CLINICAL STUDY

The occurrence of symptomatic CMV infections in heart transplant recipients

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Abstract

Background: **Human cytomegalovirus is widely spread in the human population and at present it is the most important clinical agent of opportunistic infections in immunocompromised patients. Bone marrow transplant recipients, solid organ transplant recipients and AIDS patients are at risk of the development of a severe systemic disease. Prompt detection of active infection is essential for early initiation of antiviral therapy.**
Aim: **The aim of our work was to follow the occurrence of symptomatic CMV infection in heart recipients.**
Methods: **We have used antigenemia assay, PCR and serology methods for the detection of CMV infection in heart transplant recipients.**
Results: **We found that the occurrence of symptomatic CMV infections was lower than the literary data.**
Conclusion: **Though a small group of patients was examined, we suppose that this was caused by the careful finding of CMV serostatus of donors and recipients, by using of CMV-seronegative, filtered, or leukocytes-poor blood products, by the examination of blood products and prophylaxis of risk patients, too.** ***(Tab. 1, Ref. 37.)***
Key words: cytomegalovirus, (CMV), polymerase chain reaction (PCR), CMV pp65 antigenemia, heart transplantation, therapy.

Human cytomegalovirus is widely spread in the human population and at present it is the most important clinical agent of opportunistic infections in immunocompromised patients. The key feature of CMV is its ability to remain latent in the following primary infection and to become reactivated as a consequence of attenuated immune system. Bone marrow transplant recipients, solid organ transplant recipients and AIDS patients are at risk of the development of a severe systemic disease. Approximately 50 % of transplant patients excrete CMV in body secretions (e.g., saliva and urine) at some stage after organ transplantation; this usually begins in the first month following transplant surgery. Viral shedding reaches peak levels during the second and third months following transplantation, at which time it may be associated with disease (Griffiths, 1995).

CMV genomic material has been found in monocytes/macrophages, neutrophils (Schäfer et al, 1998), lymphocytes (Schrier et al, 1985), and endothelial cells (Grefte et al, 1993). After primary CMV infection in the immunocompetent host, long-term immunity develops, controlling viral persistence – a situation that is lacking following solid-organ transplantation. While humoral immunity provides the best evidence of prior infection and the ability to transmit the virus, cytotoxic T lymphocytes are the key host defence against CMV (Fishman and Rubin, 1998). Failure to reconstitute CMV specific cellular immunity after transplantation leads to progressive CMV disease (Zeevi et al, 1998). The spectrum and severity of clinical CMV disease are dependent on the type of organ transplant, the pretransplant serologic status of the donor and recipient, the immunosuppressive regimen used, and the intensity of graft rejection. The most important risk factor for the development of CMV disease in any solid-organ transplant is primary CMV exposure (Birkeland et al, 1998). CMV infection in solid-organ transplant recipients causes a wide range of clinical mani-

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Acknowledgment. Thanks to doctors from the Department of Clinical Biochemistry in Bratislava-Petržalka for the determination of neopterin in plasma samples.

festations, from asymptomatic infection to severe, potentially lethal CMV disease. Mildly symptomatic CMV infection usually presents insidiously with fever, anorexia and malaise, without additional signs or symptoms. Prolonged fever lasting 3 to 4 weeks may be the only manifestation of symptomatic CMV infection. Myalgias, arthralgias, and arthritis may occur, but the mononucleosis syndrome accompanied by lymphoadenopathy or splenomegaly typically seen in immunocompetent hosts is rarely seen in transplant recipients. Hematologic abnormalities such as leukopenia, typically without the presence of atypical lymphocytes, and trombocytopenia, are common. This viral syndrome may be self-limited or may progress to clinically evident organ involvement. The clinical manifestations of CMV disease may be relatively nonspecific and can be difficult to differentiate from illnesses caused by a variety of other opportunistic microbes, acute graft rejection, and drug toxicity – all of which can cause fever and even organ dysfunction in transplant patients (Sia and Patel, 2000). Life-threatening complications have included severe infections of the GI tract with perforations of abdominal viscera, hepatitis, and pneumonia. Human CMV pneumonia in solid organ allograft recipients is not associated with the high mortality rates often seen in BMT recipients but can be a life-threatening disease, especially in heart and heart-lung recipients. Several recent descriptions of CMV-related death following heart and heart-lung transplantation have identified interstitial pneumonia as a major end-organ disease resulting in death of the patient (Smyth et al, 1991).

