Diagnosis of Cytomegalovirus Infections by Shell Vial Assay and Conventional Cell Culture during Antiviral Prophylaxis

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A total of 3,552 specimens for conventional cytomegalovirus (CMV) culture and shell vial assay for CMV immediate-early antigen were obtained during a prospective randomized trial for prophylaxis of CMV disease after liver transplantation. Prophylaxis with ganciclovir for 2 weeks and then high-dose acyclovir for 2.5 months was compared with high-dose acyclovir alone for 3 months. During the first 12 weeks after transplantation, when the patients were on prophylaxis, there were significantly more clinical samples positive by the shell vial assay and negative by standard culture in comparison with the number of samples obtained from weeks 13 to 24, after prophylaxis was discontinued, that were positive by the shell vial assay and negative by standard culture. In contrast, significantly fewer samples were positive by both the shell vial assay and standard culture during the first 12 weeks compared with the number obtained 13 to 24 weeks after transplantation that were positive by both methods. Samples positive by the shell vial assay only were obtained significantly more frequently from patients with asymptomatic than symptomatic CMV infections, while samples positive by both methods were obtained significantly more often from patients with symptomatic CMV infection. It was concluded that antiviral prophylaxis with high-dose acyclovir or ganciclovir and then high-dose acyclovir and asymptomatic CMV infection are associated with a decrease in the level of CMV isolation by standard cell culture in comparison with that by the shell vial assay.

Cytomegalovirus (CMV) infection remains the most frequent infection after organ transplantation (10). Demonstration of CMV in clinical samples is the principal approach for the diagnosis of CMV infection. The availability of monoclonal antibodies against CMV immediate-early antigen has allowed the identification of CMV in fibroblast cultures as early as 4 h after specimen inoculation, with a sensitivity of 80% (8). Besides producing results more rapidly, the sensitivity of the shell vial assay (SVA) is greater than that of the conventional culture method (CC), which detects between 63 and 71% of isolates (7, 11). Therefore, SVA has become widely used in the diagnosis of CMV infections.

We recently conducted a randomized trial for the prophylaxis of CMV disease after liver transplantation, comparing ganciclovir for 2 weeks and then high-dose oral acyclovir for 2.5 months with high-dose oral acyclovir alone for 3 months (13). The results showed that both high-dose acyclovir and ganciclovir decreased the overall level of recovery of CMV after transplantation. During that initial analysis for clinical efficacy, we noted a larger than usual dissociation between the level of detection of CMV by CC and the level of detection by SVA. Therefore, in the present study we determined whether the use of prophylaxis with ganciclovir or high-dose acyclovir may bias the diagnosis of CMV infection by CC or SVA. Detection of CMV by CC, SVA, or both was also compared with the development of asymptomatic or symptomatic infections.

MATERIALS AND METHODS

Study population. Between 1 February 1991 and 31 August 1991, 139 adult liver transplant recipients were enrolled with informed consent in a prospective randomized trial at the University of Pittsburgh Medical Center; the trial compared an early course of ganciclovir and then high-dose oral acyclovir (ganciclovir group; 68 patients) with high-dose oral acyclovir alone (acyclovir group; 71 patients) for the prevention of CMV disease. The study was approved by the University of Pittsburgh Biomedical Institutional Review Board. Acyclovir (800 mg; Burroughs Wellcome, Research Triangle Park, N.C.) was given four times daily and ganciclovir (5 mg/kg of body weight; Syntex, Palo Alto, Calif.) was administered intravenously twice daily. The dosages for both treatments were adjusted to the estimated creatinine clearance rate. The study addressed the period from transplantation to 24 weeks posttransplantation, which encompassed the initial 12 weeks of prophylaxis and a subsequent 12-week follow-up period. Of the 139 patients enrolled, 131 completed the drug protocol and were available for the follow-up period.

Clinical specimens cultured by CC and SVA. A total of 3,552 specimens for CMV detection by CC and SVA were obtained from various anatomical sites (Table 1), with a median of 30 specimens (range, 12 to 120 specimens) obtained from each patient. Of these specimens, 1,679 (47%) were from the ganciclovir group and 1,873 (53%) were from the acyclovir group. During the first 12 weeks after transplantation, when the patients were receiving ganciclovir or high-dose acyclovir prophylaxis, 2,957 specimens were obtained (Table 1): 1,441 (49%) from the ganciclovir group and 1,496 (51%) from the acyclovir group. From weeks 13 to 24 posttransplantation, after prophylaxis was discontinued, 615 specimens were obtained: 238 (39%) from the ganciclovir group and 377 (61%) from the acyclovir group.
A total of 2,170 specimens obtained from patients not included in the present trial were used as controls.

