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RESEARCH ARTICLE

Cytomegalovirus antigenemia is observed more frequently following allogeneic peripheral blood stem cell transplantation compared to bone marrow transplantation

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ABSTRACT

One of the major complications following hematopoietic stem cell transplantation (HSCT) is cytomegalovirus (CMV) infection. In our institution, three methods have been applied routinely for the diagnosis of CMV antigenemia (CMV-Ag): 1. Direct immunofluorescence microscopic (IFM) examination; 2. Flow cytometric (FC) analysis; and 3. Serological investigation. We were able to detect CMV-Ag by FC in 18 out of 75 transplanted cases. In 14 of these, positivity was confirmed by IFM as well. CMV-Ag was detected as positive by FC in samples from peripheral blood (14 cases) and/or bronchoalveolar lavage (BAL) fluid (4 cases). Eighteen patients had been transplanted [peripheral blood stem cell transplantation (PBSCT)/bone marrow transplantation (BMT): 16/2]. CMV-Ag was detected in 34% of PBSCTs and 7% of BMTs ($p<0.008$). Antigenemia was observed at a median of 4.5 months. In most of the patients, graft-versus-host disease (GVHD) was accompained by CMV-Ag. The ratio of acute GVHD/chronic GVHD was 6/10. Out of 18 CMV-Ag positive patients, 16 also had signs of infection. They were all positive by IFM as well. The two methods of CMV-Ag detection were correlated ($r=0.619$, $p<0.0001$). An important finding is the higher frequency of CMV-Ag and GVHD in patients who had received PBSCT.

Key words: CMV antigenemia, hematopoietic stem cell transplantation.

ÖZET

Sitomegalovir antijenemisi allojeneik periferik kök hücre transplantasyonundan sonra kemik iliği transplantasyonuna gore daha sık gözlendi

Sitomegalovirus (CMV) infeksiyonu hematopoietik kök hücre transplatasyonunu takiben görülen majör komplikasyonlardan biridir. Bizim merkezimizde CMV antijenimi tanısında rutin olarak üç yöntem kullanılmaktadır. 1) İmmunflorasan mikroskopik inceleme (IFM) 2) Akım sitometrik inceleme (ASİ) 3)Serolojik inceleme. Biz 75 transplant vakasının 18'inde ASİ ile CMV Ag saptadık. Bu vakaların 14'ünde pozitifliği IFM ile de gösterdirk. CMV Ag 'yi FC ile periferik kan örneği (14 vaka) ve/veya bronkoalveoler lavajda (4 vaka) saptadık. Onsekiz vakada transplant yapılmıştı, periferik kök hücre transplantasyonu (PKHT)/kemik iliği transplantasyonu (KİT): 16/2. CMV Ag, PKHT'nin %34 ve KİT'da ise %7 saptandı ($p<0.008$). Antijenemi median 4.5 ayda gözlendi. Hastaların çoğunda, graft versus host hastalığı (GVHH) CMV Ag'ye eşlik ediyordu: Akut GVHH/kronik GVHH oranı 6/10'idi. CMV Ag pozitif 18 hastanın 16'sında CMV infeksiyon bulguları vardı. Bunların hepsinde IFM pozitifti. CMV Ag'nin saptanmasında iki metod ($r=0.619$, $p<0.0001$). Önemli bir bulgu CMV Ag ve GVHH'nın PKHT alıcılarındaki yüksek sıklığıdır.

