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Antigenemia, immunoblotting, and enzyme immunoassay for early diagnosis of cytomegalovirus infection in renal transplant patients

L. Fischer[1], P. Rautenberg[1], H. Bienengräber[2], G. Leimenstoll[2]

[1] Institute of Medical Microbiology and Virology, Christian-Albrechts-University Kiel, Brunswiker Strasse 4, W-2300 Kiel, Germany
[2] Nephrology Section, Department of Internal Medicine, Christian-Albrechts-University Kiel, Brunswiker Strasse 4, W-2300 Kiel, Germany

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Abstract. Timely and rapid diagnosis of cytomegalovirus (CMV) infection is important for the management of transplant patients. We compared three serological assays, IgM immunoblot and IgG/IgM enzyme immunoassay (EIA), as well as the detection of CMV antigens in polymorphonuclear blood leukocytes (antigenemia), for their value in the early diagnosis of CMV infection. Thirty-one patients were monitored longitudinally for 3 months after renal transplantation. Laboratory documented CMV infection occurred in 20 patients. All of these cases showed a positive IgM immunoblot result that was confirmed by at least one of the other test assays (IgG EIA 19/20, antigenemia assay 13/20, and IgM EIA 12/20). All of the ten patients whose clinical picture was compatible with symptomatic CMV disease were positive for CMV infection according to IgM immunoblot and IgG EIA, nine were positive according to the antigenemia assay, and seven were positive according to IgM EIA. With reference to the temporal pattern, the antigenemia assay indicated CMV infection significantly earlier than the serological tests ($P \leq 0.05$). In symptomatic patients CMV antigen-positive leukocytes were, on the average, detected on the day of onset of symptoms, whereas detection by IgM immunoblot, IgG EIA, and IgM EIA followed 8, 13, and 14 days later, respectively. These results show that: (1) the CMV antigenemia assay is very useful for the early diagnosis of symptomatic CMV infections; (2) CMV antibodies, as an indicator of CMV infection, are detectable earlier and more frequently by IgM immunoblot than by IgG/IgM EIA; (3) compared to CMV antigenemia, the IgM immunoblot indicated CMV infection more often but significantly later; and (4) only a combination of several diagnostic methods allows optimal detection of CMV infections in renal transplant patients.

Key words: CMV infection, diagnosis – Renal transplantation, CMV infection – CMV early antigen, renal transplantation

Correspondence to: L. Fischer

Introduction

Cytomegalovirus (CMV) is regarded as a predominant infectious agent affecting the recipients of bone marrow [14] and solid organ transplants [7, 11, 18]. Because clinical manifestations of CMV infection are often nonspecific and may resemble those of transplant rejection or other infections, rapid and reliable identification of CMV infections is important. Studies of bone marrow transplant patients also suggest that specific treatment at an early stage of CMV infection may prevent the development of life-threatening, disseminated CMV disease [20].

A major improvement in the diagnosis of CMV infection is the development of detection systems for CMV genomic structures [24] and CMV antigens [22], to be applied either directly to clinical specimens [4, 21] or to cell culture within a few days after inoculation of clinical specimens [9]. The detection of CMV antigens in blood leukocytes in particular has been shown to be more sensitive and less time-consuming than conventional culture for the early diagnosis of CMV infection [3].

Serological procedures also have a short processing time but are hampered by the fact that the results only give an indirect indication of CMV infection. They also depend on the host's ability to mount a sufficient immune response, which may be impaired and delayed in the immunocompromised host [17]. However, promising results have been published on the detection of CMV IgM antibodies by immunoblot, as a diagnostic tool for CMV infections in organ transplant recipients [1, 15, 19].

The following study was undertaken to compare the value of the CMV antigenemia assay and the detection of CMV antibodies by enzyme immunoassay (EIA) and immunoblot for an early diagnosis of CMV infection in renal transplant recipients.

Materials and methods

Patients

Of the 37 adult patients who received a cadaver kidney transplant in 1990 at Kiel University Hospital, 31 were included in this study. Two patients with transplant thrombosis and four patients who were not

available for the entire observation period of 90 days had to be excluded from analysis. Specimens for monitoring CMV were obtained at weekly intervals.

