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Clinical evaluation in organ transplant patients of a polymerase chain reaction test for CMV DNA applied on white blood cells and serum

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Abstract A polymerase chain reaction (PCR) test for CMV DNA was evaluated for clinical usefulness. Leukocytes and serum were sampled from 36 patients who had recently undergone organ transplantation. Clinical symptoms, virus culture, and IgG and IgM antibodies were used to identify, in retrospect, patients with CMV disease certified beyond all doubt, with probable disease, with asymptomatic infection, or without infection. PCR tests for CMV DNA in leukocytes (BC-PCR) and serum (SE-PCR) were then evaluated. BC-PCR was positive in all patients with certified CMV disease but also in 31 % of the samples from patients without infection. SE-PCR was positive in 11/13 patients with certified disease and was concordant with CMV culture in 192/231 tests. Of the 39 discordant cases, 27 had a positive SE-PCR with a negative culture. The effect of ganciclovir treatment could not be predicted by any test. In conclusion, a negative BC-PCR is strong evidence against CMV disease and a positive SE-PCR strongly suggests CMV disease, but the opposite results are of little clinical help.

Key words CMV, PCR, organ transplantation · Organ transplantation, CMV, PCR · PCR, CMV, organ transplantation

Introduction

Cytomegaloviral (CMV) disease is the most frequent infection occurring in transplant recipients and may cause life-threatening symptoms. The lack of an ideal method to establish a clinically relevant diagnosis is, therefore, a problem [5, 18]. Virus culture is too time-consuming and IgG antibody titers rise too late for these tests to be useful in the clinical situation. CMV IgM antibody titers are specific but not sensitive enough. Tests of early antigens by immunofluorescence or immunoperoxidase are both rapid and sensitive [1, 9]. When quantified, high levels correlate with significant disease [1, 9]. However, in the lower range, the tests do not discriminate between subclinical viral reactivation and CMV disease. IgM immunoblot is also very sensitive, turning positive in a high proportion of asymptomatic renal transplant patients [6]. We have investigated whether the results of a recently developed polymerase chain reaction (PCR) test for CMV DNA [15], applied on blood buffy coat leukocyte preparations (BC-PCR) as well as on serum samples (SE-PCR) [16], could be used as guidelines for clinical decisions in organ transplant patients.

Patients and methods

Study design

Patients undergoing kidney or liver transplantation were followed prospectively from the day of transplantation with sampling of serum, leukocytes, and urine twice weekly for CMV serology (IgM and IgG antibodies), CMV culture on blood and urine, and PCR tests on buffy coats (BC) and serum. In the same period, such sampling was also performed on patients admitted with symptoms indicating CMV disease.

The diagnosis was re-evaluated in retrospect, based on clinical signs and symptoms, the results of CMV culture from BC, and antibody titers. This evidence was used to evaluate the results of the PCR tests.

Table 1 PCR primer sequences

Primer	Sequence (5′ → 3′)	CMV IEA1 gene coordinates (bp)
CMV1	CAAGCGGCCTCTGATAACCAAGC	732–754
CMV2	CTCTTCCTCTGGGGCAACTTCCTC	1167–1142
CMV3 (nested)	CCGATCCTCTGAGAGTCTGCTCTC	830–853
CMV4 (nested)	CAGCCACAATTACTGAGGACAGA	1018–996
CMV probe	GAGGCTATTGTAGCCTACACTTTG	901–924

Table 2 Patients grouped according to clinical, serological, and virus culture findings

Group	Number of patients	Number of sample sets	Febrile disease	CMV IgG titer rise	CMV IgM positive	CMV culture BC positive
1	7	75	None	None	None	None
2	5	57	None	3/5	1/5	3/5
3	13	73	All	All	7/13	All
4	11	39	All	5/11	4/11	1/11

Patients

Thirty-six patients were studied following transplantations performed between 1990 and 1992. Their median age was 42 years (range 19–66 years). Eleven of these patients were females. Thirty-one received kidney transplants, in eight cases combined with a pancreas graft. Five patients received liver transplants. Maintenance immunosuppression was based on cyclosporin in combination with steroids and azathioprine, but antihymocyte globulin (ATG) was used initially as induction therapy in patients receiving liver transplants, combined kidney and pancreas grafts, and in kidney recipients with HLA antibodies. Solumedrone pulses, ATG, and OKT3 were used as antirejection therapy.

CMV serology of recipients and donors

Twenty-eight recipients were CMV IgG-seropositive at the time of transplant and eight were negative. Five of the latter received grafts from seropositive donors (D+ R–) and three from seronegative donors (D– R–). Except for one seropositive liver recipient, all blood transfusions were from CMV-seronegative donors and were done using leukocyte filters.