**Virologic studies.** Weekly specimens for CMV cultures were obtained during the first 3 months after transplantation and at monthly intervals thereafter. Additional specimens for CMV culture were taken during any suspected clinical illness caused by CMV. The end points of the study were the development of symptomatic CMV infection or death.

Buffy coats were prepared from heparinized whole blood. Erythrocytes were sedimented with dextran (0.15 ml of 6% dextran [TS00; Pharmacia, Piscataway, N.J.] per ml of blood for 30 min at room temperature); the supernatant containing theuffy coat was removed and centrifuged, and the cells were washed once in phosphate-buffered saline (PBS). The cell pellet was resuspended in a few milliliters of distilled water for several seconds to lyse the erythrocytes, diluted to 30 ml with PBS, centrifuged again, and resuspended in approximately 3 ml of PBS. This cell suspension was used to inoculate tubes and vials. For urine specimens, 10 ml was centrifuged and antibiotics were added to the supernatant, which was then allowed to stand at 4°C for 30 min prior to inoculating tubes and vials. Throat swabs were swirled in 2 ml of Hanks balanced salt solution (HBSS) with 0.5% gelatin and antibiotics. The fluid was then allowed to stand at 4°C for 30 min prior to inoculation of tubes and vials. Liver and gastrointestinal biopsy specimens were placed in 2 ml of HBSS with 0.5% gelatin and antibiotics, and the ingredients were then homogenized. The homogenate was used to inoculate tubes and vials. Bronchoalveolar lavages were centrifuged, and the cells were resuspended to a final concentration of 10^9/ml in HBSS with gelatin and antibiotics. The cell suspension was then frozen and thawed once and inoculated into tubes and vials.

For CC, media were decanted from tubes of human foreskin fibroblasts (Bartels, Issaquah, Wash.), the specimens were inoculated in duplicate (0.2 ml per tube), and the tubes were incubated for 90 min at 37°C. The inoculum was then decanted and 1.5 ml of Eagle's minimum essential medium (EMEM; BioWhittaker,Walkersville, Md.) containing 2% heat-inactivated fetal bovine serum (Sigma, St. Louis, Mo.) was added to each tube. The tubes were incubated at 37°C and were screened three times per week for 3 weeks for the characteristic CMV cytopathic effect.

The **SVA** was performed as described previously (7), with minor modifications. Briefly, media were decanted from shell vial coverslip cultures of MRC-5 human fetal lung fibroblasts (BioWhittaker), overlaid with 0.2 ml of specimen (two vials per specimen), and centrifuged at 2,000 x g for 45 min at 35°C. An additional 1 ml of EMEM containing 2% fetal bovine serum was added to each vial, and the vials were incubated at 37°C for 20 h. The coverslips were then rinsed with Dulbecco’s PBS (DPBS) and fixed in acetone at room temperature for 10 min. The fixed coverslips were rinsed with DPBS and treated with 0.2 ml (per vial) of murine monoclonal antibody specific for the major immediate-early antigen of CMV (DuPont-Biotech, Rockville, MD) for 30 min at 37°C. The coverslips were then rinsed twice with DPBS, stained with goat anti-mouse immunoglobulin G (heavy and light chain specific) fluorescein isothiocyanate conjugate (Cappell, Durham, N.C.) for 30 min at 37°C, rinsed with DPBS, and counterstained for a few minutes with Evans blue dye. Following a final DPBS rinse, the coverslips were mounted on slides with buffered glycerol and read under a fluorescence microscope.

A positive culture for CMV was defined by either a positive **SVA** result (SVA+), as indicated by a typical nuclear apple green fluorescence, or a positive **CC** result (CC+), as indicated by a typical focal cell-rounding cytopathic effect, or both. CMV-specific immunoglobulin G antibody levels in serum were measured by a solid-phase fluorescence immunoassay (FIAx Test System; BioWhittaker). CMV-specific immunoglobulin G antibody levels were determined only in CMV-seronegative transplant recipients until seroconversion.

**Definitions of CMV infection.** Asymptomatic CMV infection was defined as seroconversion or a positive culture in the absence of clinical symptoms. Symptomatic CMV infection included the CMV syndrome, localized disease, or disseminated disease. The diagnosis of CMV syndrome required virus isolation by either **SVA** or **CC** along with a temperature of >38°C for 2 or more days in the absence of any other cause, with one of the following findings: atypical lymphocytosis, >3%; blood leukocyte count, <4,000/mm^3; or platelet count, <100,000/mm^3. Localized CMV disease was defined as tissue invasion in a single organ determined histopathologically, by culture of virus from a tissue specimen, or both. Disseminated CMV disease was defined as invasive involvement of two or more tissues at noncontiguous sites.