Standard immunosuppression consisted of cyclosporin A and methylprednisolone. Anti-T-cell globulin (ATG, 3 mg/kg body weight, Fresenius, Bad Homburg, Germany) or anti-CD3 monoclonal antibody (Muromonab OKT3, 5 mg/day, Cilag, Alsbach-Hähnlein, Germany) was given when the total urine output within the first 12 h after transplantation was less than 500 ml or when rejection episodes did not respond to initial treatment with pulse methylprednisolone. Intravenous CMV immune globulin (1.5 ml/kg body weight; Cytotect, Biotest) was administered prophylactically to CMV-negative recipients of CMV-positive organs during weeks 0, 1, 2, 5, 8, and 12 after transplantation and to all patients treated with ATG or OKT3 during the period of medication. The lots of CMV immune globulin used in our study had no detectable IgM antibodies according to CMV immunoblot.

CMV Antigen assay

Six milliliters of heparinized blood was mixed with 2 ml of a 6% solution of Dextran T 500 (Pharmacia, Freiburg, Germany) in 0.9% NaCl and was allowed to settle for 20 min at 37 °C. For enrichment of polymorphonuclear leukocytes, the buffy coat was centrifuged at 200 *g* for 8 min, harvested, and centrifuged twice at 500 *g* for 8 min in phosphate buffered saline (PBS, 145 mM NaCl, 10 mM Na_2HPO_4/NaH_2PO_4, pH 7.2). Cells were resuspended in PBS and centrifuged on glass slides by means of a cytocentrifuge (450 rpm for 4 min; Cytospin-2, Shandon-Elliot, UK), air-dried at room temperature, and fixed with acetone. To detect CMV antigens, each slide was incubated with a mixture of two monoclonal antibodies, C-10 and C-11 (Clonab CMV, Biotest, Frankfurt, Germany), dissolved in 1% human albumin (w/v) in PBS. These monoclonal antibodies have recently been shown to recognize a lower matrix phosphoprotein (pp65) of human CMV [8]. The slides were then incubated with peroxidase-labelled rabbit antimouse and goat antirabbit immunoglobulins dissolved in the aforementioned buffer (Dianova, Hamburg, Germany) as second and third antibodies. Incubation periods were 30 min each. The enzyme reaction was carried out with H_2O_2 (30%), diluted 1:2500 in diaminobenzidine (0.5 mg/ml; pH 5.3), followed by counterstaining with hematoxylin and mounting in glycerol gelatin. After each step of the staining procedure, the slides were washed thoroughly with PBS.

CMV IgM Immunoblot

Antigen preparation. Human diploid fibroblast cells (MRC-5) were infected with CMV strain AD169; 5–7 days later the cells were harvested, solubilized in 4 ml Laemmli sample buffer [12], sonicated with three 15-s cycles at maximum output (Branson Sonifier), and boiled for 3 min. Antigens from mock-infected MRC-5 cells served as a control.

Electrophoresis. SDS-PAGE was performed in the Laemmli buffer system at 40 volts for 15 h by using 3% stacking and 5%–17% linear acrylamide gradient separation gel. Sixty micrograms protein of the antigen preparation per 1 cm gel were loaded; prestained molecular mass markers ranging from 26 kDa to 180 kDa (Sigma, München, Germany) were included in each gel. Proteins were transferred to nitrocellulose sheets (BA 85, 0.45 µm; Schleicher & Schüll, Dassel, Germany) at 250 Volt hours in a transblot apparatus (Hoefer Scientific Instruments, Heidelberg, Germany) and stored at −30 °C.

Immunoblotting. Before use, the immunoblot strips had been incubated in blocking buffer (50 mM TRIS-HCl, 150 mM NaCl, 5% nonfat dry milk, pH 7.4). Ten microliters of each serum was tested overnight at a 1:250 dilution in blocking buffer at room temperature. To avoid interference with rheumatoid factors, the samples had been pretreated with a sheep antihuman IgG antibody preparation (RF Absorbent, Behring Werke, Marburg, Germany) prior to incubation. After three washings (50 mM TRIS-HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.4), the strips were incubated for 1 h with 2 ml of goat antihuman IgM Fc5µ fragment (Dianova, Hamburg, Germany) at a dilution of 1:2500, followed by incubation for 1 h with avidin peroxidase conjugate (Dianova, Hamburg, Germany). Incubation with 4-chloro-1-naphtole (0.3 mg/ml)/H_2O_2 (0.03% v/v in PBS) until sufficient color developed was used to detect bound enzyme.

CMV EIA

For the detection of CMV antibodies by EIA, a commercially available test kit, designed specifically for IgG and IgM, was used and performed according to the manufacturer's directions (Enzygnost CMV, Behring Werke, Marburg, Germany). For IgM determinations, all sera were pretreated with RF Absorbent (Behring Werke, Marburg, Germany) to remove IgG antibodies.

