Cytomegalovirus DNA Detection in Sera from Patients with Active Cytomegalovirus Infections

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Using a specific and sensitive polymerase chain reaction method, we detected reliably the presence of human cytomegalovirus (HCMV) DNA directly in serum samples collected at an early stage of HCMV infection, even before immunoglobulin M (IgM) antibodies were measurable. HCMV DNA was detected in serum from all patients with active HCMV infection; in 91% of these patients, HCMV DNA was found in the acute-phase serum. In 13 of 44 patients, HCMV DNA was found in serum before HCMV-specific IgM. For four kidney transplant recipients, the occurrence of HCMV DNA in serum, virus isolation from urine and leukocytes, and HCMV IgG and IgM serology were determined. We found a correlation between HCMV DNA in serum and positive virus isolation from leukocytes. In three of five congenitally infected infants, HCMV DNA and HCMV IgM were detected in the same sample. Two other infants were HCMV DNA positive, although no HCMV IgM antibodies were measurable. HCMV was found in urine from these infants either by virus isolation or with the polymerase chain reaction. Serum from one of the 22 healthy HCMV-seropositive blood donors was HCMV polymerase chain reaction positive.

Human cytomegalovirus (HCMV) infections are the major cause of illness and death in immunocompromised patients, such as allograft recipients and patients suffering from AIDS (4, 18). HCMV is the most common cause of congenital viral infection in humans, and 0.5 to 1% of infants are congenitally infected because of primary or secondary maternal HCMV infection (2).

One route of transmission of HCMV is by transfusion of blood from a seropositive donor to a seronegative recipient. HCMV is presumed to be transmitted by the donor’s leukocytes, since blood preparations that are depleted of leukocytes are less infectious. Viremia among seropositive donors has not been confirmed by virus isolation from blood (1).

The diagnosis of HCMV infection is clinically difficult. Isolation of virus by cell culture is time-consuming, but HCMV-induced early antigens can be detected by monoclonal antibodies before the development of a cytopathic effect. A recently introduced method for detection of HCMV antigens directly in leukocytes by using monoclonal antibodies is reported to correlate with ongoing HCMV viremia (23, 24).

Serology, especially detection of HCMV immunoglobulin M (IgM), is commonly used for the diagnosis of active HCMV infections. The use of serological methods for diagnosing HCMV infection in congenitally infected infants or patients with bone marrow transplants may be difficult because of transmitted or transfused IgG antibodies. The antibody response is often delayed or completely absent in immunosuppressed marrow recipients, and determination of HCMV IgM antibody in cord blood is not sensitive enough (16, 19).

Since antiviral therapy is available for severe HCMV infections, rapid methods are needed to identify infected patients early so that therapy can be instituted and monitored (7, 17).

The polymerase chain reaction (PCR), which can be used to selectively amplify and detect specific DNA sequences, is known to be a rapid and sensitive method for the detection of HCMV DNA in various kinds of specimens. Paraffin-embedded tissue specimens, urine, cervicovaginal cells, saliva, and peripheral blood leukocytes have been used (5, 11, 26, 27). The PCR for the detection of HCMV DNA compares favorably with methods such as in situ DNA hybridization, virus isolation, and Southern blotting (22, 27).

The detection of HCMV early antigen in leukocytes and/or the detection of HCMV DNA by the PCR in blood cell samples is an early diagnostic marker of imminent HCMV viremia (10, 23). We investigated the presence HCMV DNA in serum and its correlation to HCMV IgM antibodies in patients with active HCMV infection as a possible early diagnostic marker.

MATERIALS AND METHODS

Samples. Sera were collected from 44 patients, including 5 congenitally infected infants, with HCMV infection. Eleven patients were immunocompromised because of transplantation or another treatment, and patients 2, 9, 18, and 27 were renal allograft recipients described elsewhere (8, 9). Samples were taken regularly from these patients up to 34 to 46 weeks after transplantation.

Sera were also collected from 27 healthy blood donors, of which 22 were HCMV seropositive and 5 were HCMV seronegative. Sera were stored at −20°C until the samples were investigated by the PCR and serology for HCMV.