Antiviral agents currently licensed for the treatment of CMV infections include ganciclovir, foscarnet, and cidofovir. All three compounds inhibit CMV DNA synthesis by inhibiting the viral DNA polymerase (Crumpacker, 1996; Chrisp and Clissold, 1991; Lalezari et al, 1995). Ganciclovir is poorly tolerated, being associated with haematological toxicity (Noble and Faulds, 1998). Intravenous immunoglobulin has been used by various transplant centers to prevent CMV disease with noticeably discordant results. Prophylaxis with CMV-specific immunoglobulin has been shown to reduce the incidence and severity of CMV disease in seronegative renal transplant patients who receive a kidney from seropositive donor (Snydman et al, 1987). Benefit from the use of CMV-specific immunoglobulin has also been shown for patients undergoing orthotopic liver transplantation (Glowacki and Smaill, 1994). No effect could be demonstrated in patients at risk such as seronegative recipients of a graft from a seropositive donor and in heart/lung transplant recipients (Aguado et al, 1995). Fewer studies have been conducted with foscarnet, although it offers a theoretical advantage for bone marrow transplant patients in whom neutropenia associated with ganciclovir is a significant problem (Griffiths, 1995). Nephrotoxic side effects of foscarnet contraindicate its use in people with impaired renal function. Despite its reported toxicity, foscarnet is potentially useful for ganciclovir-resistant CMV infections (Noble and Faulds, 1998). Cidofovir is a novel agent for the treatment of CMV infection in patients with AIDS (Sia and Patel, 2000).

Subjects and methods

Subjects

In total, blood samples from 24 patients after heart transplantation were investigated. 22 of the patients were males, and 2 were females (the average age 44.67±14.8 years). All patients underwent transplantation at the Slovak Institute of Cardiovascular Diseases during 1998–2001. 16 patients had dilated cardiomyopathy, 7 suffered from coronary artery disease, 1 had rheumatic heart disease. Four CMV-seronegative patients received a transplant from a seropositive donor. Leukocytes for antigenemia assay were prepared from EDTA blood. For molecular assay, 200 µl plasma or leukocytes in PBS were used.

Methods

Antigenemia assay

*The early structural protei*n pp65 of CMV was detected by immunocytochemistry with peroxidase staining using a commercially available kit (CMV PO™ Kit, IQ product, Netherland). The assay was performed according to the manufacturer's instructions. Briefly, peripheral blood leukocytes were separated from fresh whole blood, counted and spotted onto slides. Following incubation with monoclonal antibodies directed against CMV pp65 and subsequent incubation with HRP conjugate, cells were stained with substrate and visualized for typical nuclear staining. If any cell gave a positive signal in a total $1x10^5$ cells analysed, the result was considered positive.

Nested PCR

Nucleic acid was extracted from leukocytes or plasma using the silica-based extraction method with a commercially available kit (QIAamp Blood Kit, Germany). This kit is designed for rapid purification of an average of 6 µg of total DNA from 200 µl of whole human blood, and up to 50 µg of DNA from 200 µl of buffy coat or $5x10^6$ lymphocytes. The CMV gB gene was used as the target, and the same primers were used for the amplification and detection of PCR products. The PCR was performed as a double PCR with two sets of primers as described previously (Schäfer et al, 1993). The outher primer set consisted of C (5' TCC AAC ACC CAC AGT ACC CGT 3') and D (5' CGG AAA CGA TGG TGT TCG 3'). The inner primer set consisted of A (5' GTC AAG GAT CAG TGG CAC AGC 3') and B (5' GTA GCT GGC ATT GCG ATT GGT 3'). The nested primer set A-B amplified a 160 bp amplimer. 10 µl of total extracted DNA (leukocytes/plasma) were analysed in a 50 µl first-round PCR using the master mixture reagents (Taq PCR Master Mix Kit, Germany). After amplifications, 2.5 µl of each first-round product was added to the nested PCR mix in a total volume of 25 µl. Amplification consisted of 35 cycles of denaturation (92 °C 1 min), annealing (45 °C 1 min), and extension (72 °C 1 min). The PCR products (10 µl) were electrophoresed on a 2 % agarose gel containing ethidium bromide, and the results were photographed under ultraviolet illumination. All reactions were performed with known negative (distilled water) and positive controls (DNA of CMV strain AD169-infected human embryonic lung fibroblasts).