Definitions

A patient or donor was considered CMV-positive when the CMV IgG EIA titer was at least 1:200, as determined immediately before transplantation. A recipient's negative or positive CMV serostatus established CMV infections as primary or recurrent. A laboratory documented diagnosis of CMV infection was made when at least two of the following assays gave positive results: (1) the presence of CMV antigen-positive leukocytes, (2) a positive IgM titer ($\geq$ 1:50) by EIA, (3) a fourfold IgG titer rise in paired sera by EIA or (4) the appearance of CMV-specific bands in the IgM immunoblot. The presence of febrile illness, with or without arthralgia, leukocytopenia, thrombocytopenia, hepatitis, or pneumonitis was the criterion for distinguishing between laboratory documented CMV infection and symptomatic clinical infection. The time of onset of symptomatic infection was defined as the day of onset of fever.

Statistical analysis

Time differences between the CMV diagnostic tests for the detection of CMV infection were analyzed using the Wilcoxon matched-pair, signed rank test.

Results

In our series of 31 transplant recipients, laboratory documented CMV infection was detected in 20 patients, with 10 patients exhibiting symptoms of CMV disease. No infection occurred in the group of CMV-negative recipients when the transplant was also CMV-negative. Sixty percent (3/5) of all CMV-negative recipients with CMV-positive transplants developed primary infection, which was symptomatic in all cases. Seropositive recipients of CMV-positive organs had an infection rate of 94% (15/16), and 25% (4/16) of those recipients showed symptoms of CMV infection. In 80% (4/5) of the seropositive patients receiving CMV-negative organs, CMV infection recurred, and symptoms were seen in 60% (3/5; Table 1).

All patients with laboratory documented CMV infection had by a positive IgM immunoblot (20/20), in combination with at least one other test assay. In addition, two seropositive recipients of CMV-positive organs exhibited a positive IgM immunoblot, while all other parameters of CMV infection remained negative. The mean period between transplantation and the first positive test was 38 ± 12 days and 40 ± 14 days for antigenemia assay and IgM immunoblot, respectively, and this difference is statistically significant ($P = 0.05$). Detection of CMV antibodies by EIA followed about 12 days later (IgG EIA 52 ± 13 days; IgM EIA 52 ± 15 days), which is also statisti-

Table 1. CMV infection parameters in renal transplant patients. + Seropositive (CMV IgG EIA titer ≥ 1:200); − seronegative (CMV IgG EIA titer < 1:200)

Pretransplant CMV serostatus (donor/recipient)	No. of patients	No. of positive results				No. of patients with symptomatic CMV infection
		CMV Antigen	IgM-Blot	IgM-EIA	IgG-EIA	
d+/r−	5	3	3	3	3	3
d+/r+	16	8	15	6	12	4
d−/r+	5	2	4	3	4	3
d−/d−	5	0	0	0	0	0
Total	31	13	22	12	19	10

Table 2. Timing of laboratory diagnosis in patients with symptomatic CMV infection. neg, Negative test results throughout observation period (90 days)

Patient	Pretransplant CMV serostatus[c] (donor/recipient)	Time between onset of symptoms (t_0) and laboratory diagnosis[a] of CMV infection in days[b]			
		CMV Antigen	IgM-Blot	IgM-EIA	IgG-EIA
		Day (n)[d]	Day	Day	Day
A	d+/r−	+ 6 (10)	+14	+14	+14
B	d+/r−	− 4 (38)	+ 9	+15	+15
C	d+/r−	− 7 (135)	− 4	+ 1	+10
D	d+/r+	− 3 (1)	− 3	neg	− 3
E	d+/r+	+ 7 (2)	+11	+11	+11
F	d+/r+	0 (16)	0	+33	0
G	d+/r+	+14 (122)	+30	neg	+34
H	d−/r+	neg (0)	− 4	neg	+18
I	d−/r+	+ 4 (3)	+26	+26	+26
J	d−/r+	−20 (18)	−12	− 4	+ 3
Total[e]		0 ± 10	8 ± 13	14 ± 13	13 ± 11

[a] Laboratory diagnosis of CMV infection: (1) presence of CMV antigen-positive leukocytes, (2) appearance of CMV-specific IgM bands, (3) positive IgM titer (≥ 1:50), (4) fourfold IgG titer rise in paired sera

[b] Number of days a laboratory diagnosis of CMV infection was made after (+) or before (−) the occurrence of symptoms

[c] + Seropositive (CMV IgG EIA titer ≥ 1:200), − seronegative (CMV IgG EIA titer < 1:200)

[d] Number of CMV Ag$^+$ polymorphonuclear leukocytes per cytospin preparation (approximately 20,000 cells evaluated)

[e] Mean ± SD

cally significant ($P < 0.05$) when compared to antigenemia assay and IgM immunoblot.