Serology. IgG and IgM antibodies to HCMV were measured in an enzyme-linked immunosorbent assay (20). HCMV infection was diagnosed when there was an HCMV-specific IgM antibody response and/or at least a fourfold rise in the titer of HCMV IgG.

Extraction of nucleic acids from serum. Nucleic acids were extracted from serum as described previously (15), with minor modifications. A 50-μl sample of serum was treated for 4 h at room temperature in a final volume of 500 μl of 10 mM Tris-HCl (pH 8.3)-0.25 mg of proteinase K (Sigma, St.

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TABLE 1. HCMV DNA findings in serum from five congenitally HCMV-infected infants

<table>
<thead>
<tr>
<th>Infant no.</th>
<th>HCMV DNA in serum</th>
<th>HCMV IgM in serum</th>
<th>HCMV findings in other samples</th>
<th>Clinical picture</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+ (0)†</td>
<td>Trace (0)</td>
<td>No sample</td>
<td>Hepatitis</td>
</tr>
<tr>
<td></td>
<td>+ (40)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>+ (2)</td>
<td>Trace (2)</td>
<td>No sample</td>
<td>Microcephalic</td>
</tr>
<tr>
<td></td>
<td>+ (66)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>+ (3)</td>
<td>(3)</td>
<td>Urine; virus isolation + (4)</td>
<td>Enlarged spleen</td>
</tr>
<tr>
<td></td>
<td>+ (30)</td>
<td></td>
<td></td>
<td>and thrombocytopenia</td>
</tr>
<tr>
<td>4</td>
<td>+ (14)</td>
<td>(14)</td>
<td>Urine; PCR + (14) and + (44);</td>
<td>Mother had a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>the urines were bacterially</td>
<td>primary HCMV</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>infected, and no virus</td>
<td>infection</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>isolation was possible</td>
<td>during the first</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>trimester</td>
</tr>
<tr>
<td>5</td>
<td>+ (9)</td>
<td>Trace (9)</td>
<td>No sample</td>
<td>Sepsis and kidney</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>and liver</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>insufficiency</td>
</tr>
<tr>
<td></td>
<td>+ (20)</td>
<td>Trace (20)</td>
<td></td>
<td>on day 5; infant</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>died on day 39</td>
</tr>
</tbody>
</table>

† Numbers within parentheses indicate the days on which the serum samples were collected.

Louis, Mo.) per ml–0.5% sodium dodecyl sulfate. trNA (Boehringer GmbH, Mannheim, Germany) was added as a carrier to a final concentration of 10 μg/ml. The nucleic acid was extracted with phenol, ethanol precipitated, and dissolved in 40 μl of distilled water.

HCMV PCR. The primers used for PCR amplification are complementary to the conserved immediate-early gene region 1 (6). The PCR was performed as a double PCR with two sets of primers as described previously (5), with minor modifications. The outer primer set consisted of C (5'- TGA GGA TAA GGC GGA GAT GT 3') and D (5'- ACT GAG GCA AGT TCT GCA GT 3'). The inner primer set consisted of A (5'- AGC TGC ATG ATG GTA GCA AG 3') and B (5'- GAA GGC TGA GTT GGT GGT AA 3'). The outer primer set C-D amplified a 242-bp amplicon, and the nested primer set A-B amplified a 146-bp amplifier.

All the reaction mixtures consisted of 10 mM Tris-HCl (pH 9.6), 10 mM MgCl₂, 50 mM NaCl, 0.2 μl of bovine serum albumin per ml, and 1 U of Thermus aquaticus (Tag) DNA polymerase (AmpliTaq; Perkin Elmer-Cetus, Emeryville, Calif.). In the first step, 0.5 mM each deoxynucleoside triphosphate and 0.15 μM primers C and D were used. In the second step, 1.0 mM each deoxynucleoside triphosphate and 0.30 μM primers A and B were used. Then 10 μl of the prepared sample was added to the C-D mixture in a final volume of 50 μl. The tubes were covered with paraffin oil and preheated for 4 min at 92°C before the incubation cycles were started. Twenty incubation cycles consisting of 30 s at 92°C, 30 s at 53°C, and 30 s at 72°C were performed in an automated thermal cycler (DNA Thermal Cycler; Perkin Elmer-Cetus). The product from the first amplification was diluted 20 times with a new reaction mixture consisting of the A-B buffer system and reamplified with 40 incubation cycles as described above.

