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Detection of cytomegalovirus infections by PCR in renal transplant patients

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Abstract

Cytomegalovirus (CMV) is the single most important infectious agent affecting recipients of organ transplants. To evaluate the incidence and the clinical importance of CMV infection in renal transplants in Brazil, 37 patients submitted to renal allograft transplants were tested periodically for the presence of cytomegalovirus DNA in urine using the polymerase chain reaction (PCR), and for the presence of IgM and IgG antibodies against CMV by enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence (IFI). The PCR-amplified products were detected by gel electrophoresis and confirmed by dot-blot hybridization with oligonucleotide probes. Thirty-two of the 37 patients (86.4%) were positive by at least one of the three methods. In six patients, PCR was the only test which detected the probable CMV infection. Ten patients had a positive result by PCR before transplantation. In general, the diagnosis was achieved earlier by PCR than by serologic tests. Active infection occurred more frequently during the first four months after transplantation. Sixteen of the 32 patients (50%) with active CMV infection presented clinical symptoms consistent with CMV infection. Five patients without evidence of active CMV infection by the three tests had only minor clinical manifestations during follow-up. Our results indicate that PCR is a highly sensitive procedure for the early detection of CMV infection and that CMV infection in renal transplant patients is a frequent problem in Brazil.

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Introduction

Cytomegalovirus (CMV) infection is an important cause of morbidity and mortality in immunosuppressed patients, particularly among allograft recipients, whose number has increased markedly in recent years. The ubiquity of this virus, its propensity to be reactivated when host defenses are compromised and its ability to disseminate to several organs are characteristics which help explain its frequent occurrence in the transplanted population. Numerous studies have shown that the majority of patients develop evidence of active CMV infection following kidney transplantation. Although most of these individuals have asymptomatic infections, others have clinical manifestations of

illness (overt CMV disease) ranging from high fever to death. The rapid and specific diagnosis of active CMV infection in these patients is important since its manifestations may resemble those of transplant rejection but require distinct management (1-6). Several procedures for the detection of CMV are available, including conventional virus culture, shell-vial, serology tests, antigenemia and PCR (7-12). In this paper we report the prevalence and clinical impact of CMV infection identified by serological tests and by PCR in a Brazilian population of kidney transplant patients.

Material and Methods

Patients

Thirty-seven patients submitted to kidney transplantation at the Kidney Transplant Unit of the Department of Internal Medicine, University Hospital, State University of Campinas, SP, Brazil, were studied prospectively over a two-year period. Informed consent was obtained from all patients and the protocol was approved by the Hospital's Ethics Committee.

The immunosuppressive regimen used during the study included corticosteroids, azathioprine, and cyclosporine. Episodes of rejection were documented by renal biopsy and treated with increased doses of oral corticosteroids for mild episodes and 500 mg intravenous pulses of methylprednisolone, or the monoclonal antibody OKT_3, or both, for moderate or severe episodes.

Clinical and laboratory records were reviewed for evidence of symptoms attributed to CMV disease.

Clinical specimens

Urine. Urine samples were obtained from renal allograft donors and recipients immediately prior to transplantation and from recipients once a month for approximately one year post-transplant.

Serum. Serum samples were collected at the same time as urine specimens and were tested by serological methods for CMV (enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence (IFI)). All specimens for a given patient were analyzed in the same batch.

Serology

Serum CMV IgG and IgM antibodies were determined by ELISA (13) and IFI (14).

