Detection of Cytomegalovirus (CMV) in Granulocytes by Polymerase Chain Reaction Compared with the CMV Antigen Test

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To compare the sensitivity and suitability of detection of active cytomegalovirus (CMV) infection by using monoclonal antibodies against CMV antigen (antigen test to detect antigenemia) and the polymerase chain reaction (PCR; to detect viral DNA) in granulocytes, 19 heart and 2 lung transplant recipients were closely monitored by these tests for at least 3 months after transplantation. All patients were CMV seropositive or had a seropositive donor. In total, 201 samples were tested; 46 were positive by both tests, 9 samples showed only antigenemia, 54 samples were positive by PCR only, and 102 samples were negative by both tests. PCR was positive earlier after transplantation in eight patients, whereas antigenemia was positive earlier after transplantation in one patient. In another four patients, both tests were positive at the same time. PCR was, on average, positive for a longer period of time. Discordant results showing a positive antigen test and a negative PCR were partly due to sampling error; some were positive by PCR on retesting. Samples which were negative by the antigen test and positive by PCR were taken at the beginning or at the end of an active CMV infection. In two patients, no active CMV infection was detected by the antigen test, cultures of urine and saliva, or serology, although PCR was positive for a long period of time in the two patients.

Recently, two new rapid tests for early detection of active cytomegalovirus (CMV) infection have been developed. These tests are detection of CMV antigen in peripheral blood leukocytes with monoclonal antibodies (antigen test; a positive result is defined as antigenemia) and detection of CMV DNA by the polymerase chain reaction (PCR).

The value of the antigen test has been investigated in kidney (1, 9) and heart transplant (10) recipients and proved to be suitable for the early detection of active infection (1, 10). Also, high-level antigenemia was found to be related to symptomatic infection (9).

Detection of CMV DNA in peripheral blood by PCR has been investigated in kidney (5) and bone marrow (2, 4) transplant recipients. PCR also proved to be an early indicator of active CMV infection and was found to be more sensitive than culture of CMV from Buffy coat cells (4, 5). PCR has also been applied to tissue specimens for the detection of CMV DNA in biopsy specimens taken from patients with CMV disease and proved to be more sensitive than in situ hybridization and slot blot hybridization with probes (4).

To compare the abilities of the CMV antigen and CMV DNA PCR methods to detect CMV infections in peripheral blood, both tests should be applied on the same samples. We performed both tests on peripheral blood granulocytes, since these cells appear to express CMV more readily than do monocytes and lymphocytes (3, 8). The aim of the study was to analyze the sensitivities and specificities of the antigen test and PCR for detection of CMV infection after heart and lung transplantations in relation to each other and to determine, in combination with results obtained from cultures and serology, which test is most suitable for the early detection of active CMV infection and the detection of CMV disease.

MATERIALS AND METHODS

Patients. Blood samples were obtained weekly or biweekly from 19 heart and 2 lung transplant recipients. The CMV carrier status of donors and recipients was determined by the detection of anti-CMV immunoglobulin G (IgG) and IgM antibodies by an enzyme-linked immunosorbent assay (ELISA; Sorin Biomedica, Saluggia, Italy). Both lung transplant recipients were CMV positive; one of the patients had a CMV-negative donor and one had a CMV-positive donor. Of the 14 CMV-seropositive heart transplant recipients, 5 had CMV-positive donors, 6 had CMV-negative donors, and 3 had donors with unknown CMV serologies. Another five heart transplant recipients were CMV negative and had CMV-positive donors; primary CMV infections, as determined by the presence of IgM anti-CMV antibodies in serum, occurred in four of these patients.

For detection of antigenemia and CMV DNA by PCR, 5 to 19 (average, 10.1) blood samples were obtained from weeks 2.6 ± 2.2 to 28.1 ± 12.5 after transplantation. In 13 patients, active CMV infection was documented by positive cultures of urine or saliva and/or an increase in IgG or IgM anti-CMV antibodies detected by ELISA (Sorin Biomedica) (1). In the other eight patients, no active CMV infection was detected by culture or serology, but in one of these eight patients only IgM was detected. Seven of the eight patients were CMV seropositive; one patient remained seronegative, although he had a CMV-seropositive donor.