To detect false-positive reactions caused by contamination, every fourth sample analyzed was a negative control. The PCR products were electrophoresed on a 2% agarose gel containing ethidium bromide, and the results were photographed.

RESULTS

HCMV in congenitally infected infants. Sera from the five congenitally infected infants were sampled during the first 14 days after delivery. HCMV DNA was found in the acute-phase sera from the five congenitally infected infants. Three of the infants had traces of HCMV IgM in the acute-phase serum (Table 1).

Infant 5 developed kidney and liver deficiencies 5 days after delivery, and the clinical symptoms indicated an infection. Serum samples collected on days 9 and 20 after delivery were clearly HCMV PCR positive. Traces of HCMV IgM were found in both sera. The IgG titers were difficult to evaluate because of HCMV IgG antibodies passively transferred by blood transfusion. No evidence of herpes simplex virus, enterovirus, or bacterial infection was found. No further samples were available, since the infant died on day 39 after delivery.

Monitoring of four renal graft recipients with PCR, serology, and virus isolation. Four renal allograft recipients were selected from previous studies, in which they had been monitored for 34 to 46 weeks with different methods for detection of HCMV infection (8, 9). All patients developed HCMV disease. Serological data for the four renal graft recipients were reported previously (8) and are presented here in Fig. 1. Three of the four patients had a primary HCMV infection (patients 2, 18, and 27; Fig. 1).

The serum from patient 2 became HCMV PCR positive before HCMV IgM was detectable. HCMV was isolated from mononuclear cells and polymorphonuclear cells coincidentally with the first positive HCMV PCR. Also, in the serum from patient 27 HCMV DNA was detected before HCMV IgM. In the next sample from patient 27, virus was isolated from mononuclear cells, but the serum was still HCMV IgM negative. Patient 18 received a blood transfusion on day 6, which gave a false increase in the IgG titer. This patient was viruric on day 31 with no sign of viremia. Serum collected at week 9 became positive coincidently for HCMV IgM and HCMV DNA. In patient 9, virus isolation from polymorphonuclear cells was positive before HCMV DNA and HCMV IgM were detected. In patients 2 and 18, the HCMV PCR was still positive for 12 to 17 weeks after the IgM had become undetectable. Virus continued to be recovered from the urine of patient 18 even after the HCMV PCR in serum became negative. Although no virus was recovered from the blood of patient 27 25 weeks after transplantation, recurrent HCMV viremia was detected by
FIG. 1. Follow-up study with HCMV PCR on serum, HCMV serology, and virus isolation from urine, polymorphonuclear cells (PMC), and mononuclear cells (MNC) from four renal graft recipients. nd, not determined.

HCMV in patients with active HCMV infection. Thirty-five patients, all with clinical symptoms of HCMV infection, were divided into four groups based on the HCMV serology in the first available serum sample (Table 2). All patients had a significant HCMV IgG titer rise, and 32 of the 35 patients had HCMV IgM.

Neither HCMV IgG nor IgM antibodies were detected in the initial serum sample collected from four patients (group I). Three patients had HCMV DNA in the acute-phase sera collected 5 to 8 days after the onset of fever. All convalescent-phase sera became HCMV IgM, HCMV IgG, and HCMV DNA positive.

The initial serum samples from 19 patients (group II) were HCMV IgM positive and had HCMV IgG titers of less than 2,000 (range, <100 to 1,600). All were HCMV DNA positive in the first serum sample. Of 19 convalescent-phase serum samples, 15 were tested by PCR; 12 were HCMV DNA and IgM positive, 2 were HCMV DNA positive and IgM negative, and 1 was both HCMV DNA and IgM negative.