PCR

CMV DNA determination in urine specimens was carried out by PCR according to the procedure of Demmler et al. (15) using three pairs of primers for amplification. Each pair was designed for the amplification of different regions of the viral genome (16). The reaction buffer contained 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 µM of each of the four deoxynucleotide triphosphates (dATP, dCTP, dTTP and dGTP), 2.4 µM of each primer, water and sample (1.5 µl), for a total volume of 100 µl. The reaction mixture was overlaid with 100 µl of mineral oil and the tubes were placed in a boiling water bath for 7 min. After boiling, 0.5 µl (2.5 U) of *Thermus aquaticus* (Taq) polymerase was added. The amplification reaction was performed in a DNA Thermal Cycler (Perkin-Elmer/Cetus, Norwalk, CT, USA). The samples were heated to 94°C for 90 s to denature DNA, cooled to 55°C for 90 s for annealing and then heated to 72°C for 120 s for extension. In the final cycle, an extension period of 7 min was done. A total of 40 cycles were performed and 5 µl of the amplified product was detected by direct analysis on 2% agarose minigels and by a dot-blot hybridization assay using oligonucleotide probes. A 159-, 400- and 435-base pair band was seen when

samples were amplified using IE, LA and MIE primers, respectively.

After electrophoresis, the remaining PCR product was tested by dot-blot. For dot-blot analysis (15), 10 µl of the reaction mixture was mixed with 190 µl of 2 M sodium chloride and then denatured by adding 10 µl of 10 M sodium hydroxide for 15 min. The sample was then neutralized by adding 100 µl of 3 M sodium acetate, pH 5.5. The DNA was immediately applied to a nylon membrane by vacuum filtration, and the membranes were dried by heating in a vacuum oven at 80°C for 1 h and hybridized with a homologous ^{32}P-labeled synthetic oligonucleotide probe in a hybridization solution containing 1% SDS and 6x SSPE buffer (0.9 M NaCl, 60 mM NaH_2PO_4, pH 7.4, and 6 mM Na_2EDTA) for 18 h. Three 10-min washes were subsequently performed in 6x SSPE and 1% SDS at room temperature. A final wash was performed in 1x SSPE and 1% SDS for 3 min at 65°C. The bound probe was detected by autoradiography at -70°C for 18 h.

Occurrence of CMV infection

Active CMV infection was defined if one or more of the following four conditions were present: 1) two or more positive results for human CMV DNA by PCR in urine, 2) CMV antibody seroconversion in a subject who was seronegative before the transplant, 3) a four-fold or greater increase in IgG CMV antibodies, and 4) a positive test for IgM CMV antibodies.

Symptoms consistent with CMV disease have been previously described (17-21), and the diagnostic criteria for symptomatic CMV disease used in our study essentially followed the recommendations made by the Workshop on human CMV disease (19). Work-up for microorganisms other than CMV included multiple bacteriological and fungal cultures of blood, urine and sputum, as well as serologic tests and multiple chest X-rays. CMV isolation was not attempted.

Results

Based on the criteria used, 32 (86.4%) of the 37 subjects had laboratory evidence of active CMV infection. In six patients, PCR was the only test, which detected a probable CMV infection. Using this technique, we identified viral DNA in 64.9% of the transplanted patients. The diagnosis was achieved earlier by PCR than by serologic tests in general, and active CMV infection occurred more frequently in the first four months after transplantation. A diagnosis of active CMV infection was obtained for 51.4% of the patients by ELISA and for 54.1% by IFI (Table 1).

Thirty-four of the 37 (91.8%) renal recipients were seropositive by ELISA before transplantation as were 32 of the 35 donors (91.4%). One hundred percent of the recipients and their respective donors were found to be CMV seropositive by IFI before the transplant.

Only one of 37 patients was seronegative for CMV (ELISA) and he received a kidney from a seropositive donor. As a result, he developed primary CMV infection after the transplant. Sixteen of the 32 patients (50%) with an active CMV infection showed clinical manifestations attributable to CMV disease (Table 2). However, CMV etiology could only be confirmed in three of these patients. All of them had pneumonia, with CMV detected by PCR in bronchoalveolar lavage in two and by lung histopathology in one fatal case. Five previously seropositive patients who did not have an active CMV infection by the adopted criteria had only minor clinical complications during follow-up.

