Diagnosis of Cytomegalovirus Infection by the Detection of Early Antigen (pp65) in Leukocytes of Kidney Transplant Patients

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Abstract

\textbf{Objective:} Cytomegalovirus (CMV) infection is a frequent complication of kidney transplantation contributing substantially to morbidity and mortality. Early diagnosis of the infection is essential to implement an effective antiviral therapy. This study is aimed at establishing the CMV antigenemia assay for the diagnosis of CMV infection, and for monitoring the effectiveness of ganciclovir therapy in kidney recipients in Kuwait. \textbf{Methods:} Leukocyte-enriched fractions of 215 blood specimens from 92 kidney recipients were processed for the detection of CMV-specific protein (pp65) by using a commercial kit (Incstar Corp., USA). Plasma fractions were also tested for the presence of CMV IgM antibodies, and selected samples (n = 15) were processed for virus isolation in MRC-5 diploid cell culture. \textbf{Results:} Regarding symptomatic (n = 35) and asymptomatic patients (n = 57) the assay showed an 86% positive and a 79% negative predictive value, compared to the 55% and 70% values, respectively, for CMV IgM antibodies. The number of positive cells was parallel with the severity of symptoms. Using a cutoff level of >5 antigen-positive leukocytes/50,000 cells, the assay reached 93% positive and 96% negative predictive values. There was a very good agreement between the assay and virus isolation. Ganciclovir therapy could be monitored effectively with the assay, and in 11 symptomatic patients treated with ganciclovir, a prompt decrease in the number of antigen-positive cells was noted. \textbf{Conclusion:} The CMV antigenemia assay is a valuable tool for the early diagnosis of symptomatic CMV infection and monitoring antiviral therapy in kidney transplant patients.
Introduction

In transplant patients, active cytomegalovirus (CMV) infection frequently occurs and cause serious disease [1]. The incidence of clinical manifestation varies between 40 and 90% [2]. Antiviral therapy, when implemented early in a CMV infection, reduces the mortality of CMV disease substantially [3, 4]. In kidney and other transplant patients, it is essential to diagnose active CMV infection as early as possible. Rapid diagnostic tests are of practical value to differentiate CMV infection from other infections and to guide the use of antiviral therapy [5].

Isolation of infectious virus in cell culture is still considered the gold standard in the diagnosis of CMV infection [6]. However, the potential of virus isolation for the early diagnosis of CMV infection is limited by the long time requirement and the relatively low sensitivity of the method [7]. Polymerase chain reaction may have a strong impact on the diagnosis of CMV infection, but there is a need for further standardization and simplification [8].

Primary CMV infection may be diagnosed by serological methods. Although IgM detection is reliable to identify a recent CMV infection in healthy individuals, in immunocompromised patients, such as transplant recipients, CMV infection is produced in approximately 50–75% of the cases [6].

For the diagnosis of an active CMV infection, the detection of the CMV structural antigen (pp65) in polymorphonuclear leukocytes seems to be the best approach [9, 10]. The method now known as CMV antigenemia assay (AA) has been extremely useful for diagnosing active CMV infection in transplant patients [11–13].

In Kuwait, where several hundreds of transplant patients are under clinical surveillance, rapid and reliable laboratory diagnosis of CMV disease is essential. Therefore, we studied the usefulness of the AA for early diagnosis of CMV infection in kidney transplant patients.

Materials and Methods

Study Population. We enrolled 92 kidney transplant patients (35 females and 57 males), who received their transplants during 1996 and 1997. Their age spanned from 18 to 60 years, and 78% were between 30 and 45 years of age. All patients received an immunosuppressive regimen of cyclosporin A, azathioprine and steroids. One blood sample was collected from 38 patients, and two or more from 54 patients. From 15 of those 32 patients who developed any of the symptoms associated with CMV disease (unexplained fever, arthralgia, leukopenia, thrombocytopenia, elevated aminotransferase level, pneumonitis, hepatitis, retinitis, gastrointestinal ulceration), serial samples at different intervals were collected after transplantation and patients were monitored for 6–20 weeks. Eleven of these patients were treated with ganciclovir. Blood samples were collected in EDTA-containing tubes and transferred to the laboratory within 2–3 h. All the samples were processed for antigen detection on the same day. Plasma fractions of the samples were tested for the presence of CMV-IgM. Part of the separated cells were stored at −70°C for virus isolation.

CMV-AA. From each patient 5 ml of blood was collected and processed immediately to isolate leukocytes by the use of dextran sedimentation method. Following incubation and centrifugation, the cell pellet was suspended in phosphate-buffered saline (PBS), erythrocytes were lysed with 0.8 M ammonium-chloride, centrifuged, washed in PBS, counted and spotted onto glass slides (50,000 cells per spot). They were then dried and fixed in acetone bath, stained with Incstar CMV-vu Kit according to recommended procedures. The number of cells containing the CMV-specific pp65 antigen were counted with a light microscope (× 400).

