VIROLOGY

Detection of Cytomegalovirus DNA, Viral Antigens, and Antibodies to It in Patients after Organ Transplantation

M. S. Dolgikh, A. M. Vedyakov, E. Yu. Feoktistova, S. M. Adueva,* N. E. Makarova,* A. A. Kushch,* and B. Pustovoit**

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 127, No. 5, pp. 560-563, May, 1999
Original article submitted May 21, 1998

Cytomegalovirus (CMV) DNA was determined by the PCR method, anti-CMV IgM and IgG were analyzed by enzyme immunoassay, and infective activity of CMV was evaluated by the rapid culture technique with monoclonal antibodies in the blood of patients, who underwent organ transplantation. In cases when the results coincided, PCR was much more sensitive, while the rapid culture technique was more reliable in predicting the course and outcome of CMV disease. Enzyme immunoassay failed to detect active infection by the moment of examination.

Key Words: *organ transplantation; cytomegalovirus (CMV) infection; PCR diagnosis; anti-CMV IgM; infective activity of CMV*

According to serological findings, infection caused by human cytomegalovirus (CMV) occurs in 44-85% recipients of organ and bone marrow graffs and is a serious threat for patients after allotransplantation [6,9].

Several methods are used for the diagnosis of CMV infection including gene diagnosis (detection of CMV DNA), detection of antigenemia (detection of CMV proteins in blood cells in a cytological preparation), virological, and serological methods. Recently PCR has been used for the diagnosis of CMV infection in patients with organ allografts [2,5,10,11,13,15]. However, many problems in the interpretation of diagnostic tests are still unsolved, in particular determination of the optimal terms for the beginning of specific antiviral therapy, evaluation of its efficacy, and prediction of the disease course.

Institute of Transplantology and Artificial Organs, Ministry of Health of the Russian Federation; *D. I. Ivanovsky Institute of Virology, Russian Academy of Medical Sciences, Moscow; **Institute of Virology, University of Leipzig, Germany

We compared the diagnostic value of three markers of CMV infection: DNA (detected by PCR), antibodies (detected by enzyme immunoassay, EIA), and infective activity of the virus (evaluated by the rapid culture technique).

MATERIALS AND METHODS

A total of 193 patients with kidney or heart allografts and patients on hemodialysis were examined in 1996-1997. The patients were treated at Institute of Transplantology, Moscow Nephrology Center, and Clinical Hospital No. 7, Moscow. Fifty-five blood donors were controls. The majority of patients were administered three-component immunosuppressive therapy (cyclosporine, azathioprine, and prednisolone) after organ transplantation.

PCR was carried out with blood, saliva, and urine samples. Blood leukocytes were isolated by selective lysis of erythrocytes. DNA was isolated using SiO_2 adsorbent (Sigma) and by phenol extraction from 10^6 leukocytes or 100 μl whole blood.

0007-4888/99/0005-0507$22.00

PCR samples contained DNA isolated from 10^5 leukocytes or 10 ml whole blood. In analysis of the saliva and urine, DNA was isolated from the precipitate after centrifugation of 1-3 ml saliva or 5-10 ml urine. The CMV genome site was amplified using primers to CMV immediate early (IE) gene region [4,12].

Standard reaction mixture for PCR consisted of reaction buffer (67 mM Tris buffer, 16.7 mM ammonium sulfate, and 0.1 mM 2-mercaptoethanol), 3 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate (dNTP): dATP, dCTP, dTTP, dGTP, 12-25 pM primers, 0.15 mg/ml BSA, and test DNA. Mineral oil was the upper layer. Taq polymerase was added after 3-5-min preheating of the reaction mixture at 95°C. The reaction was performed using MC-2 or Cyclotemp amplifiers (Russia).

The reaction products were separated by electrophoresis in 2% agarose with 0.001% ethidium bromide, followed by visualization in UV light. PCR products were identified and the sensitivity of the method was improved by two-stage amplification (nested PCR). Semiquantitative evaluation of CMV DNA in the blood was carried out by amplification of the sample after 10-fold dilution to a final point at a known sensitivity of the method (less than 10 copies of viral DNA for 2 PCR rounds and less than 100 viral DNA copies for 1 PCR round). The sensitivity of the method was verified using CMV DNA (Sigma).