Anahtar sözcükler: CMV antijenemi, hematopoietik kök hücre transplantasyonu

INTRODUCTION

Cytomegalovirus (CMV) viremia with its associated infectious complications continues to be a major problem observed among patients undergoing high-dose chemoradiotherapy and hematopoietic stem cell transplantation (HSCT). CMV is a latent virus belonging to the beta herpes virus family. Origin of CMV infection can be endogenous or exogenous. Until full immune reconstitution, risk of CMV infection is high and is even perturbed by over-immunosuppression or graft-versus-host disease (GVHD). Allogeneic (allo)-HSCT, acute and chronic GVHD, and pre-transplant CMV-seropositivity are important risk factors for CMV infection. In previous studies, age, sex, disease status before HSCT, pre-conditioning, CMV, or GVHD prophylaxis regimens could not be established as being associated with the frequency of CMV infection [1,2]. The methods used in the detection of CMV antigenemia (CMV-Ag) are: viral culture methods, immunofluorescence, and detection of CMV DNA or mRNA by polymerase chain reaction (PCR) [3,4]. In our institution, three methods have been in routine use: 1. Direct immunofluorescence staining and microscopic evaluation of peripheral buffy coat cells stained with a fluorescent-labeled Moab against pp65; 2. Flow cytometric (FC) examination of peripheral blood and/or bronchoalveolar lavage (BAL) fluid after an incubation with an anti pp65 Moab. FC enables quantitative measurement of CMV-Ag in lymphocytes, granulocytes and mononuclear cells separately. The ability of detection in samples with low counts such as detected in BAL is an advantage of FC; and 3. Serological investigation - CMV immunoglobulin IgG and IgM antibodies were determined using enzyme immunoassay kits.

The aims of the current study were 1) to compare the results of the two methods used for the detection of CMV-Ag in patients receiving allo-HSCT, and 2) to evaluate the effect of stem cell source and GVHD on the incidence of CMV-Ag.

MATERIALS and METHODS

Patients: Eighteen out of 47 peripheral blood stem cell transplantation (PBSCT) and 28 bone marrow transplantation (BMT) patients, who were suspected to have CMV infection based on clinical findings, between January 1998-October 1999, were included in this study. The data of the patients studied are summarized in Table 1. All patients had busulphan 4 mg/kg/day po for four days and cyclophosphamide 60 mg/day iv for two days as a conditioning regimen, and one patient had whole body irradiation + cyclophosphamide chemotherapy. Patients had cyclosporin A (CsA) and short-term methotrexate (MTX) protocol for GVHD prophylaxis. All patients had received granulocyte colony-stimulating factor (G-CSF) starting at a dose of 5 μg/kg, iv infusion from the $+1^{st}$ day, until the neutrophil counts reached $>1x10^9/L$ on three consecutive days. In the PBSCT and BMT groups, fluconazole 200 mg/day and acyclovir 1 g/day were administered from day –8 to day +180. Ciprofloxacin 400 mg/day was given from day –8 till neutrophil engraftment and also trimethoprim-sulfamethoxazole was administered from day –8 to day 0.

I- Quantitative pp65 antigenemia detection using immunofluorescence microscope (IFM):

The CMV-Ag assay consists of direct staining of nucleated cells with monoclonal antibodies against the lower matrix protein pp65. The results are expressed as the ratio of antigen-positive cells relative to the total number of cells in each slide. Assay characteristics: The antigenemia assay consists of four steps i) isolation of polymorphonuclear leukocytes (PMNLs) by dextran separation and preparation of slides, ii) fixation, iii) immuno-staining, and iv) detection and quantification. The CMV-Ag assay has been developed using a cocktail of two monoclonal antibodies (C10/C11) directed against CMV lower matrix protein pp65. The CMV Brite Test Kit is based on the application of the C10/C11 cocktail over the slides of isolated leukocytes. In the first step, leukocytes are purified and cytocentrifuged on a slide, fixed, and permeabilized to allow subsequent detection of CMV pp65 antigen. The presence of the CMV-antigen is detected by the C10/C11 monoclonal antibodies and visualized by means of a specific secondary FITC-labeled anti-mouse Ig G antibody. CMV-antigen-positive leukocytes exhibit homogeneous yellow-green polylobulated nuclear staining under a fluorescence microscope. A positive result consisted of one or more CMV-antigen-positive cells per set of duplicate slides [5,6].

