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Detection of Cytomegalovirus mRNA and DNA Encoding the Immediate Early Gene in Peripheral Blood Leukocytes From Immunocompromised Patients

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Detection of cytomegalovirus (CMV) DNA in peripheral blood leukocytes has been shown to be a sensitive marker of CMV infection. However, the specificity with regard to its clinical significance is less clear, since infections considered to be latent may be detected. In this report, the presence of CMV immediate early antigen (IEA) DNA and mRNA in peripheral blood leukocytes detected by PCR was investigated and related to the appearance of CMV pp65 antigen, CMV serology, and clinical status. Thirty-seven consecutive samples were submitted to the laboratory from 36 immunocompromised patients, on a routine basis for analysis because of a potential risk of CMV infection. To facilitate differentiation between DNA and mRNA, primers were chosen in exons 2 and 3 of the immediate early gene of CMV. Keratin type I mRNA and the ssu rRNA gene served as internal controls. Thirty specimens were CMV antibody positive, of which 11 were also CMV IEA DNA positive. Two of seven seronegative samples were CMV IEA DNA positive. No relation was found between serology and the presence of CMV IEA DNA as determined in 37 samples. Five of 32 samples that could be analyzed were positive for CMV IEA mRNA, of which four were also positive in the pp65 antigen detection technique. A clear relation was found between the presence of CMV IEA mRNA and CMV pp65 antigen in leukocytes and with the clinical findings as well ($P < 0.01$). It is concluded that detection of CMV mRNA may have a role in diagnosis of an active clinically relevant CMV infection. © 1994 Wiley-Liss, Inc.

KEY WORDS: AIDS, early diagnosis, IEA, pp65, PCR, transplantation.

INTRODUCTION

Human cytomegalovirus (CMV) infections are a major cause of morbidity and mortality in patients with decreased cellular immunity, in particular in recipients of organ transplants and patients with the acquired immunodeficiency syndrome (AIDS) [Griffiths and Grundy, 1988; Editorial, 1989; Drew, 1992a,b]. The diagnosis of CMV infection often poses great challenges. Clinical symptoms are mostly nonspecific, which adds to the importance of virological diagnosis. Isolation of the virus is time consuming and may be unrelated to disease, since shedding of the virus frequently occurs. The immune response is often delayed or completely absent in immunocompromised patients. To rely on a specific IgM response, commonly used for diagnosis of a primary CMV infection, may therefore be difficult. To overcome these problems, rapid virus isolation and detection methods have recently been developed. These procedures, such as a rapid centrifugation assay for virus isolation [Gleaves et al., 1985; Rothbarth et al., 1987] and antigen detection of CMV in leukocytes [Van der Bij et al., 1988; Jiwa et al., 1989], are of great value in patient management, especially since antiviral drugs such as ganciclovir and foscarnet have become available for treatment of severe CMV illness. In addition, molecular biological methods have become available. Detection of specific CMV DNA sequences by the polymerase chain reaction (PCR) has been reported [Demmler et al., 1988; Cassol et al., 1989; Einsele et al., 1991). Although the presence of CMV DNA in leukocytes in general did not correlate clearly with the presence of antibodies, the detection of latent infections has been reported [Rowley et al., 1991; Bitsch et al., 1992]. This means that the specificity of CMV DNA detection, defined as the ability to detect active viral replication, remains questionable. Therefore an evaluation of the role of specific CMV mRNA detection, which is more likely to correlate with an active viral infection, has been performed. In this report the presence of CMV

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DNA and mRNA in peripheral blood leukocytes is compared with serology and specific CMV antigen detection and the possible clinical significance is discussed.

MATERIALS AND METHODS

Collection of Specimens

Thirty-seven blood samples were obtained from 36 patients clinically at risk of infection with CMV. The series consists of samples submitted consecutively as received by the laboratory on a routine basis. All patients had an acquired or induced immunodeficiency (22 heart, liver, or kidney transplant recipients; nine patients with AIDS, two patients with leukemia, one patient with a myelodysplastic syndrome, one patient with primary Epstein Barr virus mononucleosis, and one patient with Kaposi sarcoma). Serum or plasma was prepared by centrifugation of the samples and stored at −70°C (plasma) or −20°C (serum). Ethylene diaminotetraacetic acid (EDTA) blood samples were used for isolation of nucleic acids and pp65 antigen detection in leukocytes. One milliliter of EDTA blood was added to 9 ml storage medium [4 M guanidiniumthiocyanate (GuSCN), 2.6 g Triton X-100 per 100 ml, and 22 ml 0.2 M EDTA per 100 ml] and placed at −70°C.