Amplification of DNA of types 1 and 2 herpes viruses, Epstein—Barr virus, and hepatitis B virus with primers for CMV genome was carried out to control the specificity of amplification. No specific amplification products were detected.

Antibodies to CMV (IgM and IgG) were analyzed by EIA using Medix Biotech or DRG kits.

CMV antigens were analyzed using monoclonal antibodies to tegument protein pp65 and immediate early protein IEp72 obtained previously [1]. Diploid fibroblasts from human embryo lung (Cell Culture Collection of D. I. Ivanovsky Institute of Virology) were grown on slides in 24-well plates (Costar). Material from patients was put into wells for 1 h, after which virus-containing material was washed out and the cells were cultured for 48 h at 37°C and then fixed in cold acetone. Virus proteins were detected by indirect fluorescent analysis. To this end, a mixture of affinity-purified monoclonal antibodies to pp65 and p72 was incubated with cells for 1 h at 37°C. Bound antibodies were detected by FITC-labeled rabbit antimurine antibodies (N. F. Gamaleya Institute of Epidemiology and Microbiology). Samples containing no less than 5% fluorescent cells were considered as positive.

RESULTS

A total of 232 blood samples, including 198 samples from 96 patients after kidney allotransplantation, 9 from 3 patients after heart transplantation, and 25 from 16 patients on hemodialysis were analyzed for CMV DNA by the PCR method and for CMV-specific IgG and IgM by EIA. PCR was positive in 43% patients with allotransplantats and in 11% patients on hemodialysis. In none of 55 blood donors CMV DNA was revealed.

Anti-CMV IgM were detected in 120 blood samples (51.7%) and anti-CMV IgG in 209 (90.1%). Data of PCR (CMV DNA) and EIA (anti-CMV IgM) coincided in 55.6% cases. CMV DNA was detected in 82 (35%) blood samples from 30 patients without anti-CMV IgM by the moment of analysis (Fig. 1,*b*). CMV disease was diagnosed in 10 patients; they were treated with gancyclovir. Twelve patients had subfebrile temperature and 8 had no clinical signs of infection. Forty-seven blood samples (20%) contained no CMV DNA but contained anti-CMV IgM; there were no clinical symptoms by the moment of analysis (Fig. 1, *a*).

In 37 patients gancyclovir therapy considerably reduced (more than 100-fold) or eliminated CMV DNA from the blood and improved clinical state. On the other hand, in the majority of cases the titers of anti-CMV IgM and IgG increased and antibodies circulated for a long time (Fig. 1 and 2). In a patient with a symptomatic CMV infection, CMV DNA was detected 2 weeks before detection of anti-CMV IgM and/or IgG or increase in their titers during activation of the disease (Fig. 2). In other patients in this group the appearance of CMV DNA in the blood in the course of infection preceded the appearance of antibodies to CMV. These findings are in line with other reports, demonstrating that the appearance of CMV DNA in the blood precedes the development of clinical symptoms, while a notable increase in the level of IgM and IgG antibodies to CMV is observed 0.5-2 weeks

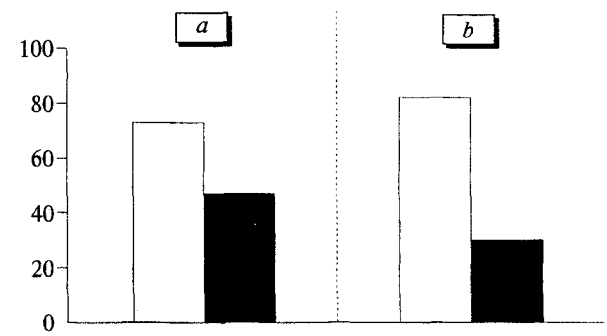

Fig. 1. Number of samples containing (*a*) and not (*b*) IgM antibodies to cytomegalovirus (CMV) detected by enzyme immunoassay and containing CMV DNA detected by PCR. White bars: samples containing CMV DNA; black bars: samples containing no CMV DNA.