Serology

We detected anti-CMV IgM and IgG in human serum using a commercial automated enzyme-linked fluorescent assay (Biomerieux VIDAS). Test serum (100 µl) was introduced into a sealed strip containing all the reagents and was placed in the VIDAS instrument together with a solid-phase receptacle and was tested by using the CMVG program or CMVM program. The solid-phase receptacle serves as an antigen-coated solid phase as well as the pipetting device. When the assay was completed, the fluorescence was read and analysed by computer and the result IgG was expressed in arbitrary units (AU) per millilitre. Reading of less than 4 AU/ml was considered negative according to the manufacturer's recommendations. Samples with reading of more than 400 AU/ml were retested 1:4 in saline solution to obtain a reliable quantitative result. The result IgM was qualitative rather than quantitative. The manufacturer considered a fluorescent signal index of 0.9 or more to be a positive result and an index of between 0.7 and 0.9 to be an equivocal result, and these criteria were adopted in this study.

Detection of neopterin

Detection of neopterin in plasma samples was done in the Department of Clinical Biochemistry in Bratislava-Petržalka.

Results

24 heart transplantations were performed at the Slovak Institute of Cardiovascular Diseases in Bratislava during 1998–2001. We have been making CMV screening before transplantation for this institute using VIDAS IgM and IgG since 1997. We completed CMV monitoring in these patients introducing CMV antigenemia assay and nested PCR in May 1999. CMV monitoring is made once a week. It starts on the seventh day after transplantation until the patient is discharged from the hospital (the average hospital time after HTx is 21 days) and then during the control hospitalizations (the first month – weekly, the second month – once per two weeks, the third to sixth month – monthly, later if necessary).

Four of 24 patients were at the highest risk of diseases, because they were CMV-seronegative and they received the organ from CMV-seropositive donors. We proved symptomatic CMV infection using laboratory tests only in one of the 24 patients. It was a 56 years old man with the highest risk of disease (D^+/R^-). The laboratory results of tests are shown in Table 1. 56 days after transplantation we proved CMV DNA in plasma. CMV DNA persisted 20 days in this patient. 84 days after transplantation the sample was negative. The patient was given ganciclovir prophylacticaly, but on the sixth day after heart transplantation (HTx) it was substituted for by a specific immunoglobulin G. 70 days after HTx the patient had a fever and CMV disease was manifested. CMV DNA and specific production of IgM were proved by laboratory tests 70 days after HTx. Antibodies IgG proved on the fourteenth day were caused by giving specific IgG. These antibodies disappeared 63 days after HTx and 126 days after HTx we found seroconversion in the class IgG. These antibodies were produced because of CMV infection. Antigenemia assay could not be classified because of endogenous peroxidase and this method was negative from other samples. The raised level of neopterin correlated with CMV DNA (Tab. 1). After therapy initiation with ganciclovir the patient's condition improved, CMV DNA already disappeared in plasma and the level of neopterin decreased (normal levels: 0–9.9 nmol/l). IgM antibodies have persisted until the present in this patient (3.5 years).

Discussion

Cytomegalovirus has long been recognized as a major cause of life-threatening complications in transplant recipients. There is an increased interest and perceived need for the use of techniques that will allow early detection of viral activation before any symptomatology, making the use of pre-emptive therapy possible in those at greatest risk of disease.

The most commonly used method to detect CMV infection was conventional cell culture on human fetal lung fibroblasts. The problem with this procedure is that cytopathic effect evolves,

Tab. 1. The profile of CMV investigations in 56 years old patient after HTx.

Methods	pp 65	PCR	Antibodies			Neopterin (nmol/l)	Therapy
No days after HTx			IgM	IgG (IU/ml)			
14	neg	neg	neg	pos	16	NP	anti-CMV IgG
28	neg	neg	neg	pos	23	NP	anti-CMV IgG
56	NC	pos	neg	eqv	5	39.5	anti-CMV IgG
63	neg	pos	neg	neg		41.3	
70	neg	pos	pos 1.37	neg		49.4	Ganciclovir
77	neg	pos	pos 1.91	neg		49.4	Ganciclovir
84	neg	neg	pos 2.02	neg		17.3	Ganciclovir
105	neg	neg	NP	NP		15.1	
126	neg	neg	pos 1.9	pos	8	16.4	
207	NP	neg	pos 1.73	pos	26	NP	

Note: NC — not classifiable, NP — not performed

most of the time, very slowly: it can take up to 21 days to visualize the cytopathic effect of CMV infection in cultured cells. A more rapid way to detect CMV is to centrifuge leukocytes in a shell vial. Immediate early antigens are detected 48 h later by using a monoclonal antibody, which recognizes the 72-kDa protein (Gleaves et al, 1985). Alternatively, it is possible to detect CMV antigens, in blood, by using monoclonal antibody to the pp65 CMV protein (The et al, 1995). The problem of antigenemia assay is that CMV disease develops in some patients without previous positivity of antigenemia or positivity of antigenemia precedes only a very short time the disease (Boeckh et al, 1996). Nucleic acid amplification by PCR methods has become the most widely used diagnostic tool for CMV infection. Most of these procedures are highly specific and excellent positive predictive value (Boeckh and Boivin, 1998). However, they may provide contrasted results regarding their sensitivity, their negative predictive value and the lag time between the first positive test and appearance of clinical symptoms.