Detection of pp65 Antigen

CMV pp65 antigen detection in polymorphonuclear and mononuclear leukocytes was carried out immediately after submission of the sample to the laboratory. The modified assay was performed at room temperature unless stated otherwise and based on procedures described previously [Van der Bij et al., 1988]. Briefly, 2 ml EDTA blood was mixed with 6 ml 4.2% dextran [4.2% Dextran T500 (Pharmacia, Uppsala, Sweden) in 120 mM glucose, 28 mM EDTA, and 170 mM NaCl] and allowed to stand for 45 min at 37°C at an angle of 45°. The supernatant was collected and centrifuged for 10 min at 800*g*. Erythrocytes were lysed as described, and the suspension was centrifuged for 5 min at 500*g*. The pellet was washed in PBS and resuspended in PBS. Leukocyte concentration was adjusted between 1×10^6 and 1×10^7 cells/ml. Next, 100 μl cell suspension was placed on an ethanol/ether (1:1) pretreated slide. To adhere the cells to the slides, they were left for 1 min at room temperature without allowing them to dry completely. The moist slides were fixed for 15 min in a methanol:acetic acid mixture (20:1) at room temperature. After fixation, slides were washed three times with PBS over a 10 min period, and a monoclonal antibody directed against the CMV pp65 antigen (Clonab, Biotest, Dreieich, Germany) of CMV diluted in PBS/0.05% Tween-20/2% BSA (PTB) was applied and a coverslip was placed on top. Slides were incubated upside down for 45 min at 37°C. After removing the coverslip, slides were washed three times with PBS during 10 min. One hundred microliters of rabbit antimouse IgG labelled with peroxidase (Dakopatts, Glosstrup, Denmark) diluted in PTB was applied and incubated as described previously. After washing three times during 10 min, staining was carried out with diaminobenzidine (DAB; 0.05 g in 100 ml 0.5 M Tris, pH 7.6, 1 ml imidazole, 160 μl H_2O_2) for 10 min with continuous shaking. Slides were washed with tapwater for 3 min. Citifluor (City University, London, England) was added and a cover slide was placed on the slide, which was examined by microscopy for positive staining (magnification ×400). To obtain the best results, drying of slides was avoided at all times.

Detection of CMV Antibody

The presence IgG and IgM antibodies against CMV was determined with a commercially available ELISA (Sorin Biomedica, Saluggia, Italy) and confirmed by an indirect immunofluorescence assay [Hekker et al., 1979].

Extraction and Detection of RNA and DNA

RNA and DNA were simultaneously extracted from blood using a procedure previously described [Boom et al., 1990]. Briefly, 1 ml blood was mixed with 9 ml storage buffer. One hundred microliters of silica suspension (Celite, Janssen Chimica, Geel, Belgium) was added and mixed with blood and storage buffer. After 15 min of incubation at room temperature, the suspension was centrifuged for 10 min at 1,000*g*, and the pellet was resuspended in 1 ml storage buffer. The suspension was centrifuged for 15 sec at 12,000*g*, and the pellet washed twice with 4 M GuSCN in 0.1 M Tris buffer, pH 6.8; twice with 70% ethanol; and once with acetone, followed by drying in a vacuum desiccator. RNA and DNA were eluted in 100 μl sterile RNase-free water supplemented with 1 U RNasin (Promega, Madison, WI) and collected after 3 min of centrifugation at 12,000*g*. Extracted nucleic acid samples were stored at −70°C.

Amplification of RNA and DNA

Primers. CMV primers were chosen in the major immediate early antigen gene. Primer 1 was located in exon 2 (position 1487–1506), and primer 2 was located in exon 3 (position 1770–1751) [Akrigg et al., 1985]. This allows discrimination between amplified DNA originating from mRNA [170 base pairs (bp)] and DNA (284 bp). As an internal control, a PCR was done on the human keratin type I mRNA and for DNA on the small subunit rRNA (ssu rRNA) gene. Keratin primers were located in exon 1 (position 494–513) and exon 3 (position 2531–2512) [Marchuk et al., 1985]. Amplified product originating from mRNA was 218 bp. Primers on the ssu rRNA gene were located at position 430–452 and position 626–604 [Falcinelli et al., 1993], yielding a product of 197 bp. All primers were synthesized with an Applied Biosystem DNA synthesizer (model 380).

cDNA synthesis. cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (M-MLV RT; Gibco BRL, Gaithersburg, MD) and random primers (Pharmacia, Uppsala, Sweden) in a volume of 30 μl using the buffer provided by the manufacturer, 1 μl random primers 0.4 μg/μl, 2.5 μl 0.1 M

dithiothreitol (DTT), 1.25 μl 10 mM of all four dNTPs, 1 μl RT (200 U), and 1 μl RNAs in (1 U; Promega, Madison, WI). Samples were preheated at 80°C and then quenched on ice, and 5 μl was added to the reaction mixture. Ater incubation at 42°C for 1.5 hr, RT was heat inactivated for 10 min at 90°C.