**Statistical analysis.** Differences between **SVA** and **CC** as a function of time, the type of disease (asymptomatic or symptomatic), and the type of prophylaxis given were assessed by chi-square analysis.

**RESULTS**

**Isolation of CMV from clinical specimens compared with time post-organ transplantation.** Of the 3,552 specimens, CMV was detected in 287 (8%) of them. Table 2 shows the distribution of specimens with each type of culture result. The data show that there were significantly more SVA+ and CC+ **SVA**-coated buffy coats than **SVA**- and CC- nonblood samples (P = 0.02). Conversely, there were more urine specimens than other specimens that were SVA+ and CC+ and SVA- and CC+ (P < 0.0001). During the first 12 weeks after transplantation, significantly more specimens were positive for CMV by **SVA** only (SVA+ and CC-; P = 0.02) (Table 3). In contrast, during weeks 13 to 24 significantly more specimens were positive by both methods (SVA+ and CC+; P = 0.003). No difference in the frequency of SVA- and CC+ and that of SVA+ with contaminated CC samples was found during these two periods of time (Table 3).

<table>
<thead>
<tr>
<th>Specimen type</th>
<th>No. (%) of specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td>buffy coat</td>
<td>urine</td>
</tr>
<tr>
<td>Patients on prophylaxis</td>
<td>1,034 (35)</td>
</tr>
<tr>
<td>Patients not on prophylaxis</td>
<td>208 (34)</td>
</tr>
<tr>
<td>Total</td>
<td>1,242 (35)</td>
</tr>
</tbody>
</table>

* Percentage of total specimens.
The results obtained during weeks 13 to 24 were similar to those found in the control group.

Isolation of CMV compared with antiviral treatment and clinical status. A total of 83 (29%) specimens from the ganciclovir group and 204 (71%) from the acyclovir group were positive for CMV during the entire study period. There were no differences in the frequencies of SVA+ and CC−, SVA+ and CC+, and SVA− and CC− between the two groups. The median time from transplantation to the first isolation of CMV was 45 days (range, 17 to 151 days) in the acyclovir group and 78 days (range, 19 to 140 days) in the ganciclovir group (P = 0.02).

Symptomatic CMV infection developed in 26 patients (20 in the acyclovir group and 6 in the ganciclovir group). Asymptomatic infection was found in 35 patients (23 in the acyclovir group and 10 in the ganciclovir group). Of 287 positive specimens, 134 (47%) were from asymptomatic patients and 153 (53%) were from symptomatic patients (P = not significant). There were significantly more positive specimens of the SVA+ and CC− type from asymptomatic than from symptomatic patients (P = 0.04). In contrast, more positive specimens of the SVA+ and CC− type were obtained from symptomatic patients (P = 0.003). No difference was found between the type of CMV-related disease and the frequency of SVA− and CC+ specimens and SVA+ with contaminated CC type specimens (Table 4).

Viremia was found in 26 patients, 6 of whom (5 in the acyclovir group and 1 in the ganciclovir group) had no symptomatic infection. In contrast, six patients with localized disease did not develop viremia. No differences in buffy coat specimens positive for CMV were observed between patients with asymptomatic infections (64% SVA+ and CC−, 29% SVA+ and CC+, 7% SVA− and CC+) and those with symptomatic infections (57% SVA+ and CC−, 26% SVA+ and CC+, 17% SVA− and CC+).

**DISCUSSION**

We found that antiviral prophylaxis either with high-dose acyclovir or with ganciclovir and then high-dose acyclovir after liver transplantation influences the frequency of detection of CMV by different methods. Significantly more SVA+ and CC−-type specimens than SVA+ and CC+ type specimens were detected during the 12-week period when patients were on prophylaxis. Usually, between 60 and 70% of specimens positive for SVA yield CMV by CC (7, 11, 15), similar to that found in our study after treatment was discontinued. No difference in the incidence of SVA− and CC− was observed between either period of time. This suggests that a negative SVA result with a positive CC result is not influenced by antiviral drug administration. The SVA− and CC− results may have been due to factors such as expression of an immediate-early antigen that is not detectable by our monoclonal antibody (16) or, most likely, to sampling variability because of the presence of a low viral inoculum in the specimens (5).