We have introduced CMV antigenemia assay and CMV nested PCR for monitoring of CMV infections in heart transplant recipients in our laboratory. We did not prove the positivity of antigenemia assay, not even in the patient with symptomatic CMV disease. It is possible that an antigenemia was positive before the detection of CMV DNA in plasma, some time between 28th and 56th day when samples for CMV were not taken from this patient. On the other hand some authors compared CMV PCR from PMNL with CMV antigenemia assay and they found that on average PCR was positive earlier and for a longer time than antigenemia (Alain-Albertini et al, 1991; Gerna et al, 1991).

Discrepant results between the CMV antigenemia assay and CMV cultures have been previously reported and appear to be the consequence of antiviral therapy or sample variability (Gerna et al, 1991). Erice et al (1992) demonstrated that the CMV antigenemia assay is more sensitive than shell vial cultures of PMNL fractions for rapid diagnosis of CMV viremia. This dissociation between CMV antigenemia and culture results observed during ganciclovir therapy could possibly be due to a ganciclovir-induced block in viral DNA replication (and consequent inhibition of infection of fibroblasts in culture) combined with intact expression of CMV antigens in infected PMNL. On the basis of these observations, some investigators have recommended that the CMV antigenemia assay should be used for monitoring patients during ganciclovir therapy (Gerna et al, 1991).

In one study (Ehrnst et al, 1995) the pp65 antigenemia test was found to be the sensitive method for assessment of CMV activation, although the assay gave a better positive predictive value for identification of CMV disease, than whole blood and plasma qualitative PCR assay. Since the antigenemia test is dependent on the type and quality of cells available from a peripheral blood sample, results can be unreliable or difficult to interpret for severely neutropenic patients (particularly BMT recipients).

In our laboratory we have found that nested PCR was better than antigenemia assay from direct method of CMV detection. We did not prove significant positive antigenemia. To compare CMV detection using PCR, samples to be tested by the antigenemia assay should be processed within 6h after collection because delays may diminish pp65-positive cell counts (Gerna et al, 1992). Nested PCR is more sensitive than antigenemia, but this method has higher risk of contamination with amplicons after the first amplification.

Variables, which contribute to infection in the posttransplant period, include donor and recipient serologic status, type of immunosuppression, source of allograft (living – related versus cadaveric), HLA matching of donor and recipient, and type and amount of blood product used. Perhaps the most important contributing factor to the development of severe CMV disease in the posttransplant period is the serologic status of the donor and recipient. Individuals with primary infection almost always develop more significant disease than those with secondary or superinfections (Smyth et al, 1991).

In our work is described the case of 56 years old patient after HTx with high risk of CMV disease (D^+/R^-). The patient was given ganciclovir prophylacticaly in dose 5 mg/kg/BID i.v. Ganciclovir was substituted with specific hyperimmune CMV IgG because of acute humoral rejection (treated with cyclophosphamid) and low values of trombocytes on the sixth day. CMV disease was manifested as an interstitial pneumonia in this patient on the 70th day after transplantation. By laboratory tests we found the presence of CMV DNA using PCR in plasma, the increased levels of neopterin, the start of production of specific IgM and seroconversion IgG. Neopterin is a marker of inflammatory diseases and infections with HIV or CMV and opportunistic infections. Neopterin is also a marker in the diagnosis and monitoring of malignant diseases. High neopterin concentrations were also observed during the monitoring of solid organ and bone marrow allograft recipients (Müler et al, 1991).