PCR. PCR was carried out as described earlier [Giesendorf et al., 1992]. Seven and one-half microliters of the cDNA synthesis reaction product was amplified in order to detect mRNA. Ten microliters of the eluent of the nucleic acid extraction was used in order to detect DNA. Annealing was carried out at 55°C for 1 min, extension at 72°C for 2 min, and denaturation at 95°C for 1 min. This cycle was repeated 30 times in a Biomed PCR Processor (Theres, Germany).

Detection of amplified product. The amplified product was visualized after electrophoresis on a 2% agarose gel under ultraviolet light and identified by Southern blotting and hybridization [Sambrook et al., 1989] with an appropriate ^{32}P-labelled oligonucleotide probe (CMV IEA: position 1688–1717; Keratin type 1: position 1871–1900; ssu rRNA: position 563–589).

Determination of sensitivity. To establish the sensitivity of the cDNA synthesis and subsequent PCR, RNA transcripts of a plasmid containing the major IEA gene of CMV were used (kindly provided Drs. T. Kievits, Organon Teknika, Boxtel, The Netherlands). Starting with 10^6 RNA molecules, serial tenfold dilutions were made and subject to cDNA synthesis and subsequent PCR.

Statistical Analysis

Statistical analysis was carried out using Fisher's exact test. P values < 0.05 were considered significant.

RESULTS

Thirty-seven samples from 36 patients were analyzed for the presence of CMV pp65 antigen, CMV mRNA, CMV DNA, and anti-CMV immunoglobulin. Thirty samples were CMV antibody positive. Nineteen of these were negative and 11 were positive for CMV DNA. Seven samples were seronegative for CMV; five of these were also CMV DNA negative and two were CMV DNA positive (Table I). The internal ssu rRNA DNA control was positive in all samples. Statistical analysis failed to demonstrate a relation between the presence of CMV DNA and the presence of CMV antibody ($P > 0.5$).

The efficiency of the cDNA synthesis and subsequent CMV-specific PCR was measured by processing recombinant CMV RNA transcripts from the IEA region. In a dilution series, ten to 100 RNA molecules could be detected reproducibly (Fig. 1).

Because the internal keratin RNA control was negative in five samples, the remaining 32 specimens were further analysed for the presence of CMV mRNA (Table I). Four samples were positive for both pp65 antigen and mRNA, and 27 were negative in both tests. Sample 4 was positive in the CMV mRNA detection technique, while no pp65 antigen could be observed. Statistical analysis showed a P value < 0.01 when correlating the CMV mRNA test and the CMV pp65 antigen detection method. No relation was found when comparing the presence of CMV DNA with the presence of pp65 antigen nor with the clinical diagnosis.

The five CMV mRNA-positive samples were obtained from four patients. Specimen 2, obtained from a patient with bronchial carcinoma and an immunocytoma after heart transplantation, had only one cell positive in the pp65 antigen detection technique. A diagnosis of a primary CMV infection had been made 1 month earlier based on seroconversion and positive CMV pp65 antigen detection. The numbers of positive cells with the pp65 antigen detection technique in specimens 20, 22, and 28 were 10, 20, and 30, respectively. In these cases, clinical diagnosis in one patient (specimen 28) was CMV gastritis after heart transplantation, in the others (specimens 20 and 22) AIDS with CMV esophagitis and retinitis. The sample positive for CMV mRNA and negative in the pp65 antigen detection technique (specimen 4) was obtained from a kidney transplant patient with retinitis and episodes of high fever (Table I). None of the patients with a negative CMV mRNA test had other laboratory evidence of symptomatic CMV infection, and in none of these patients was a clinical diagnosis of CMV-associated disease made, neither at the moment when the sample was obtained nor in the recent past.