Table 1. Characteristics of transplant patients

Case	*Age/Sex*	*Disease*	*CMV-antibodies before transplant*		*Source of transplant*
			Ig G	*Ig M*	
1.SK	32/M	CML	(+)	(-)	PBSCT
2.MG	42/M	CML	(+)	(-)	PBSCT
3.GK	24/M	CML	(+)	(-)	BMT
4.HŞ	33/M	CML	(+)	(-)	PBSCT
5.AA	35/M	CML	(+)	(-)	PBSCT
6.AG	35/M	CML	(+)	(-)	PBSCT
7.IY	42/F	CML	(+)	(-)	PBSCT
8.AD	32/M	MM	(+)	(-)	PBSCT
9.VÇ	26/M	CML	(+)	(-)	PBSCT
10.SP	39/F	CML	(+)	(-)	PBSCT
11.AP	23/M	ALL	(+)	(-)	BMT
12.HT	23/M	AML	(+)	(-)	PBSCT
13.BK	21/M	MDS	(+)	(-)	PBSCT
14.DK	33/M	AML	(+)	(-)	PBSCT
15.SK	35/M	AML	(+)	(-)	PBSCT
16.NŞ	25/F	CML	(+)	(-)	PBSCT
17.EŞ	28/F	CML	(+)	(-)	PBSCT
18.HT	26/F	AML	(+)	(+)	PBSCT

CMV: Cytomegalovirus. **CML:** Chronic myeloid leukemia. **MM:** Multiple myeloma. **ALL:** Acute lymphoblastic leukemia. **AML:** Acute myeloid leukemia. **MDS:** Myelodysplastic syndromes. **PBSCT:** Peripheral blood stem cell transplantation. **BMT:** Bone marrow transplantation.

II- Quantitative detection of pp65 CMV-Ag by flow cytometry (FC)

CMV late antigen was identified by indirect staining of peripheral blood leukocytes (PBL) with a Moab reactive to CMV late (pp65) proteins (Clonab CMV, Biotest) [7,8]. CMV-Ag was detected both on the surface and the cytoplasm of PNLs.

Surface antigen detection:

100 µl of ETDA blood sample was incubated with either 10 µl of isotypic control or Clonab for 20 minutes, and then labeled with a FITC conjugated secondary antibody. Following an erythrocyte lysing procedure, fluorescent cells were collected and analyzed by a FacSort (BDIS, USA) using the Cell Quest software.

Cytoplasmic antigen detection:

For cytoplasmic staining, cells were incubated for 10 minutes in cell lysis solution (BDIS, USA) to lyse erythrocytes and obtain the permeabilization of leukocytes before the addition of Moabs. Following this initial procedure, the above-mentioned protocol was applied except for the second lysing step. For the evaluation of surface and cytoplasmic staining, isotype control tubes were used to set the markers. Cells reacting brighter than those in the control tube were evaluated as positive. Negative and positive controls were used for each assay to overcome the problem of false positivity and negativity.

TREATMENT

Decision of CMV infection was based on the presence of fever, and pulmonary (cough, infiltrates), hematological (myelosuppression) and hepatic (enzyme elevations) symptoms or findings, and CMV-Ag was detected by at least two methods. After fulfilling the above criteria, patients received gancyclovir 10 mg/kg/day for 21 days. Therapy duration was a minimum of 21 days and was further extended in cases with persistent antigenemia.

Table 2. Clinical characteristics of patients

Sex: F/M	5/13
Age: median (range)	32 (21-40)
Diagnosis: CML/AML/ALL/MM/MDS	11/4/1/1/1
Sources of Transplant: PBSCT/BMT	16* (89%)/2 (11%)
Frequency of CMV-Ag in PBSCT/BMT (%)	34/7**
Antigenemia Time From Transplant: Median (Months)	4.5 (2-24)
GVHD Accompanied: acute GVHD/chronic GVHD	6/10
Clinical Sings of Infection (+/-)	16/2
CMV-Positive: IFM (+)/ FC (+)	14/18***
Gancyclovir Treatment	16

*0.001, **p<0.008, ***p<0.0001. **CML:** Chronic myeloid leukemia. **AML:** Acute myeloid leukemia. **ALL:** Acute lymphoblastic leukemia. **MM:** Multiple myeloma. **MDS:** Myelodysplastic syndromes. **CMV:** Cytomegalovirus. **PBSCT:** Peripheral blood stem cell transplantation. **BMT:** Bone marrow transplantation. **GVHD:** Graft-versus-host disease. **IFM:** Immunofluorescence microscope. **FC:** Flow cytometry.