In ten patients IgM immunoblot and IgG EIA identified symptomatic CMV infection that occurred at a mean period of 39 ± 15 days after transplantation. The antigenemia assay identified CMV infection in nine of these patients and IgM EIA in seven. The discrepancy in the positive results seen for the antigenemia assay and the IgM EIA occurred in the group of seropositive recipients with recurrent infection. The antigenemia assay detected CMV infection in symptomatic patients earlier than the other tests in seven cases ($P < 0.05$) and prior to the appearance of symptoms in four cases (Table 2). CMV identification by IgM immunoblot preceded the development of symptoms in four cases but became positive in three patients 14, 26, and 30 days after the first clinical signs, with corresponding time periods of 6, 4, and 14 days, for the antigenemia assay. A comparison among the serological tests in symptomatic patients showed no statistical difference; however, the IgM immunoblot preceded IgM and IgG EIA in five cases. IgM and IgG EIA were never the first serological assays to indicate CMV infection.

Sera exhibiting positive results with the IgM immunoblot reacted with a total of 12 antigens with molecular weights ranging from 24 kDa to 160 kDa. Prior to transplantation the IgM immunoblot was negative in all patients. Individual sera showed reactions with a minimum of one and a maximum of nine polypeptides (median six). The 150 kDa polypeptide was detected in all cases (22/22; 100%); other polypeptides with a high incidence of positive reactions had molecular weights of 38 kDa (81%), 53 kDa (76%), 96–100 kDa (62%), and 80–85 kDa (57%). No characteristic pattern could be observed in the immunoblot reactions either in relation to the results of the other tests or in relation to primary, recurrent, asymptomatic, or symptomatic CMV infection. Figure 1 shows an IgM immunoblot reaction pattern, together with the longitudinal course of the CMV diagnostic results, in a patient with a recurrent, asymptomatic CMV infection. A transient antigenemia occurred on day 27 after transplantation, together with CMV-specific bands in the IgM immunoblot, as first indicators of CMV infection. A fourfold IgG titer rise followed 11 days later and the IgM EIA remained negative throughout.

Discussion

This study compared the detection of CMV antigens in blood leukocytes with three serological assays (CMV IgG EIA, CMV IgM EIA, and CMV IgM immunoblot) for the early diagnosis of CMV infection in renal transplant pa-

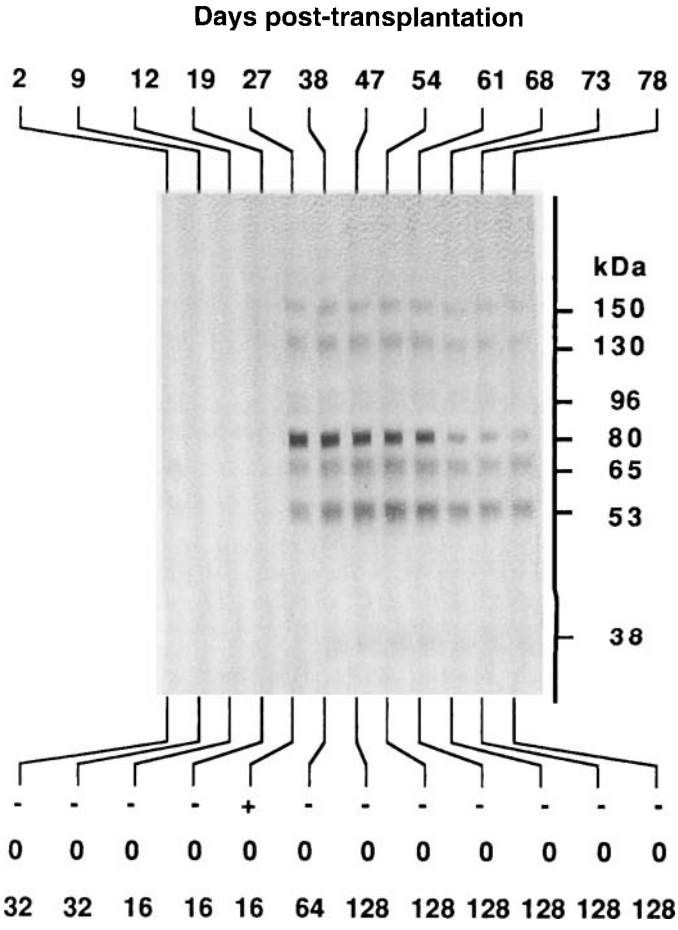

Fig. 1. Time course of CMV IgM immunoblot, CMV IgM/IgG EIA, and CMV antigenemia assay in an asymptomatic transplant recipient (CMV serostatus: recipient-positive; donor-positive). CMV infection is documented by CMV antigenemia and appearance of IgM bands in the CMV immunoblot on day 27, and a fourfold CMV IgG titer rise in the EIA on day 38. – Absence of CMV antigenemia; + presence of CMV antigenemia