DISCUSSION

Symptomatic CMV infection in immunocompromised patients is traditionally diagnosed by culture of CMV from leukocytes, since shedding of the virus in urine and throat frequently occurs without clinical disease [Metselaar et al., 1987; Bower et al., 1990]. Serological methods, in particular the detection of IgM antibodies, are often applied but frequently have been shown to be insufficiently sensitive. CMV culture from blood cells is a good parameter for an active CMV infection but is hampered by technical difficulties. The pp65 antigen detection in peripheral leukocytes [Van der Bij et al., 1988; Jiwa et al., 1989] proved to be a good replacement for CMV culture from blood, although this method has some technical disadvantages as well. Therefore, the use of CMV DNA and mRNA as early markers for viremia and active CMV infection is under evaluation at present. Reports have shown varying relations between CMV DNA detection, CMV culture or antigen detection, serology, and clinical manifestations of CMV infection [Gerna et al., 1991; Bitsch et al., 1992; Boland et al., 1992; Delgado et al., 1992; Van Dorp et al., 1992; Zipeto et al., 1992]. A general finding has been that although a good or even excellent sensitivity has been obtained, CMV DNA detection has not been shown to be specific in the sense of detecting exclusively clinically relevant infections. Positive signals produced by latent infections may occur with this method. The presence of CMV mRNA in blood can be used as a marker closely related to active stages of CMV infection. However, until now CMV mRNA detection has only rarely

TABLE I. Clinical Data and Outcome of Serology (CMV IgG and IgM), CMV pp65 Antigen Detection (CMV pp65 ag.), CMV IEA mRNA PCR (CMV IEA mRNA), Keratin mRNA PCR (Ker. mRNA), CMV IEA DNA PCR (CMV IEA DNA), and ssu rRNA DNA PCR (ssu DNA)*

No.	CMV pp65 ag.	CMV IgG	CMV IgM	CMV IEA mRNA	Ker. mRNA	CMV IEA DNA	ssu DNA	Clinical status
1	–	+	–	–	+	+	+	Leukemia
2	+	–	+	+	+	+	+	HTx, g.r. fever, bronchial carcinoma, immunocytoma
3	–	+	–	–	+	+	+	LTx, elevated liver enzymes
4	–	–	–	+	+	+	+	RTx, retinitis, fever
5	–	+	–	–	+	–	+	AIDS
6	–	+	–	–	+	+	+	HTx, g.r.
7	–	+	–	–	+	–	+	*Cryptococcus* meningitis, Kaposi sarcoma
8	–	+	+	–	+	–	+	AIDS
9	–	+	–	–	+	–	+	HTx, g.r.
10	–	+	–	nd	–	+	+	HTx
11	–	+	+	nd	–	+	+	RTx, g.r., fever
12	–	+	+	–	+	–	+	RTx
13	–	+	–	–	+	+	+	RTx
14	–	–	–	–	+	–	+	AIDS, lobular pneumonia, candida infection
15	–	+	+	–	+	+	+	AIDS, lobular hepatitis, retinitis
16	–	+	–	–	+	–	+	Acute myeloid leukemia, vasculitis
17	–	+	–	–	+	–	+	RTx
18	–	–	–	–	+	–	+	HTx
19	–	+	–	–	+	–	+	Primary EBV mononucleosis
20	+	+	–	+	+	+	+	AIDS, esophagitis, retinitis
21	–	–	–	–	+	–	+	RTx
22	+	+	–	+	+	+	+	AIDS, esophagitis, retinitis
23	–	+	–	–	+	–	+	AIDS, otitis media, lymphadenopathy
24	–	+	+	–	+	–	+	HTx
25	–	+	–	–	+	–	+	AIDS
26	–	+	+	–	+	–	+	HTx, g.r.
27	–	+	+	–	+	–	+	HTx, sec. EBV infection, immunocytoma
28	+	+	–	+	+	+	+	HTx, gastritis
29	–	–	–	–	+	–	+	AIDS
30	–	–	–	–	+	+	+	AIDS, epilepsy
31	–	+	+	–	+	–	+	RTx, tuberculosis
32	–	+	–	–	+	–	+	Myelodysplastic syndrome
33	–	+	–	–	+	–	+	HTx, seizure
34	–	–	–	–	+	–	+	HTx, sternum infection *Staphylococcus aureus*
35	–	+	–	nd	–	–	+	HTx
36	–	+	+	nd	–	–	+	HTx
37	–	+	+	nd	–	–	+	HTx

*Keratine and ssu rRNA serve as internal mRNA and DNA controls, respectively. Abbreviations: No., sample number; nd, not done; HTx, heart transplant patient; LTx, liver transplant patient; RTx, renal transplant patient; EBV, Epstein Barr virus; CMV, cytomegalovirus; AIDS, acquired immunodeficiency syndrome; IEA, immediate early antigen; g.r., graft rejection.

been evaluated [Rowley et al., 1991]. In this report it has been shown that, in immunocompromised patients, CMV DNA detection by PCR in blood did not correlate with the presence of CMV pp65 antigen or with CMV mRNA or with serology. A lack of agreement of CMV DNA detection with the presence of antibodies has been reported by others as well [Bitsch et al., 1992; Delgado et al., 1992]. This implies that CMV DNA cannot be used as a reliable marker of either latency or active CMV infection.