Prolonged unexplained fever occurring without the discernible involvement of other organ systems, but usually associated with malaise, anorexia, fatigue, night sweats, myalgias and arthralgias, was the most

Table 1 - Results of serology (ELISA and IFI) and PCR on urine during follow-up of 37 kidney transplant patients who presented active CMV infection.

D/R = Donor/receptor; + = positive reaction; - = negative reaction; *the numbers after transplantation indicate the months in which PCR and/or serology were indicative of active CMV infection; SC = seroconversion; Not detected = no seroconversion and/or increase in IgG antibody levels during follow-up.

Patients	Before transplantation		After transplantation*				
						Seroconversion and/or increase in IgG antibody levels detected by	
	IgG ELISA D/R	PCR D/R	PCR	IgM ELISA	IgM IFI	ELISA	IFI
1	+/+	-/-	7,8	-	-	Not detected	8
2	+/+	-/-	-	-	-	4	9
3	+/+	-/-	1,2,3,9	-	-	Not detected	Not detected
4	-/-	-/+	1	1	-	Not detected	Not detected
5	+/+	-/-	2,4,6	-	-	2,3,4,6	2,4,6
6	+/+	-/-	1,3,5,6	-	-	Not detected	Not detected
7	+/+	-/-	-	-	-	Not detected	Not detected
8	+/+	-/-	3,6	1,2,3,4	-	1,2,3,4,6	Not detected
9	+/+	-/-	5,7	-	-	Not detected	7
10	+/+	-/+	2	-	-	Not detected	Not detected
11	+/+	-/-	2,3	-	-	Not detected	Not detected
12	+/+	-/+	3,4,5,7	2,3,4,5,7	-	Not detected	Not detected
13	+/-	-/-	2,3,4,5,6	1,2,3,4,5,6	2,3,4	SC(2)	SC(2)
14	+/+	-/-	-	-	2,4	2,4,10	Not detected
15	+/+	-/-	1,2,3,4	-	-	Not detected	Not detected
16	+/+	-/-	4,6,10	-	-	2,4,6,10	2,4,6,10
17	+/+	-/+	5	-	-	Not detected	5,9,10,11
18	-/+	-/-	-	-	-	Not detected	Not detected
19	+/+	-/-	-	-	3,4	3,4	Not detected
20	+/+	-/-	-	-	-	Not detected	Not detected
21	+/+	-/-	2,3,6,8	-	8	Not detected	Not detected
22	+/+	-/+	3,4,8	-	-	3,4	Not detected
23	+/+	-/+	1	-	1	3,6,7	Not detected
24	+/+	-/-	3,4	-	-	Not detected	Not detected
25	-/-	-/-	-	-	-	Not detected	Not detected
26	+/+	-/-	-	-	2,7	Not detected	Not detected
27	+/+	-/+	3	-	7	Not detected	Not detected
28	+/+	-/-	-	5,9,11	5,9,11	5,9,11	Not detected
29	+/+	-/-	3,5	3,4	-	Not detected	Not detected
30	+/+	-/-	-	-	-	4	Not detected
31	+/+	-/+	3,4	4,5	5	5	4,5
32	+/+	-/-	-	-	-	3,5,6	1,2,3
33	+/+	-/-	-	-	6	Not detected	5,6
34	+/+	-/+	3,5	-	5	5	Not detected
35	+/+	-/-	-	-	-	Not detected	Not detected
36	+/+	-/+	1,6	-	-	6	6
37	+/+	-/-	3	-	-	3	3

common symptom observed in patients who developed CMV infection (11 patients) (Table 2). All of these patients had fever ≥38°C for at least one week without other detectable infectious causes. All patients with active CMV infection had at least one of the following manifestations in addition to fever: 1) leukopenia (white blood cells <4,000/mm^3); 2) thrombocytopenia (platelets <100,000/mm^3); 3) hepatitis (serum alanine aminotransferase >40 IU) and 4) atypical lymphocytosis ≥3%).