Isolation of peripheral blood granulocytes. Granulocytes were isolated from heparinized blood samples after mononuclear cells were removed by Ficoll density gradient centrifugation (1,000 × g, 20 min). The bottom fraction, which

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contained granulocytes and erythrocytes, which were enriched for granulocytes by dextran sedimentation (20 min at room temperature). After sedimentation, the top layer was removed and centrifuged (800 g, 10 min). Residual erythrocytes were lysed by hypotonic shock (10 min, 4°C). The granulocytes were washed (600 g, 10 min) and used for detection of CMV antigen, PCR, and culture.

**Antigen test for detection of antigenemia.** For detection of antigenemia, cytospin preparations were made from the granulocytes (1.5 × 10⁶ cells per cytospin preparation). From each blood sample, two cytospin preparations were used to detect antigenemia; one of the cytospin preparations served as a control. The slides were fixed in water-free acetone (Riedel-de Haen, Seelze, Germany) for 10 min, air dried, and incubated with a combination of two monoclonal antibodies against CMV antigen pp65 (monoclonal antibodies C10 and C11; kind gifts from T. H. The, Groningen, The Netherlands). After 1 h of incubation, the slides were washed and incubated with peroxidase-conjugated goat anti-mouse IgG (Tago, Burlingame, Calif.) for 45 min at room temperature. After this incubation, the slides were washed and specific binding was visualized with 3-amin-9-ethylcarbazole (Sigma, St. Louis, Mo.) in citric acid buffer (pH 5.4) with 0.015% H₂O₂. Counterstaining was performed with Mayer hematoxylin (Riedel-de Haen). The number of positive cells per slide was counted by using a microscope with a ×250 magnification. The sensitivity was estimated to be one positive cell per 0.5 × 10⁵ to 1 × 10⁵ cells.

**PCR.** The granulocytes isolated from the blood samples were stored in methanol at −20°C until use. Before PCR, the methanol was removed by centrifugation and evaporation. Pretreatment of the samples consisted of overnight incubation with proteinase K (Boehringer, Mannheim, Germany) (a 200-μg/ml solution of proteinase K containing 0.25% Tween 20; 37°C). For amplification, two primers were used (5′ primer sequence, 5′-TGC TCA CGC ACA TTG ATC AC-3′; 3′ primer sequence, 5′-GTA CTG GGC G-3′), both of which were located within the fourth exon of the CMV immediate-early gene, resulting in a 273-bp DNA fragment after amplification.

Samples were denatured at 100°C for 10 min and after that they were specifically amplified in a 50-μl reaction mixture containing 5 μl of sample, 11.1 mM Tris-HCl, 11.1 mM MgCl₂, 55.6 mM NaCl, 4 mM deoxyribonucleotide triphosphates (Boehringer), 0.25 μg of each primer, and 0.5 U of ampli-Taq polymerase (Cetus, Norwalk, Conn.). Each sample was amplified in 35 cycles of 95, 60, and 72°C in a thermal cycler (Perkin-Elmer Cetus, Couda, The Netherlands).

As a positive control for the presence of sufficient DNA from each sample, amplification of the β-globin DNA was also performed at the same time (7). Procedures for avoiding contamination were strictly followed. Negative controls included reaction mixtures without DNA. A check for false-positive and false-negative results was done by performing PCR again and then hybridizing the gels with an internal probe.

The sensitivity of the protocol used in this study was determined from experiments on CMV-infected fibroblasts; it was concluded that the sensitivity of the protocol was 1 infected cell in 10⁶ uninfected cells.

**Analysis of PCR amplification products.** Amplification products were analyzed by gel electrophoresis in a 2% agarose gel (Pronarose; SphaeroQ, Leiden, The Netherlands) in TAE (Tris-acetate-EDTA) containing 0.15 μg of ethidium bromide per ml. A molecular size reference (φX DNA HaeIII; Promega, Madison, Wis.) was also run on each gel next to the lanes in which the CMV-specific products were run. After electrophoresis, the gels were photographed under UV illumination. Some gels were dried and some gels were blotted onto a nylon membrane (Hybond-N⁺; Amersham International plc., Buckinghamshire, England). The parts of the dried gels that contained the CMV-specific PCR bands were used for hybridization with the internal probe.