The initial serum samples from 12 patients with reactivated HCMV infection had HCMV IgG titers of >4,000 range, (4,100 to 140,000). Five of these were HCMV IgM negative (patient group III), and seven were HCMV IgM positive (group IV). Three patients with only a fourfold HCMV IgG titer rise as serological evidence belong to group III. The initial serum samples from all five patients in group III were HCMV DNA positive. Four of these five patients had HCMV DNA in the first serum sample, whereas the fifth patient had HCMV DNA later (140 days after transplantation).

### TABLE 2. HCMV DNA findings in 27 healthy blood donors and in the initial serum samples from 35 patients with serologically verified HCMV infection and clinical symptoms

<table>
<thead>
<tr>
<th>Group</th>
<th>Serological data</th>
<th>No. of samples</th>
<th>No. of HCMV DNA-positive samples/total</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>HCMV IgG titer, &lt;100; HCMV IgM negative</td>
<td>4</td>
<td>3/4</td>
</tr>
<tr>
<td>II</td>
<td>HCMV IgG titer, &lt;2,000; HCMV IgM positive</td>
<td>19</td>
<td>19/19</td>
</tr>
<tr>
<td>III</td>
<td>HCMV IgG titer, &gt;4,000; HCMV IgM negative</td>
<td>5</td>
<td>5/5</td>
</tr>
<tr>
<td>IV</td>
<td>HCMV IgG titer, &gt;4,000; HCMV IgM positive</td>
<td>7</td>
<td>6/7</td>
</tr>
<tr>
<td>V</td>
<td>Healthy blood donors; HCMV IgG titer, &lt;100</td>
<td>5</td>
<td>0/5</td>
</tr>
<tr>
<td>VI</td>
<td>Healthy blood donors; HCMV IgG titer, &gt;1,000</td>
<td>22</td>
<td>1/22</td>
</tr>
</tbody>
</table>

* The initial sample was PCR negative, but convalescent-phase serum collected 30 days later was PCR positive.

* The initial sample was PCR negative, but convalescent-phase serum collected 24 days later was PCR positive.
were immunocompromised. Three serum samples were collected on days 5 through 8 after the onset of illness, and two serum samples were collected after 2 and 4 weeks, respectively. Three convalescent-phase serum samples were tested by the PCR and; two were HCMV DNA and IgM positive, and one was HCMV DNA and IgM negative.

HCMV DNA was detected in the initial serum sample of six of the seven patients belonging to group IV. HCMV IgM was detected before HCMV PCR was positive in the serum from one patient. Five convalescent-phase sera were studied by PCR; four of them were HCMV DNA and IgM positive, and 1 serum sample was HCMV IgM positive and PCR negative.

HCMV DNA was detected by PCR in the first serum sample collected, even before HCMV IgM antibodies were measurable, in eight patients (groups I and III). In 25 patients, HCMV IgM and HCMV DNA were detected in the same serum. Three serum samples contained HCMV DNA, although a fourfold IgG titer rise was the only serological evidence of an active HCMV infection (group III). The majority (22 of 27) of the convalescent-phase serum samples were both HCMV PCR and IgM positive.

**HCMV DNA in sera from healthy blood donors.** Sera from five HCMV seronegative healthy blood donors (group V) were HCMV PCR negative, as were 21 of 22 samples from HCMV-seropositive healthy blood donors (group VI). The one PCR-positive serum sample was HCMV IgM negative. Unfortunately no later serum samples were available. DNA from this serum was prepared four times, and HCMV PCR was repeatedly positive. Blood from one seropositive donor had traces of HCMV IgM but was HCMV PCR negative.