The discordant results between CC and SVA found in the present study could be due to a sampling bias since 83% of the specimens were collected in the first 12-week period. However, the similar distribution of results obtained during weeks 13 to 24 and in the control specimens suggests that other factors account for these findings. Discordant results between CC and SVA have been reported during ganciclovir therapy (17). However, our study suggests that high-dose acyclovir therapy also influences the detection of virus positivity. There was no difference in the overall frequencies of SVA and CC positivity between the ganciclovir and acyclovir groups. Furthermore, the median time to the first isolation of CMV in the ganciclovir group was 78 days, when the patients were receiving only high-dose acyclovir. These results suggest that high-dose acyclovir therapy affects CMV detection in vitro, as evidenced by an increased prevalence of SVA positivity in the absence of viral replication, as detected by CC.

**TABLE 2. Comparison between CC and SVA for detection of CMV in clinical specimens**

<table>
<thead>
<tr>
<th>Test result</th>
<th>No. (%) of CMV-positive specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Buffy coat</td>
</tr>
<tr>
<td>SVA+, CC−</td>
<td>36 (59)</td>
</tr>
<tr>
<td>SVA+, CC+</td>
<td>16 (26)</td>
</tr>
<tr>
<td>SVA−, CC+</td>
<td>9 (15)</td>
</tr>
<tr>
<td>SVA−, cont.</td>
<td>0</td>
</tr>
<tr>
<td>SVA−, CC−</td>
<td>1,181</td>
</tr>
<tr>
<td>Total</td>
<td>1,242</td>
</tr>
</tbody>
</table>

* cont., standard CC was contaminated.

**TABLE 3. Comparison between CC and SVA during antiviral therapy, without treatment, and in control specimens**

<table>
<thead>
<tr>
<th>Test result</th>
<th>No. (%) of CMV-positive specimens</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With therapy</td>
<td>Without therapy</td>
</tr>
<tr>
<td>SVA+, CC−</td>
<td>83 (49)</td>
<td>41 (35)</td>
</tr>
<tr>
<td>SVA+, CC+</td>
<td>54 (32)</td>
<td>59 (50)</td>
</tr>
<tr>
<td>SVA−, CC+</td>
<td>23 (14)</td>
<td>15 (13)</td>
</tr>
<tr>
<td>SVA−, cont.</td>
<td>9 (5)</td>
<td>3 (3)</td>
</tr>
<tr>
<td>Total</td>
<td>169</td>
<td>118</td>
</tr>
</tbody>
</table>

* Comparison between without-therapy and control specimens with therapy specimens.

**NS**, not significant.

* cont., standard CC was contaminated.

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that are SVA\(^{+}\) and CC\(^{-}\) have a lower viral load than those that are SVA\(^{+}\) and CC\(^{+}\). These results are similar to those obtained by the detection of CMV in buffy coats by the antigenemia test. By that test, the number of antigen-positive cells is significantly higher in patients with symptomatic infections than in patients with asymptomatic infections (5, 6, 20).

Also, virus isolation by CC is dependent upon higher numbers of antigen-positive cells (5).

High-dose acyclovir was shown to prevent clinical CMV disease in kidney transplant recipients (1). However, our clinical trial (13) and another recent study (19) have demonstrated a lower level of effectiveness of high-dose acyclovir when compared with that of ganciclovir alone or ganciclovir and then high-dose acyclovir in liver transplant recipients. The in vitro results obtained in the present study with urine specimens might explain these discrepancies. Urine specimens showed the highest rate of CC positivity. The CMV titer is significantly higher in urine than other specimens (12), and there are higher levels of bound β₂-microglobulin (14), which may enhance CMV infectivity (9). On the other hand, the average levels of renal excretion of acyclovir is quite variable, ranging between 30 and 90\% (2), in contrast to the more than 90\% elimination of ganciclovir (3). The inhibitory effect of acyclovir in vivo might depend on the CMV inoculum, the concentration of the drug, or both.

In summary, antiviral prophylaxis decreases the level of CMV isolation by CC in comparison with that by SVA, probably lowering the viral burden. However, use of SVA alone cannot be recommended, since 14\% of the specimens in the present study were positive only by CC. An alternative in these circumstances may be to prolong the period of culture, which has been shown to increase the positivity of CC (6). On the other hand, our results suggest that the effects of antiviral drugs will need to be considered in the interpretation of the results of other techniques for diagnosing CMV infection, such as the antigenemia assay and viral nucleic acid amplification.

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REFERENCES