**Hybridization.** For hybridizations, dried gels were denatured by treating them with a 0.5 M NaOH solution containing 0.15 M NaCl for 30 min. After neutralization with 0.5 M Tris-HCl (pH 8.0)–0.15 M NaCl, the gels were prehybridized for 1 h at 42°C in a solution containing 5X SSPE (1X SSPE is 0.18 M NaCl, 10 mM NaPO₄, and 1 mM EDTA [pH 7.7]), 5X Denhardt’s solution, and 0.1% sodium dodecyl sulfate (SDS). Hybridization was performed by using an oligoprobe located within the amplification product from the PCR (sequence, 5′-GGT CAC TAG TGA CGC TTG TAT GAT GAC CAT GTA CCG G-3′) which was labeled with 3²P by using T4 polynucleotide kinase (Promega). The labeled probe and gels were incubated overnight at 42°C, washed in 2X SSC, 0.25% SDS, for 1 h, rinsed in 1X SSC, 0.25% SDS, and dried. Autoradiography of the filters was recorded on radiosensitive film at −80°C overnight (X-Omat AR; Kodak, Rochester, N.Y.).

**Culture ofuffy coat cells to detect viremia.** For the detection of viremia, granulocytes were isolated by using sterile culture techniques. The granulocytes were suspended in RPMI (GIBCO, Uxbridge, England) containing 10% fetal calf serum (Flow Laboratories, Irvine, England), 2 mM glutamine, 100 IU of penicillin per ml, and 100 μg of streptomycin per ml. Approximately 1 × 10⁶ to 5 × 10⁶ cells were inoculated onto a monolayer of fetal fibroblasts in 6 wells of a 96-well plate and were incubated for 2 h at 37°C in a 5% CO₂ atmosphere. Then, the cultures were washed and incubated with fresh RPMI (supplemented with fetal calf serum and antibiotics) for another 2 to 3 days. Infection of the fetal fibroblasts with CMV was determined by the detection of early antigen fluorescent foci method, as follows. The cells were fixed with methanol at −20°C for 10 min. The plates were washed with phosphate-buffered saline and incubated for 2 h with a monoclonal antibody against CMV immediate-early antigen (Biosoft, Paris, France); the monoclonal antibody was diluted 1:200 in medium containing 1% albumin and 0.1% azide. The plates were washed again and incubated for another 1 h with fluorescein-conjugated goat anti-mouse IgG (Becton Dickinson, Mountain View, Calif.). Positive cultures were detected with a fluorescence microscope.

**RESULTS**

**Comparison of antigen test and PCR.** Detection of CMV antigen and detection of CMV DNA by PCR were compared for 201 samples (Table 1). Hybridization of the PCR products was performed on 44 samples, CMV was always detected by PCR in samples with high-level antigenemia. However, 9 of 34 samples with low-level antigenemia gave negative PCR results, whereas 54 of 156 samples negative for CMV antigen were positive by PCR. Some of the samples with inconsistent results were investigated further. This was done by hybridization of the PCR products with an internal probe and by reperforming PCR on the frozen cells of the original blood sample. Results are shown in Table 1 (see below).
TABLE 1. Comparison of detection of CMV antigen by monoclonal antibodies and detection of CMV DNA by PCR

<table>
<thead>
<tr>
<th>Antigena</th>
<th>No. of samples</th>
<th>Hybridizationc</th>
<th>PCR</th>
<th>Viremiaa</th>
<th>No. of examples</th>
<th>No. positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;10</td>
<td>11</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>&lt;10</td>
<td>25</td>
<td>0</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>&lt;10</td>
<td>54</td>
<td>2</td>
<td>16</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Negative</td>
<td>102</td>
<td>1</td>
<td>14</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>201</td>
<td>5</td>
<td>44</td>
<td>32</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Number of CMV antigen-positive granulocytes per $10^5$ granulocytes.
b A total of 194 samples were also tested by culture to detect viremia.
c Hybridization of PCR products.

Time to detection of CMV DNA or CMV antigen after transplantation. To analyze which detection method was positive first, patients were divided into four groups, according to the results obtained by the antigen test and PCR.

PCR was found to be positive earlier, on average, than antigenemia in patients with high numbers as well as in those with low numbers of antigen-positive cells (Table 2). In all 13 patients in whom positive results were obtained by both tests, PCR was positive earlier in eight patients, antigenemia was positive earlier in one patient, and both tests were positive at the same time in four patients. In both patient groups with high- and low-level antigenemia, PCR was positive, on average, for a longer period of time than antigenemia (Table 2).