tients. The incidence and time course of CMV infections in our study group, as well as the distribution of asymptomatic and symptomatic infection according to the CMV serostatus of donor and recipient, were similar to those already reported in the literature [10, 18, 23, 25].

The CMV IgM immunoblot proved to be very sensitive and showed the highest number of positive results. However, two samples that were IgM immunoblot-positive were negative according to both of the other laboratory methods and might be considered false-positive. Detection of CMV IgM antibodies by immunoblot was particularly frequent in seropositive transplant recipients (19/21). As reported by others, a positive IgM in this patient group was not predictive of a symptomatic infection [13, 17]. Compared with immunoblot, the EIA used in our study showed a much lower overall detection rate for CMV IgM antibodies (22/31 versus 12/31, respectively). Among the ten patients with discordant results who remained negative according to CMV IgM EIA, there were three who developed symptomatic CMV infection as confirmed by positive results for IgM immunoblot, IgG EIA, and antigenemia. In addition to a higher frequency of detection, the immunoblot identified CMV IgM antibodies significantly earlier than the EIA. This striking difference in the detection of CMV IgM antibodies between EIA and immunoblot has also been observed by Basson et al. [1]. In their study of renal transplant patients, CMV IgM antibodies in primary and recurrent CMV infection were detected by EIA in only 71 % and 54 % of the cases, respectively, as compared with immunoblot. This phenomenon could be explained by differences in antigen preparation, by the separation of viral proteins, which might allow a lower quantity of antibodies to be visualized on the immunoblot, or by differences in the antibody-detecting system. Crossreactions of the immunoblot with antibodies against other herpes viruses were excluded in preliminary experiments of our study. Furthermore, the commercial EIA used in our study has been reported to detect CMV IgM antibodies in recurrent infections of renal transplant recipients less frequently than one other commercial EIA and than an in-house EIA [6]. This might also contribute to the different performances of IgM EIA and IgM immunoblot.

The earliest indicator of CMV infection in our study was the CMV antigenemia assay. In symptomatic patients, CMV antigen-positive cells could normally be detected on the day of onset of symptoms, while the serological tests did not show evidence of CMV infection for at least another week. These observations are in keeping with the results reported by others, and the CMV antigenemia assay is generally regarded as a major improvement in the clinical management of CMV infections [2, 4, 5]. However, no CMV antigenemia could be detected in one patient who developed a symptomatic CMV infection as determined by positive serological results and clinical presentation. Despite a number of studies in which the CMV antigenemia assay has been shown to be a highly sensitive method for diagnosing CMV infection, there are occasional reports of failures. In a prospective study, Miller et al. [16]

could not demonstrate CMV antigenemia in ten transplant patients with symptomatic CMV infection. Their presumption that processing delays might have been responsible for these negative results is supported by a recent report that a delay of 6 h in the transport of blood samples to the laboratory decreased the viral antigen detection rate by at least 80% [8]. In another study, 1 out of 17 transplant patients with symptomatic CMV infection remained CMV antigen-negative, despite positive results for CMV shell-vial assay and CMV IgM antibodies [26]. We believe, therefore, that the CMV IgM immunoblot provides additional information that can be helpful in the management of the individual transplant patient. Given the high sensitivity of the CMV IgM immunoblot, a positive result might be the only early indicator for CMV as a cause of an unspecific illness in a transplant patient, whereas a negative IgM immunoblot would argue strongly against CMV infection.

In conclusion, the CMV antigenemia assay proved to be very useful in providing an early diagnosis of CMV infection in symptomatic patients. Of the various serological tests for CMV infection, the IgM immunoblot appeared to be superior to both IgG and IgM EIA in terms of early diagnosis. However, no single diagnostic test proved to be a perfect diagnostic tool. Thus, it appears that only a combination of tests (e.g., CMV antigenemia assay and CMV IgM immunoblot) allows for an optimal detection of CMV infection in transplant patients.

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