STATISTICAL ANALYSIS

Comparison of data was performed by the chi-square or Fischer's exact test. CMV laboratory tests were compared using the linear correlation coefficient. In all cases, the confidence interval (CI) was 95%. Values of $p < 0.05$ were considered to be significant.

RESULTS

In the flow cytometric analysis of BAL fluid, CMV-Ag was positive in four of 18 patients. The positivity of CMV-Ag was found at posttransplant 2nd – 24th months (median 4.5). Among the CMV-Ag–positive patients, 16 had undergone allo-PBSCT while two had undergone allo-BMT. In the allo-PBSCT group, CMV positivity was observed more frequently than in the allo-BMT group ($p<0.008$, Table 2). CMV-Ag was detected by FC in 18 patients and by IFM in 14 patients. IFM was not applicable in two patients. There was a correlation between the two methods ($r=0.619$, $p=0.0001$, Table 2). Clinical signs of infection were present in 16 of 18 patients with CMV-Ag positivity. All 16 patients who received gancyclovir for three weeks achieved clinical improvement and CMV-Ag was determined by either of the two methods. The two patients lacking signs of clinical infection did not receive therapeutic intervention. CMV-Ag was accompanied frequently by GVHD (acute GVHD/chronic GVHD: 6/10).

DISCUSSION

In this study, we observed an increased frequency of CMV-Ag among patients transplanted from peripheral blood compared to bone marrow. Another important finding was the coincidence of GVHD and CMV-Ag. GVHD and CMV infection are still major complications observed in patients following allogeneic transplantation. Delayed immune reconstitution may result in transplant-related mortality from infectious complications. The recovery of immunocompetency after allogeneic stem cell transplantation is a complex process, which is dependent on a large number of pre- and post-transplant factors. Recent studies have shown more rapid immune reconstitution after allo-PBSCT compared to allo-BMT. Among various explanations for the faster recovery, the high number of lymphocytes infused to the patients may be the most logical one [9-11]. Such a rapid immune recovery could indeed contribute to the apparent therapeutic advantage of PBSCT when compared with BMT. However, recent randomized trials have demonstrated an increased frequency of chronic GVHD following PBSCT [12,13]. Due to infusion of primed stem cells and mature myeloid cells, neutrophil and platelet reconstitution is uniformly reported to occur faster after allo-PBSCT than BMT [12,13]. Thus, neutropenia-related infections are observed less among PBSCT. PBCST allografts contain a three- to four–fold higher number of CD34+ cells, approximately eight-fold higher than both lymphoid

subsets, and NK cells when mobilized with G-CSF [14]. Despite the fact that a higher number of lymphocytes are infused following allo-PBSCT, B cells, CD4+ lymphocytes (reversed CD4/CD8 ratio) and NK cells remain low in number during the first year persistently. Contrary to common qualitative deficiencies encountered, analysis of T cell activation was shown to be normal in one study [15,16]. The balance between quantitative and qualitative analysis is kept normal and results in NK deficiency [11,16].

In animal models and in clinical studies, G-CSF induces T cells to produce interleukin–4 (IL-4) and IL–10, rather than IL–2 and IL-gamma. IL–4 and IL–10 (the profile of type 2 helper T cells) have been shown to down-regulate inflammatory responses, including those involved in GVHD [10,11,16].

Mobilization of stem cells by rhG-CSF blocks the ability of donor monocytes and dendritic cells to produce IL–12, which plays a pivotal role in the initiation of protective Th1 immunity against bacteria, viruses, and fungi. Antigen presentation in the absence of IL–12 promotes Th2 responses, thereby exerting a strong immunosuppressive effect in recipients of stem cell transplant [11]. All of those factors establish a compromised immune defense and may result in increased incidence of viral infections. The association between GVHD and CMV-Ag has been very well documented. Thus, following PBSCT, despite faster immune reconstitution, a protective factor, increased frequency of GVHD, a susceptibility factor, combat against each other.