For six patients, both the antigen test and PCR remained negative, and for five of these patients, no other evidence of CMV reactivation was seen. Two patients had a negative antigen test and a positive PCR; results for these two patients are shown separately in Table 2. In one of these two patients, anti-CMV IgM was detected, but no other evidence for active CMV infection was present.

Retesting of PCR in discordant samples. Antigenemia was detected in nine samples from five patients, but PCR was negative (Table 1). All these samples had low numbers of antigen-positive cells (one or two positive cells per cytospin preparation). Eight of these samples from four patients were retested to determine whether the negative PCR could be due to sampling error. The 8 blood samples were divided into 30 samples and were again tested by PCR. Three of the eight blood samples were positive on retesting (Table 1).

TABLE 2. Average weeks after transplantation when PCR and antigen test were positive

<table>
<thead>
<tr>
<th>Antigenb</th>
<th>No. of patients</th>
<th>Total no. of samples</th>
<th>Avg wk after transplantation to first detection</th>
<th>Avg no. of wk tests were positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;10</td>
<td>6</td>
<td>76</td>
<td>4.7</td>
<td>16.7 11.0 11.0</td>
</tr>
<tr>
<td>&lt;10</td>
<td>7</td>
<td>75</td>
<td>4.7</td>
<td>9.0 1.8 1.6</td>
</tr>
<tr>
<td>Negative</td>
<td>6</td>
<td>36</td>
<td>4.7</td>
<td>0 0 0</td>
</tr>
<tr>
<td>Negative</td>
<td>2</td>
<td>14</td>
<td>1.5</td>
<td>12.0 0 0</td>
</tr>
</tbody>
</table>

a Patients in whom high (>10/10^5) positive cells (>10), low (<10/10^5 [<10]), and no antigenemia were detected.
b Antigen test positive.
c Patients with negative antigen test and negative PCR.
d Patients with negative antigen test and positive PCR.

Another 54 samples were positive by PCR and negative by the antigen test. Fourteen of these samples were from two patients; all of the samples from these patients were negative by the antigen test. A few samples from another three patients (27 samples) were positive by the antigen test, but nearly all samples were positive by PCR (Table 3, groups b). The patients were divided into groups with high-level antigenemia, low-level antigenemia, and no antigenemia; patients with discordant results are group b.

Hybridization of PCR amplification products to confirm the specificity of PCR. Hybridization of the products from PCR was done for five patients; results for two of the patients were discordant in comparison with the antigen test results (Table 1). Hybridization results were positive for 29 PCR-positive samples, and 1 PCR-positive sample was negative by hybridization. Three of the 14 PCR-negative samples were positive by blotting. PCR, blotting, and antigen detection results for one patient are shown in Fig. 1.

Relation between symptomatic infection and culture of buffy coat cells for the detection of viremia. Only two patients had symptomatic CMV infection; all other patients were asymptomatic. Both symptomatic patients had positive PCR results and high-level antigenemia at the time that they showed symptoms of CMV infection. Five samples were positive for culture (viremia; Table 1); only two of these samples were positive for antigenemia and only three were positive for

TABLE 3. Correlation between PCR and antigen test in groups with high- and low-level and no antigenemia

<table>
<thead>
<tr>
<th>Antigen (group)</th>
<th>No. of patients</th>
<th>Total no. of samples</th>
<th>PCR</th>
<th>Antigen test</th>
<th>PCR and antigen test</th>
<th>Antigen test and PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;10</td>
<td>6</td>
<td>76</td>
<td>65</td>
<td>41</td>
<td>60</td>
<td>97</td>
</tr>
<tr>
<td>&lt;10 (a)</td>
<td>4</td>
<td>48</td>
<td>6</td>
<td>19</td>
<td>33</td>
<td>11</td>
</tr>
<tr>
<td>&lt;10 (b)</td>
<td>3</td>
<td>27</td>
<td>96</td>
<td>19</td>
<td>19</td>
<td>100</td>
</tr>
<tr>
<td>Negative (a)</td>
<td>6</td>
<td>36</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Negative (b)</td>
<td>2</td>
<td>14</td>
<td>86</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Percent of PCR-positive samples that were antigen positive.