Collection of blood and serum samples

EDTA blood, serum, and urine samples were collected prospectively, scheduled twice weekly, from 19 patients, starting on the day of transplantation in all but 3. The median number of samples obtained per patient was 9.4 (range 5–16). In the remaining 17 patients, testing was initiated several weeks post-transplantation when CMV disease was suspected and then continued with a median of 4.0 tests per patient (range 2–8). After 1 year CMV IgG antibody titers were determined in all initially seronegative patients.

Serology and virus isolation

The antigens for assay of antibodies against CMV were prepared as previously described [10]. Determinations of IgG were performed by ELISA and determinations of IgM by immunofluorescence.

For CMV culture, BC and urine samples were inoculated on monolayers of human fibroblasts. After fixation of fibroblasts with glutaraldehyde, suspected cytopathic plaques were confirmed by ELISA using monoclonal IgG antibodies DDG9 and CCH2, reactive with CMV IE and E antigens (Dakopatts, Glostrup, Denmark).

PCR

Serum samples and BC white cell blood preparations were analyzed by PCR. The PCR was performed in a two-step reaction, first with a pair of outer primers, and then with a pair of inner (i.e., nested) primers. The primers used in the nested PCR assay were selected from the fourth exon of the CMV IEA 1 gene (Table 1). The primers were synthesized and purified as described by the manufacturer (Scandinavian Gene Synthesis, Köping, Sweden). The amplification was performed in the Gene Amp PCR system 9600 (Perkin-Elmer Cetus, Göteborg, Sweden). With the first primer set, amplification was performed by 30 incubation cycles of thermal denaturation at 92 °C for 15 s, primer annealing at 66 °C for 10 s, and primer extension at 72 °C for 15 s. Each extension time was increased by 1 s on each subsequent cycle. With the second primer set, DNA amplification was performed by 40 cycles, as described above. After the second amplification, the reaction mixture was analyzed by electrophoresis and the results photographed under ultraviolet illumination. Strict precautions were taken to avoid contamination [11, 12].

The sensitivity of the PCR analysis was 5 fg of CMV DNA, corresponding to approximately 20 viral genomes.

The total time required from sampling to result was 8 h for the SE-PCR and 30 h for the BC-PCR, the difference depending on separate methods for DNA extraction.

Definitions of CMV infection and disease

Based on clinical symptoms and microbiologic tests other than BC-PCR and SE-PCR, patients were allocated to one of the four following groups:

No CMV infection (group 1) – asymptomatic patients lacking microbiologic evidence of CMV activity, i.e., with negative BC cultures, absence of IgM antibodies, and no rise in IgG antibody titres.

Asymptomatic CMV infection (group 2) – patients with microbiologic signs of CMV infection in the absence of clinical signs or symptoms.

CMV disease (groups 3 and 4) – a clinical syndrome with fever and leukopenia (or intolerance to azathioprine), often with other organ involvement. When both BC culture and CMV IgG serology (seroconversion or fourfold or greater increase in titers) were positive, CMV disease was considered *certified* (group 3). When the laboratory confirmation was incomplete, the patient was assigned to the group *probable CMV disease* (group 4).

Table 2 summarizes the evidence in each group.

Antiviral treatment

The PCR data were not available for clinical decisions. Ganciclovir was used as treatment in all patients judged to have CMV disease, except for one who received foscarnet. The doses of both drugs were adjusted to renal function, most patients receiving 2.5 mg/kg of ganciclovir IV every 12 or 24 h. Treatment was given for a median of 10.7 days (range 8–19 days).

Results

Following the creation of these groups, the tests for BC-PCR and SE-PCR were evaluated.

Patients with no CMV infection ($n = 7$)

These patients, who were followed prospectively, did not develop clinical or laboratory signs of CMV infection. Three D– R– and one D+ R– remained CMV IgG-negative, even after 1 year, and three seropositive patients had constant IgG titers. IgM antibodies were not demonstrated in any of 76 serum samples, and CMV was never cultured from a total of 79 BC preparations.

Viral cultures from 62 urine samples were negative for CMV, but 5 were positive. These were obtained from four different patients: on day 2 post-transplantation in one patient, on days 1 and 3 in another, on day 4 in a third patient and on day 14 in the fourth. Those with early positive urine cultures were the three D– R– patients.

By definition, none of these patients had febrile disease, pulmonary, gastrointestinal or CNS manifestations, but two had temporary leukopenia and one had slightly elevated liver enzymes, considered to be side effects of the medication.