Pre-emptive antiviral therapy based on the early detection of CMV infection is an important strategy for the prevention of CMV disease following allo-BMT. Recent studies have shown that the intensity of viral load is correlated with the occurrence of CMV-related symptoms. Methods for the detection of CMV infection include antibody serology, viral culture, modified rapid viral culture (shell vial and related techniques) from various body fluids (blood, urine, BAL), detection of CMV antigens in blood (pp65 antigenemia assay), and qualitative and quantitative CMV genome detection [4,17,18].

The humoral and cytotoxic T lymphocyte responses to CMV are directed predominantly against glycoprotein B and the structural phosphoprotein pp65. The dominant target antigen was found to be the major late CMV matrix protein pp65 [7,19]. This pp65 antigenemia is one of the most widespread methods for quantifying CMV viral load, but the conventional procedures used, indirect immunofluorescence and quantification by fluorescence microscopy, are relatively time-consuming, and the interpretation of slides involves a subjective element [7].

Flow cytometry was used to detect a CMV-specific antigen (pp65) in CMV-infected fibroblast cells and in leukocytes of renal transplant recipients. FC gave positive results when the number of antigen-positive cells was five or more per 5×10^4. Moreover, the percentage of antigen-positive cells by FC correlated well with symptomatic CMV infections [8,20].

During active infection, CMV disseminates in the blood, and viremia has been described as a major risk factor for the progression to clinical disease, particularly in allo-BMT recipients [21]. Accordingly, quantification of the viral load in persistently infected hosts may provide a method to predict the development of CMV disease and help to differentiate symptomatic infection from asymptomatic shedding. Preventive strategies increasingly use CMV load as a surrogate marker for disease and initiate antiviral treatment based on the systemic viral load. Sensitive techniques, such as the pp65 antigenemia assay or the quantitative PCR assay, allow the detection and quantification of systemic CMV. Both assays provide a good estimation of the systemic CMV burden. Owing to the high sensitivity of these assays, CMV is also detectable in patients with asymptomatic infections. However, patients with symptomatic infections often have a higher viral load and can therefore be discriminated [21,22].

The pp65 antigenemia assay determines the systemic CMV load and consists of direct staining of PMNL with monoclonal antibodies against the lower matrix protein pp65 [5,23]. This determination has classically been made by very difficult and time-consuming microscopic observation of immunostained cells [23]. Recent studies have evaluated FC for the direct detection and quantification of CMV antigens in

PMNL from transplant recipients [7,24]. A correlation was found between the degree of DNA load and the level of antigenemia detected by FC in renal transplant recipients. Although the sensitivity of the method was somewhat lower than that of the slide method, it might be sufficient to predict the disease. Honda et al. [25] also identified specific CMV-infected cell populations in peripheral blood lymphocytes from CMV-infected patients by FC. Using monoclonal antibodies directed against immediate-early CMV antigen or against several cell membrane markers to phenotype-infected peripheral blood cells from BMT recipients, these authors developed a rapid and quantitative FC method for the detection of immediate-early CMV antigen. The detection of CMV antigens specifically in the PMNL from transplanted patients with CMV pneumonia suggests that the FC antigenemia assay would be useful for predicting CMV-associated disease in transplant recipients [25].

Detrick et al. [24] found that FC identification of CMV-Ag correlates with isolation of CMV in HSCT patients and may be a predictive test for the rapid detection of CMV in the blood.

CONCLUSIONS

In this study, we were able to detect CMV-Ag by two techniques. FC identification of CMV-Ag correlated with clinical findings in HSCT patients. Although not as sensitive or specific as PCR, this technique may be a predictive test for the rapid detection of CMV infected cells in the blood. In conclusion, these two methods can be used in parallel to confirm CMV–Ag, and it must be kept in mind that FC evaluation is more sensitive to over- diagnose CMV-Ag. Treatment decision must be based on both clinical findings and laboratory confirmation. Another important finding is the more frequent occurrence of CMV-Ag in PBSCT compared to BMT.

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