Virus Isolation. Diploid fibroblast cell monolayers grown in LabTek 4-chamber slides were inoculated with polymorphonuclear blood leukocytes stored at −70°C. Before inoculation, cells were treated with 3 cycles of freezing and thawing. Two chambers were used for each sample. After inoculation cultures were incubated for 2 h at 37°C, fed with a medium and incubated further for 3–4 days in the presence of 5% CO₂. Then the cells were fixed in cold methanol, washed with PBS, incubated immediately with a
monoclonal antibody to CMV early antigen (Biosoft, Paris, France) for 2 h. Finally, fluorescein-conjugated antimouse IgG was added to the cells and incubated for 1 h. Cells showing greenish fluorescence were detected by UV microscopy.

**Serology.** CMV-specific IgM antibodies (CMV-IgM) were detected with the use of conventional ELISA (Sorin, Biomedica), and/or immunofluorescence tests and confirmed with CMV-IgM blot assay (Genlabs Diagnostics, Singapore).

**Statistics.** For calculating the positive predictive value (PosPV) and the negative predictive value (NegPV) the following formulae were used: PosPV = (number of patients with positive test result and CMV-related disease/total patients with positive test result) × 100. NegPV = (number of patients with negative test result and without CMV-related symptoms/total patients with negative test result) × 100.

**Results**

**Presence of CMV Antigen in Leukocytes.**

Among the 92 patients, 35 had symptomatic infection and 32 of them were positive for the pp65 antigen. Among the 57 asymptomatic patients, 12 patients had CMV antigen in their leukocytes (table 1). Accordingly the PosPV and NegPV of the assay are 86 and 79%, respectively. However, in 15 patients monitored for a longer period of time (6–20 weeks) by testing 3 or more consecutive samples from each patient, the presence of antigen correlated even more closely with the clinical condition. All of the 11 patients who had symptomatic infection during the follow-up period were positive by the AA.

**Comparison of AA and IgM Detection.**

From the 92 patients 215 samples were collected and tested both with the AA and the IgM test. CMV-IgM could be detected only in 42% of the 79 antigen-positive samples. On the other hand, there was a relatively good agreement between the two tests regarding the 136 antigen-negative samples; 85% of them were negative for the presence of CMV-IgM (table 2).

When symptomatic and asymptomatic patients were compared (table 3), the result showed that IgM antibody could be detected in only 17 of the 35 symptomatic patients. The PosPV was 55% and the NegPV of the test (70%) approximated that of the AA (79%).

| Table 1. Presence of CMV pp65 antigen in leukocytes of kidney recipients |
|--------------------------|---------|---------|---------|
| AA                       | Patients | Samples | Patients |
|                         | with symptoms | without symptoms |                   |
| Positive                 | 43 (47%) | 79 (37%) | 31 (89%) | 12 (21%) |
| Negative                 | 49 (53%) | 136 (63%) | 4 (11%) | 45 (79%) |
|                          | 92       | 215      | 35/92 (38%) | 57/92 (62%) |

| Table 2. Correlation between AA and CMV-IgM test |
|--------------------------|---------|---------|
| AA                       | IgM test |
|                         | positive | negative |
| Positive                 | 33 (42%) | 46 (58%) |
| Negative                 | 20 (15%) | 116 (85%) |
| Total                    | 53       | 162      | 215      |

Cytomegalovirus Infection in Kidney Transplantation Patients
**Table 3.** Presence of pp65 antigen and CMV IgM antibodies in patients with and without clinical symptoms

<table>
<thead>
<tr>
<th>Patients</th>
<th>symptomatic</th>
<th>asymptomatic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>positive</td>
<td>negative</td>
</tr>
<tr>
<td>AA</td>
<td>31/35 (89%)</td>
<td>4/35 (11%)</td>
</tr>
<tr>
<td>CMV-IgM</td>
<td>17/35 (49%)</td>
<td>14/35 (40%)</td>
</tr>
</tbody>
</table>

**Table 4.** Number of pp65 antigen-positive leukocytes in symptomatic and asymptomatic patients

<table>
<thead>
<tr>
<th>Patients</th>
<th>Positivity scale</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>very low</td>
</tr>
<tr>
<td>Symptomatic (n = 35)</td>
<td>n</td>
</tr>
<tr>
<td>n</td>
<td>6</td>
</tr>
<tr>
<td>Asymptomatic (n = 57)</td>
<td>n</td>
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<tr>
<td>n</td>
<td>10</td>
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Positivity scale: very low = 1–5 positive cells; low = 6–10 positive cells; high = 11–50 positive cells; very high = >50 positive cells.

**Comparison of AA and Virus Isolation.** In selected samples AA was compared to virus isolation. A strong agreement between the two methods was noted. Only one antigen-positive sample was negative in virus isolation.