In this group, BC-PCR tests were positive in 23/75 samples (31 %), one obtained on the day of transplantation. Only one of seven patients, the D+ R–, was consistently negative. The three D– R– patients had one, two and three positive tests each. The positive BC-PCR coincided with only one of the five positive urine cultures. SE-PCR was positive in 2 of 75 samples (2.6 %), each being the last sample in the series obtained from one patient and, therefore, not possible to classify as a false-positive.

We conclude from this set of tests that CMV DNA can often be detected by BC-PCR in the absence of clinical disease, serologic evidence of CMV infection, or positive BC cultures. Therefore, a positive BC-PCR does not per se indicate present or threatening CMV disease. In contrast, SE-PCR is very rarely positive in patients without clinical or other laboratory signs of CMV infection.

Patients with asymptomatic CMV infection ($n = 5$)

These patients had laboratory signs of CMV infection but did not develop clinical CMV disease. The indications of CMV infection were IgG seroconversion in one D+ R–, a significant increase in IgG titers in another three seropositive patients, one of whom also had a positive IgM titer, and positive BC virus cultures in three patients, one, four, and seven times, respectively.

In this group, each patient had at least two positive BC-PCR tests. Thirty-four of the 57 tests (60 %) were positive. Two patients had positive tests on the day of transplantation and another on day 3 post-transplantation.

SE-PCR was positive in samples from two of the five patients, both with a series of positive cultures on BC. In both patients, SE-PCR was negative for more than a week after the first positive BC culture but then became positive and remained so until the culture turned negative.

This series of tests reinforces the notion that the BC-PCR test is too sensitive for a positive test to be of value in the clinical situation. Reliance on SE-PCR would have indicated treatment in two patients who did not develop disease.

Patients with certified CMV disease ($n = 13$)

This group included three D+ R– and ten seropositive patients. All patients had fever equal to or higher than 38 °C, ten had leukopenia and/or azathioprine intolerance, seven had aminotransferase enzymes equal to or greater than 1.0 μkat/l, four had gastrointestinal disease, three lower respiratory tract symptoms, and two CNS symptoms. Signs of disease, usually fever, appeared 38.0 ± 15.9 days post-transplantation. BC cultures were always positive at the time of disease. Four patients were IgM-positive at the time of disease, and three more turned positive later. A significant increase in IgG titers was recorded in the follow-up of all patients.

In this group of patients, BC-PCR was positive in 66/73 tests (85 %). Six of seven negative tests were obtained on days 0–22 post-transplantation, and one during ganciclovir treatment. Thus, all patients had positive BC-PCR at the time they developed symptoms. All but one remained positive throughout follow-up (median 67 days post-transplantation).

SE-PCR was positive in 42/71 tests (59 %). All patients had at least one serum test performed in the interval between onset of symptoms and start of treatment. This was positive in 11/13. There was no definite time re-

Table 3 Results of PCR CMV on serum and CMV culture on buffy coats (231 concomitant samples obtained from 36 organ transplanted patients)

	SE-PCR	
	Negative	Positive
CMV culture		
Negative	162	27
Positive	12	30

lationship between BC culture and SE-PCR. Three patients were SE-PCR-negative while the corresponding BC culture was positive, but in another SE-PCR became positive 2 weeks before the BC culture.

These tests show that a negative BC-PCR is rarely, if ever, found in patients with active CMV disease. SE-PCR is a sensitive, but not infallible, marker of CMV disease.

Eleven of the 13 patients with certified CMV disease were treated with ganciclovir for a median of 11.1 days (range 8–19 days). Two were not treated. The interval between first symptoms and start of treatment was 8.4 days (median). There was no mortality. SE-PCR remained positive in all seven patients tested during the 1st week of ganciclovir treatment and turned negative after 2 weeks in all five tested.

Following treatment, five patients had some indication of CMV activity. Three patients had a relapse of CMV disease and received a second course of ganciclovir starting 15–16 days after the first. They had become negative in BC cultures as well as SE-PCR during, or shortly after, the first course, but SE-PCR again turned positive before the second. Two other patients had fever and prolonged fatigue, respectively, both with persistently positive SE-PCR and BC culture.

The other patients treated did not have further clinical symptoms of CMV disease; two with positive SE-PCR tests spontaneously normalized after 2 or more weeks, while two with negative tests stayed negative.

This series of tests shows that SE-PCR is a strong marker of active CMV disease but cannot be used to predict the prolonged effect of therapy.