Discussion

Cytomegalovirus infection remains a major cause of morbidity and mortality in the immunocompromised patient, and is the most serious problem in organ allograft recipients. The possibility of using specific antiviral therapy to treat CMV infections makes a timely diagnosis imperative (21-34). To overcome the time disadvantage of a cell culture assay, investigators have used PCR to amplify CMV specific sequences and have demonstrated its usefulness for monitoring CMV infection in renal transplant patients (25,26).

To our knowledge, this is the first study in Brazil to use PCR to detect CMV infection in renal transplant patients. With this technique, we identified viral DNA in 64.9% of the transplanted patients. The findings of Rowley et al. (25) and Chen et al. (22) suggest that the PCR assay is only capable of detecting CMV viral DNA in patients with an active infection and that it is not positive in seropositive healthy individuals or in seropositive transplant patients without other evidence of active CMV infection. Remarkably, we did not find positive CMV PCR in over 200 urine samples from seropositive healthy adults used as controls (data not shown). The results of our study are comparable to those of Olive et al. (26) who concluded that PCR was more sensitive than ELISA and cell culture in detecting CMV in renal transplant patients.

The predominant clinical feature detected in patients with evidence of active CMV infection was fever (70%). Other relevant clinical manifestations included hepatitis with negative tests for hepatitis viruses A, B and C, leukopenia, and interstitial pulmonary in-

Table 2 - Clinical features of patients who developed CMV infection.

(+) Fatal illness.

Patients	Clinical findings in probable and confirmed* CMV infection	Days after transplantation
6	Fever, myalgias and cough	150
10	Compromised renal function, without cellular rejection	60
12	Unexplained fever	210
13	Fever, leukopenia, splenomegaly	30
14	Generalized lymphadenopathy	13
16	Fever, myalgias and cough	300
17	Compromised renal function, without cellular rejection	150
22	Hepatitis with leukopenia	90
23(+)*	Fever, dyspnea and interstitial pulmonary infiltration	30
27	Leukopenia	90
28	Unexplained fever	330
29*	Fever and cough	45
31	Fever, myalgia and fatigue	150
32*	Fever and interstitial pulmonary infiltration	30
34	Fever, myalgia and fatigue	45
36	Prolonged unexplained fever	30

filtrates associated with detection of CMV in bronchoalveolar lavage and in a lung biopsy by PCR. We also observed compromised renal function in two cases for which a graft biopsy did not demonstrate a well-defined cellular rejection.

In agreement with data in the literature, the clinical manifestations observed in patients who were CMV seropositive before transplantation were not severe and resolved spontaneously, except for one patient (No. 23) who died.

In a previous study conducted in Brazil (35), a CMV infection was diagnosed in 5.3% of the patients based exclusively on serological methods (complement fixation and IFI). Another study using viral cultures and serological methods (complement fixation and IFI) (36) detected CMV infection in 26% of the patients. In these two studies, as well as in the present one, there was an absolute predominance of live over dead donors. The different frequencies of CMV infection obtained in these studies probably reflect methodological differences. The first of them (35) was retrospective and based the diagnosis of CMV infection on serological methods that are regarded as somewhat unreliable. The frequency of CMV infection in the present study was greater than in previous studies (35,36), probably because of the high sensitivity of PCR in detecting CMV together with a longer follow-up period.

In the present study, 32 patients showed evidence of active CMV infection. In 13 (40.6%) of them, renal function was compromised one year after the transplant. In five, the graft was unsuccessful, two died and three underwent hemodialysis or peritoneal dialysis. In five of the 37 patients who showed no evidence of active CMV infection or any relevant clinical problems, renal function was maintained one year after the transplant. In view of the small number of patients studied, these results do not permit statistical analysis but show a tendency towards more frequent loss of renal function in patients with active CMV infection.

The utilization of techniques such as PCR that permit the rapid diagnosis of CMV infection improves the chances for implementation of specific antiviral treatment. The finding that 86.4% of the patients developed CMV infection underscores the frequency of this problem in renal transplant patients in Brazil.

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