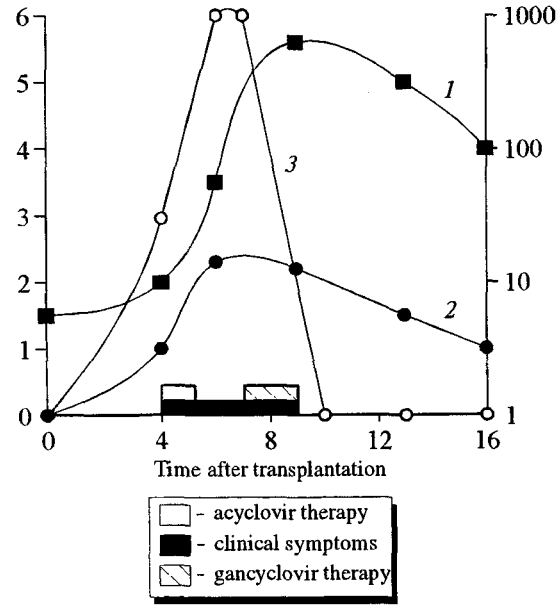

Fig. 2. Time course of cytomegalovirus (CMV) DNA, IgM and IgG antibodies to CMV in the blood of patients after allotransplantation. Ordinate: left: optic density at 490 nm, arb. units; right: DNA dilutions. *1*) anti-CMV IgG; *2*) IgM; *3*) DNA.

after clinical manifestations [13,15]. Moreover, according to serological studies, CMV infection is highly prevalent (30-100%) among healthy population [8,14]. In our experiments IgG were detected in 12 out of 14 (85%) blood donors. These data and the absence of antibodies at the early stages of the infectious process and long circulation of IgM because of inadequate immune response impede the interpretation of serological findings in recipients [13,15]. Administration of hyperimmune globulin used for treating posttransplantation patients can lead to erroneous conclusions on patient's serological status.

Infective activity of CMV was studied by the rapid culture technique and the data were compared with the findings of PCR of CMV DNA. A total of 117 samples from 78 patients were analyzed. The results of both methods coincided in 81 samples (69%). Negative results coincided 2 times more often than positive (55 and 26 samples, respectively). If the results of both tests were negative, there were no symptoms of disease. In the majority (62%) of cases both DNA and infective activity of the virus were detected and clinical symptoms associated with CMV infection were observed. In 37% cases positive results of both tests were not associated with clinical symptoms by the moment of analysis. This is in line with other reports that 8-39% patients after organ transplantation develop CMV disease [7,9].

Fourteen patients were tested 3-6 times by two methods in parallel. PCR was positive in 29 samples, and the viral activity was detected in 16 of these. In 5 patients positive results of PCR coincided with the positive results of the culture method and were paralleled by pronounced clinical symptoms. In 3 patients with CMV disease, CMV DNA was detected 10-17 days before the infectious virus. In 5 patients the positive results of PCR and negative results of culturing were not paralleled by clinical manifestations during the observation period (9 weeks after surgery). Presumably, the immune system of these patients suppressed viral replication and the development of the infection.

In patient K. analysis of the material collected on day 31 after kidney transplantation by PCR and rapid culture technique gave positive results; cimevene therapy was prescribed (Fig. 3). Four days after the first course of treatment, acute deterioration (fever with intestinal hemorrhage) was observed. CMV DNA and infective activity of the virus were detected again. Repeated course of therapy, after which PCR and culturing gave negative results, stopped hemorrhage and improved the patient's clinical status.

In acute CMV disease, DNA and infective activity of CMV were detected in the blood, saliva, and urine. In the absence clinical symptoms, tests gave controversial results for different biological material. In one urine sample the result of PCR was negative, while the culture method gave a positive result. Control amplification with primers for C8 complement component gene site showed that the negative result of PCR was associated with inhibition of Taq polymerase. Similar results are reported by other authors [3,7,10]. Therefore, the results of PCR analysis of CMV DNA in urine samples can be false-negative.

