMV antigenemia for early diagnosis of infection in this group of high-risk liver transplant recipients.

References