**Number of Antigen-Positive Leukocytes and Clinical Symptoms.** The number of antigen-bearing cells varied extensively, from 0 to several hundred. For rating the positivity the following arbitrary scale has been formulated: 1–5 cells = very low positive; 6–10 cells = low positive; 11–50 cells = positive, and >50 = high positive. Grouping antigen-positive leukocytes detected in symptomatic and asymptomatic patients by this scale shows that detecting just a few cells (1–5) among the 50,000s screened did not give a definite indication of an ongoing CMV infection. On the other hand, the presence of more than 5 positive cells was strongly associated with clinical symptoms. With a cutoff of more than 5 cells as a positive test, the AA has a 93% PosPV and a 96% NegPV (table 4).

**Positive AA and Ganciclovir Treatment.** Eleven patients have received ganciclovir treatment for various periods of time (from 2 to 8 weeks). One patient became antigen-positive 8 weeks after transplantation and was symptomatic. Ganciclovir therapy was started and continued for 2 weeks. When the patient was tested 4 and 8 weeks later he was asymptomatic and negative in the AA. Four patients followed the same pattern becoming antigen-negative and asymptomatic after the antiviral treatment. Four other patients had a somewhat different course. At the time when clinical symptoms were present, a high number of antigen-positive leukocytes could be detected. Their number reduced gradually but positive cells were still present when patients became asymptomatic. One patient who had a multiple, concomitant disease process including pneumococcal meningitis responded to ganciclovir treatment, and the initially high number of positive cells (175) was reduced to 4. Another patient who responded to ganciclovir treatment (antigen-positive cells could not
be detected at week 12 after transplantation) presented with central nervous system lymphoma and became critically ill. There were 4 patients with symptoms who did not receive antiviral treatment. Two of them were very low positive (1–2 cells) in the AA and they became asymptomatic spontaneously. The other 2 patients had an increase in the number of antigen-positive cells and presented with CMV-compatible symptoms. Unfortunately, their follow-up could not be continued further because they left the country.

**Discussion**

Detection of the CMV-related pp65 antigen in peripheral leukocytes of transplant patients has become an increasingly important assay for the diagnosis of CMV infection [14, 15]. The CMV AA has substantial advantages: it gives rapid results (available within 5–6 h), correlates exceptionally well with symptomatic CMV infections, and can be used for monitoring the effectiveness of antiviral therapy [16]. The assay is relatively easy to perform and inexpensive when compared to either cell culture or PCR methodologies [17].

In this study, the diagnostic significance of the AA was determined on 92 kidney transplant patients with and without symptomatic infections. Among the 35 symptomatic patients there were 4 AA-negative patients. All of them presented only with one clinical symptom (unexplained fever) which may not have been related to CMV infection. The 31 patients who had more than one CMV-related symptom were positive by the AA. Among the 57 asymptomatic patients, 12 had the antigen in their leukocytes, however, the number of antigen-positive cells never exceeded 10. If we used a cutoff value >10 positive cells none of the asymptomatic patients would fall into the positive range. Indeed quantification seems possible [18, 19] and it is important in relation to patient management [10]. The higher the number of antigen-positive cells the closer is the association with more severe symptomatic CMV infections.

Although in this study only 15 selected samples were tested for both the AA and virus isolation, the result showed a good agreement between the two tests. There was only 1 AA-positive case with negative virus isolation but the number of antigen-positive cells was very low (2 cells only).

The scale upon which the positivity of the AA is determined may differ from one laboratory to the other, however, it does not jeopardize the value of the assay. Since several modifications of the assay are available [20], it would not be reasonable to set a unified rating scale.

A comparison between AA and CMV-IgM showed that only 49% of symptomatic cases had CMV IgM antibodies, which means that the PosPV of the IgM test is much lower (55%) than that of the AA (86%). It may be due to several factors like antibody status before transplantation, source of grafts, immunological compatibility, immunosuppressive therapy etc., which influence the production of IgM in organ transplant patients [18].

It is of interest that the 4 patients with the highest number of antigen-positive cells did not produce detectable CMV IgM antibodies.

The assay has an obvious impact on monitoring ganciclovir therapy. In all the 11 patients treated with the drug, the number of CMV antigen-positive cells decreased promptly as the clinical conditions of the patients improved. The 4 patients without treatment had a relatively low number of AA-positive cells. Nevertheless, 2 patients were symptomatic when they had more than 5 positive cells. Unfortunately their follow-up could not be continued.
In summary we conclude that the CMV AA is a valuable laboratory method for diagnosing CMV infections and monitoring anti-CMV therapy in kidney transplant patients.

Acknowledgment

This study was supported by the office of the Vice-Rector for Research, Kuwait University, Kuwait, grant No. MI 096.

References