Patients with probable CMV disease (n = 11)

This group differed from those with certified disease in that the patients had clinical signs but not as convincing microbiologic evidence of CMV disease. All patients had fever. The symptoms included leukopenia in six and pulmonary involvement in three. The general clinical picture motivated ganciclovir treatment in eight patients and foscarnet in one. Four developed CMV IgM antibodies and five others had a significant increase in IgG titers. BC culture was positive in one.

In this group of patients, BC-PCR was positive in 22/39 tests (56 %), and all patients had at least one positive test. However, three were negative on presentation, including the only patient with a positive BC culture.

SE-PCR was positive in 3/40 tests, one from each of three patients, all in the interval between onset of symptoms and treatment. In all three cases the test turned negative after 6 days.

This series of tests is more difficult to interpret than the previously presented groups because the CMV diagnosis was not established in each case. However, since one must assume the majority to have had CMV disease, the proportion of positive SE-PCR is low and suggests that the test is not sensitive enough to discover all cases that will benefit from treatment. This is then also true for CMV culture on BC because only one of the CMV cultures in this group was positive.

The total experience with SE-PCR as compared to CMV culture on BC is presented in Table 3. The majority of discordant cases were obtained from patients with increasing or decreasing disease activity.

Discussion

Several groups have already reported their experience with PCR tests for CMV DNA in solid organ transplant patients [2–4, 7, 14, 17]. The common experience is that the test applied on leukocytes is extremely sensitive, probably even more so than the early antigens tests [2, 17]. Thus, the test is positive in a high proportion of asymptomatic patients. This is in accordance with our experience. We even found positive tests in three seronegative patients who received kidneys from seronegative donors and seronegative blood transfusion, who did not develop disease and who were seronegative at the 1-year control. False-positive PCR tests have been reported previously [17]. Contamination cannot be ruled out in spite of the extensive precautions taken, but the possibility of virus presence in spite of negative IgG titers must also be considered. In our patients this is supported by the positive urine CMV cultures in some cases.

For clinical use, extreme sensitivity of a test is not an advantage. The purpose of testing is not to detect the virus, which is obviously present in the vast majority of patients, but to find indications of significant involvement. One way is to develop a quantitative PCR method [7]. This still requires too extensive resources to be used in the clinical routine. As an alternative, we have studied whether the presence of CMV DNA in serum is a better indicator of viremia than its presence in leukocytes [16]. Our experience supports this hypothesis. The sensitivity of the SE-PCR is close to, and slightly better than, that of virus culture on BC. This conclusion can only be drawn if one accepts as a fact that there is no golden standard to the diagnosis, but that one must rely on a series of indications [18]. The number of patients with CMV-like disease, only partly confirmed by serologic tests and with negative BC culture and SE-PCR, suggests that the sensitivity of both tests is too low.

The PCR technique has also been used in attempts to monitor antiviral treatment, but without success [17]. One obstacle might be that demonstration of virus in vitro must be performed with methods unaffected by any presence of antiviral drugs in the specimens. It has been pointed out that isolation of CMV is uncertain in patients treated with acyclovir [8]. Ganciclovir inhibits herpes virus DNA polymerase, but the fact that the tests remained positive during treatment shows that ganciclovir does not significantly interfere with the polymerase used in our PCR test.

Although CMV can be detected during treatment, the result could not be used to evaluate or predict the effect of treatment. Virus DNA was found to disappear from serum during treatment and then to reappear, causing disease, but it was also found to persist for weeks after treatment and then spontaneously vanish. On the other hand, in this series, the second treatment course always efficiently controlled the relapse. The risk of recurrence cannot be estimated with other tests, including virus culture [17]. However, recently, quantitative CMV antigenemia has been reported to predict the response to antiviral treatment in liver transplant patients [13].

We propose the following guidelines with regard to the use of our PCR tests for CMV DNA in kidney or liver transplant patients:

1. A positive PCR test for CMV DNA on buffy coat does not, by itself, indicate CMV disease
2. A negative PCR test for CMV DNA on buffy coat is strong evidence against CMV as the cause of a febrile disease.
3. A positive PCR test for CMV DNA on serum in a patient with a febrile disease strongly suggests CMV as the causative agent (in the absence of bacterial infection). In an asymptomatic patient, a positive test alone does not necessarily herald significant CMV disease and would not, in a kidney or liver transplant patient, indicate the need for active treatment.
4. A negative PCR test for CMV DNA in serum does not rule out CMV viremia.

When combined, the two applications of the test could prove valuable in making decisions on initiating anti-CMV treatment of recently transplanted patients with fever.

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