FIG. 1. Results of PCR, blotting, and antigen detection for one patient monitored every 2 weeks. Ant pos/10^6 cells, number of antigen-positive cells per 10^6 cells; Tx, transplantation.

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CMV by PCR. Of the 189 samples that were negative by culture, 43 (23%) were positive for antigenemia and 86 (46%) were positive for CMV by PCR.

DISCUSSION

Detection of CMV antigenemia and CMV DNA by PCR in peripheral blood samples has proved to be useful for monitoring CMV infections after transplantation (1, 4, 5, 9, 10). A direct comparison of these tests is interesting, although the target molecules are quite different: by the antigen test, CMV antigen is detected; by PCR, CMV DNA is detected. This should be taken into account when the two tests are compared. However, by performing both tests on the same peripheral blood samples, clinical application of the two tests can be evaluated.

We applied both tests on 201 samples from 19 heart and 2 lung transplant recipients; samples were obtained regularly after transplantation. Other tests, like culture of CMV from granulocytes, urine, and saliva and CMV serology, were also performed. Detection of CMV in peripheral blood granulocytes by PCR occurred earlier in most patients than did detection of CMV antigenemia. PCR also remained positive for a longer period of time.

The relation of the antigen test and PCR with culture and the detection of CMV from buffy coat cells was poor, but the number of viremic samples was low. It is necessary to have larger numbers of viremic samples to draw solid conclusions regarding the relation between culture and PCR and antigenemia. However, when positive cultures are obtained from the buffy coat cells, one would expect CMV DNA to be present and to be detected by PCR.

High-level antigenemia has been shown to be related to symptomatic infection (9). In both patients with symptomatic infections in this study, high-level antigenemia was found. Not all patients with high-level antigenemia, however, were symptomatic. We found a good correlation between antigenemia and PCR in samples with high-level antigenemia, and this indicated that both tests are suitable and equally sensitive for the detection of a symptomatic, active CMV infection.

Inconsistent results between the detection of CMV antigen and CMV DNA were seen in samples that had low-level antigenemia and that were negative by PCR and in samples that were negative by the antigen test but positive by PCR. Some of the samples with low-level antigenemia and negative PCR results showed these results because of sampling errors and were found to be positive on retesting. Some of the inconsistent results of a positive PCR and a negative antigenemia were due to the greater sensitivity of PCR, especially for those samples from patients who were found to be positive for CMV by PCR before and after antigenemia was detected. These results confirm results of studies in bone marrow transplant recipients that indicated that PCR detects CMV DNA before antigenemia can be detected.

However, in our study, most of the discordant samples that were positive by PCR but negative for antigenemia were from five patients, most of whose samples were positive by PCR, whereas the antigen test was negative or was positive in only a few blood samples. PCR results were specific, as shown by hybridization of the PCR products from two patients. In three patients with CMV-positive cultures of urine or saliva, the higher sensitivity of PCR may explain these findings. However, in two other patients with negative antigen tests and no other evidence of CMV reactivation, there must be another explanation. Since PCR is a very sensitive technique for the detection of DNA, latent CMV DNA in minute amounts is probably detected while no significant or detectable amounts of viral antigens are present, and therefore, the antigen test is negative. Viral particles may have been phagocytosed, and the CMV DNA present in phagosomes was detected by PCR. The presence of phagocytosed CMV in granulocytes, however, has thus far been detected only during active CMV disease (6).

In conclusion, both PCR and antigenemia are very sensitive techniques for the detection of active CMV infection; however, the correlation between CMV disease and culture of CMV from buffy coat cells is poor. The advantage of the antigen test is that it yields quantitative results that are related to symptomatic infection.

Inconsistent results can be explained by sampling error (antigen positive, PCR negative) or by the greater sensitivity of PCR compared with that of the antigen test (PCR positive, antigen negative). However, PCR was also found to be positive in patients without further evidence of active CMV infection, which indicates that, after transplantation, CMV DNA can be present in peripheral blood granulocytes, even though CMV antigen and overt CMV infection cannot be detected. If so, it would be interesting to analyze in which form CMV is present in peripheral blood granulocytes and whether CMV RNA is present in those cells.

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REFERENCES