In addition, detection of CMV mRNA was carried out, using the exon–intron structure of the IEA gene (Fig. 2). In four of five CMV mRNA-positive cases, pp65 antigen detection was also positive, and no other cases of positive antigen detection were found. Although pp65, the lower matrix protein, is not a genuine late antigen, it has its most abundant expression after initiation of viral DNA replication [Alison and Stenberg, 1989; Grefte et al., 1992]. This may explain the greater sensitivity of the mRNA test. the striking relation between the results of CMV mRNA and the pp65 antigen assays supports the possible value of CMV mRNA detection, which is also demonstrated by the relations with clinical diagnoses in this series. This result appears to contrast with a previous study in which CMV mRNA was found to be insufficiently sensitive [Rowley et al., 1991]. The possible discrepancy could be due to a different primer selection and a different amplification efficiency. With our primers, which were located in the immediate early gene, a sensitivity of ten to 100 RNA molecules could be obtained. An additional difference to be noted is the dissimilarity of mRNA and DNA discrimination. Rowly et al. [1991] used extensive DNase treatment, whereas in this study discrimination

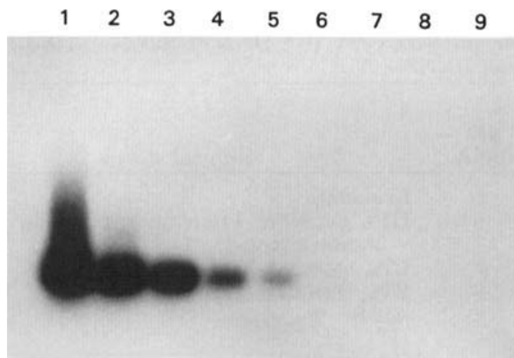

Fig. 1. Sensitivity of CMV immediate early gene RNA PCR assay. Southern blot and autoradiography of cDNA PCR products from serial tenfold dilutions of recombinant CMV RNA encoding the unspliced IEA gene (284 bp). **Lane 1:** 10^6 RNA molecules. **Lane 2:** 10^5. **Lane 3:** 10^4. **Lane 4:** 10^3. **Lane 5:** 10^2. **Lane 6:** 10^1. **Lane 7:** 10^{-1}. **Lane 8:** 10^{-2}. **Lane 9:** Negative control.

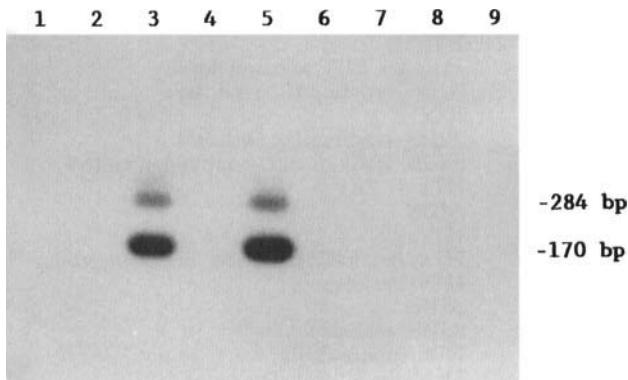

Fig. 2. Results of CMV mRNA detection in peripheral blood leukocytes using Southern blotting and autoradiography of eight consecutive samples. **Lanes 1–8:** Samples 18–25. **Lane 9:** No template. Product sizes are indicated: 284 bp DNA originates from CMV DNA, 170 bp originates from CMV mRNA.

between mRNA and DNA was achieved by using the intron–exon structure of the IEA gene, making the use of such an additional DNase treatment unnecessary. The method used also facilitates incorporation of an internal control after cDNA synthesis for the presence of RNA in every consecutive specimen obtained.

In conclusion, the detection of CMV mRNA may be of value in the diagnosis of active CMV infection in immunocompromised patients. Further studies should involve larger series of patients and, technically, the amplification of a late antigen gene in addition to the IEA gene used in this study. Evaluation of quantitative PCR may provide interesting information with regard to management of patients undergoing antiviral therapy. The method of mRNA detection described demonstrates that molecular biological detection of CMV merits further study to establish the role of this approach in the diagnosis of active and clinically relevant infections.

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