Our findings indicate that PCR is a highly sensitive and specific diagnostic method for detecting the

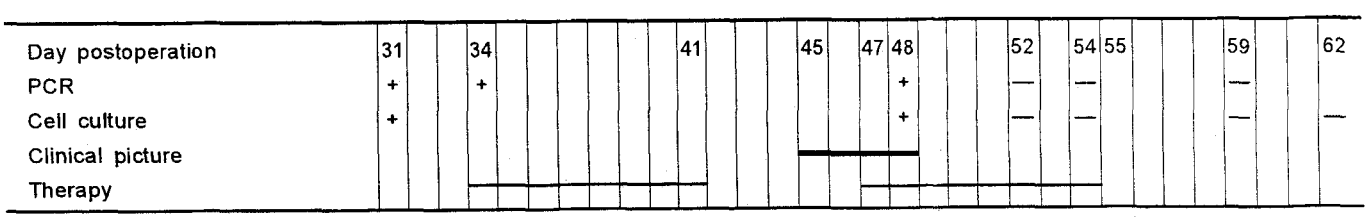

Fig. 3. Monitoring of cytomegalovirus DNA and infective activity of the virus in the blood of a patient after kidney transplantation. +) positive, —) negative result; fine lines: gancyclovir therapy; bold line: clinical manifestation of the disease.

viral genome before clinical manifestation of the disease. Detection of IgM and especially IgG to CMV in the majority of cases gave a retrospective information and did not reflect the true intensity of viral reactivation by the moment of analysis. The data indicate that the rapid culture technique is less sensitive but more prognostically valuable in comparison with PCR. Combined use of these two methods more completely characterizes the infectious process, helps to monitor drug treatment, and more accurately predicts the outcome of CMV infection.

REFERENCES

1. N. E. Makarova, A. A. Kushch, L. A. Ivanova, *et al., Vopr. Virusol.*, **41,** No. 1, 28-32 (1995).
2. M. M. Abecassis, A. J. Koffron, M. Kaplan, *et al., Transplantation,* **63,** No. 2, 275-279 (1997).
3. S. J. N. Choi, N. H. Kim, C. K. Cho, *et al., Transplant. Proc.*, **28,** No. 3, 1523-1524 (1996).
4. G. J. Demmler, G. L. Buffone, C. M. Schimbor, and R. A. May, *J. Infect. Dis.*, **158,** 1177-1184 (1988).
5. G. Gerna, D. Zipeto, M. Parea, *et al., Ibid.,* **164,** 488-498 (1991).
6. S. Goral and J. H. Helderman, *Transplant. Proc.*, **26,** No. 5, Suppl. 1, 5-6 (1994).
7. P. Grossi, L. Minoli, E. Percivalle, *et al., Transplantation,* **59,** No. 6, 847-851 (1995).
8. M. Hirata, P. I. Terasaki, and Y. W. Cho, *Ibid.*, **62,** No. 1, 34-37 (1996).
9. M. Ho, *Transplant. Proc.*, **26,** No. 5, Suppl. 1, 173-179 (1994).
10. M. P. Landini, *Progress in Medical Virology*, Ed. by J. L. Melnick, Basel (1993), pp. 157-177.
11. J. S. M. Peiris, C. E. Taylor, J. Main, *et al., Nephrol. Dial. Transplant.*, **10,** 1198-1205 (1995).
12. A. Rolfs, I. Schuller, U. Finckh, and I. Weber-Rolfs, *PCR: Clinical Diagnostics and Research*, Berlin - Heidelberg (1992), p. 69.
13. C. A. Schmidt, H. Oettle, R. Peng, *et al., Transplantation,* **59,** No. 8, 1133-1138 (1995).
14. G. E. Tegtmeier, *Semin. Liver Dis.*, **6,** 82-95 (1986).
15. C. Wolff, M. Scourtopolous, D. Hornschemeyer, *et al., Transplantation,* **61,** No. 5, 750-757.