**DISCUSSION**

In this study, HCMV DNA detected by the PCR in serum samples was shown to correlate with HCMV serology, indicating an active infection. The PCR has been used to detect HCMV in blood, saliva, urine, tissue specimens, and bronchoalveolar lavage cells. Since the PCR is highly sensitive, it is important to select relevant specimens for diagnosing active HCMV infection. HCMV DNA can be detected frequently in urine and throat washings from HCMV-seropositive immunosuppressed patients without clinical disease. This may indicate that the patients are at risk for developing an active HCMV infection, whereas the presence of HCMV DNA in blood appears to correlate with an active HCMV infection (10, 26). Other studies have shown a strong correlation between the detection of early HCMV antigens in leukocytes and viremia (23, 24). In a recent study, HCMV DNA was detected in sera from four bone marrow transplant recipients and four patients with leukemia, all of whom suffered from pneumonia (13).

In 13 of 44 patients with active HCMV infection, HCMV DNA was detected in the serum by the PCR before HCMV IgM was detected. HCMV DNA was detected in four of nine immunocompromised patients before serological evidence of HCMV infection. The acute- and convalescent-phase sera from all patients with serological evidence of HCMV infection were PCR positive. The PCR detected HCMV DNA in 38 of 40 of the first collected samples. One acute-phase serum sample was both HCMV PCR and HCMV IgM negative. In sera collected on day 33 after transplantation and on day 3 after illness from a kidney and pancreas transplant recipient with reactivated HCMV infection, HCMV DNA was not detected in the HCMV IgM-positive acute-phase sample. This could be due to antiviral treatment, which depletes the virus of virions, or to HCMV-antibody-virion complexes that decrease the number of free circulating virions.

HCMV DNA was detected in the initial serum sample from two infants, although no HCMV IgM antibodies were measured. One mother was known from clinical findings and HCMV serology to have had a primary HCMV infection. In renal graft recipients, detection of HCMV DNA by the PCR correlated to positive virus isolation from blood cells. One patient was viremic with no sign of viremia. Transmission of HCMV via the kidneys has been reported (12), so this viremia might be caused by reactivation of the HCMV strain from the transplanted kidney. The virus was isolated from polymorphonuclear cells from another patient before HCMV DNA and HCMV IgM were detected. Since the patient was HCMV seropositive before transplantation, these results might indicate reactivation of the patient's own HCMV strain, which had just begun to replicate in the cells. Thus, there may not have been enough HCMV spread into the serum for PCR detection. Blood cells from a patient at the late stage of HCMV infection was virus isolation negative but still HCMV DNA positive. A sample collected later became virus isolated, possibly from leukocytes that had been removed from the PCR during the viremia-free interval before recurrent HCMV viremia (10).

We found no HCMV DNA in sera from seronegative blood donors, whereas 1 of 22 serum samples from seropositive blood donors was PCR positive for HCMV. Transmission of HCMV has been well documented in cases of blood transfusion (1). Studies have estimated that 6 to 8% of HCMV-seropositive blood donors have HCMV-specific IgM at the time of donation (3, 25). Three percent of blood donors excrete HCMV at the time of blood donation (14); our subject may belong to this category. This donor might have just started or ended an HCMV reactivation. Since only a few HCMV IgM-positive blood donors transmitted HCMV to the recipient (1, 21), reactivation in a healthy seropositive person might result in viremia with a low number of virions in the circulation.

The PCR-amplified HCMV DNA in serum might have been derived from virions or infected cells that had been lysed so that viral nucleic acids were released. Since our PCR has a detection limit of 20 genomes (5), more than 1.6 HCMV genomes per µl of serum is needed for a positive amplification. This limit allowed HCMV genomes from active HCMV infections, but usually not HCMV DNA from healthy seropositive donors, to be identified.

The PCR detected HCMV DNA in serum from patients with primary HCMV infection as well as reactivated HCMV infection. Since HCMV DNA can be found in sera with weak or no HCMV IgM, convalescent-phase sera are not needed in such cases. In congenitally infected infants and bone marrow transplant recipients with weak serological responses, the PCR is a good method for the detection of HCMV DNA in serum. Successful ganciclovir treatment resulted in the disappearance of HCMV DNA from leukocytes detected by the PCR (10). The PCR might therefore be a good method for monitoring antiviral treatment responses directly in patient sera.

**ACKNOWLEDGMENT**

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