Detection of the CMV nucleic acid is an alternative to the antigenemia assay. Qualitative CMV-DNA amplification does not correlate well with development of clinical disease. The test is, in most hands, too sensitive and can detect latent viral genomes without relevance to clinical disease (Boeckh et al, 1997). Therefore it is necessary to show increased amounts of DNA either in plasma or in leukocytes by quantitative amplification. The virus load, defined as number of CMV-DNA copies, is found to be well correlated to symptomatic CMV infection (Peiris et al, 1995). Because DNA is relatively stable, it may remain at high levels for some time in spite of successful treatment, and emergence of drug-resistant mutants may not be detected. High CMV DNA titres in blood are associated with clinical symptoms in transplant patients. CMV infections occurring early in the posttransplantation period, that is, within 2 months of transplantation, appear to have higher viral loads, most probably because immunosuppression is more intense, and are usually associated with disease. The maximum CMV DNA level during infection is significantly higher in patients who are experiencing primary infection compared to those who have asymptomatic and/or reactivation CMV infection (Manez et al, 1996; Roberts et al, 1998). After the initiation of antiviral therapy, CMV DNA levels in blood fall rapidly, correlating with the disappearance of clinical symptoms. In contrast, patients unre-

sponsive to therapy have persistent high levels of viral DNA (Peiris et al, 1995).

We detected CMV DNA using nested PCR in 56 years old patient 56 days after the transplantation. CMV DNA persisted in plasma 20 days, CMV DNA decreased after the initiation of antiviral therapy and CMV DNA was not detected in plasma 84 days after heart transplantation.

Serologic methods are often of limited value in the transplant recipient because of the inability of these assays to distinguish between infection and invasive disease. The presence of CMV-specific IgM antibodies is also of questionable importance because a significant number of allograft recipients will generate IgM antibodies in the posttransplant period, even in the absence of invasive disease. Conversely, the lack of an IgM antibody response in the presence of CMV infection in the posttransplant period is often associated with severe immunosuppression and poor outcome (Britt and Alford, 1996).

IgM can persist a long time after primary infection in the transplant recipients. IgM has been persisting 3.5 years in the 56 years old patient. It means that serology is an insensitive marker of active CMV infection in the organ transplant population and is therefore of limited diagnostic usefulness. CMV serology is useful for determining the history of CMV infection in potential organ donors and allograft recipients and it is also useful for screening potential blood donors (Schmidt et al, 1995).

A lot of measures for preventing CMV infection were developed for reduction of the frequency of CMV infection and CMV disease: selection of allograft from CMV-seronegative donors for CMV-seronegative recipients; use of CMV-seronegative, filtered, or leukocyte poor blood products; a passive immunization with immune globulin; prophylaxis with antiviral agents; preemptive therapy. For toxicity of ganciclovir it is given only to patients with high risk of CMV disease (D^+/R^-).

The incidence of CMV disease ranges from 8 % to 55 %, depending on the type of transplanted organ. Symptomatic infections occur in approximately 9 to 35 % or 25 % of heart transplant recipients (Ho, 1994; Grossi et al, 1995; Patel and Paya, 1997). Symptomatic CMV infection was defined as an episode of fever (>38 °C) for 2 days or more, or leukopenia, thrombopenia, hepatitis, intestinal ulceration, or interstitial pneumonia (Dorp et al, 1990). Symptomatic CMV infection occurs predominantly during the first 4 months after transplant, and late CMV disease has rarely been reported (Hebart et al, 2000).

We observed the occurrence of CMV disease in 24 patients after HTx. Heart transplantation was performed in these patients at Slovak Institute of Cardiovascular Diseases in Bratislava during 1998–2001. In this small group of transplanted patients (24) CMV disease was manifested only in one patient (4 %). We found lower occurrence of symptomatic CMV infections in these patients in comparison with literary data. Because it was a small group of patients the results show limited predictive value. In our opinion the incidence is lower thanks to the careful finding of CMV serostatus of donors and recipients, the use of CMV-seronegative, filtered, or leukocyte-poor blood products in seronegative recipients, the examination of blood products and prophylaxis of risk patients, too. Knowledge of the CMV serostatus of the donor and recipient pretransplantation will allow selecting correct prophylactic approach.

The weekly measurement of the systemic CMV load during the first 3 months after transplant is useful to predict CMV disease in solid-organ transplant recipients. Although good data have been obtained for renal, liver, and heart transplant recipients using the CMV antigenemia assay, more data are needed to define breakpoints for CMV DNA in different patient populations by using molecular methods (Boeckh and Boivin, 1998).

Several authors have advocated the use of a combination of methods (CMV antigenemia assay, shell vial cultures, and conventional cultures) to achieve maximum sensitivity for detection of CMV viremia (Gerna et al, 1991).

On the basis of our results, we recommend the PCR assay as the preferred method for rapid diagnosis of CMV viremia. But, quantitative PCR could be an addition for the diagnostics and the prediction of CMV disease in our laboratory and for distinguishing the latent and active CMV infection and monitoring antiviral therapy in transplant recipients.

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Received April 22, 2003.
Accepted October 6, 2003.