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Full Length Research Paper

Investigatıon of Epstein-Barr virus serology and DNA in bone marrow transplant recipients

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The aim of this study was to investigate Epstein-Barr virus (EBV) serology and EBV DNA in the patients with bone marrow transplantation. In the current study, 128 patients with bone marrow transplantation (BMT) were included in Hematology-Oncology Department of Medical Faculty, Erciyes University between June 2005 and October 2009. In order to evaluate EBV serology and DNA, 357 samples obtained from the patients were studied with enzyme-linked immunosorbent assay and real-time polymerase chain reaction (PCR). The components of EBV serology were anti-Ebstein-Barr nuclear antigen (EBNA)-1 IgG, anti-viral capsid antigen (VCA) IgG and IgM. EBV seropositivity was found in 98.4% of the samples. EBV DNA positivity was detected in 13.3% of the patients. High viral load was only seen in one patient among all the EBV DNA positivity. No patient showed any symptoms regarding EBV virus and admitted with post-transplant lymphoproliferative disease. In conclusion, for the diagnosis and follow up of EBV infections in patients with BMT, detection of EBV DNA by PCR method is also beneficial in addition to serological tests regarding EBV.

Key words: Epstein-Barr virus, serological markers, real-time PCR, bone marrow transplant recipients.

INTRODUCTION

Epstein-Barr virus (EBV) is a member of Herpesviridae family. It place in Gammaherpesvirinae subfamily of Lymphocryptovirus genus. EBV infects nearly all the adults population worldwide and remains lifelong as a persistant infections in human body same as other herpes viruses. It enters the body via oropharynx (Epstein and Crawford, 2005) and spreads out the whole body by infecting B cells after multiplying in the epithelial cells. Although, it mainly spreads by person to person an intimate contact but it is also transmitted by blood products, transplantation and sexual relationship (Hess, 2004). EBV can also cause certain diseases which is highly morbid and mortal in the immunocompromised patients. The spectrum of the disease can vary from an asymptomatic infection resembling in infectious mononucleosis to the lymphoma with malign B cell (Lennette, 1995; Linde and Falk, 2007). It is known that the EBV infection is associated with some malignancies such as a post-transplant lymphoproliferative disease (PTLD) in the recipients (Epstein and Crawford, 2005).

Cancer risk at the bone marrow transplant (BMT) recipients is 4 to 7 times higher than general population. There is a serious immune deficiency in the first year after the transplantation. PTLD is seen mostly in this period, especially in the first 5 months. The mean PTLD incidence in allogeneic BMT recipients is 1%. After the first year, the incidence is lower than 5 cases of 10.000 patients per year (Wagner et al., 2002a). EBV infection is one of the main risk factors leading to PTLD (Wagner et al., 2002b). Virus isolation, immunohistochemical methods, serological and molecular tests are used for the diagnosis of EBV infection. Any diagnostic method used in the immunosuppressed patients must detect the EBV replication early and have a high predictive value to make a preemptive treatment possible (Gärtner et al., 2000). Serological tests include EBV specific tests which are commented variously in addition to nonspecific tests such

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Table 1. The demographical features of the patients.

The characteristics of the patients	n (%)
Age (mean ± SD years)	32.7 ± 13.2 (min15-max69)
Sex	
Male	78 (60.9)
Female	50 (39.1)
Transplantation type	
Autologous BMT	32 (25)
Allogeneic BMT	96 (75)
Diagnosis of patients before transplantation	
Hodgkin disease	13 (10.2)
Non-Hodgkin disease	3 (2.3)
Leucemia	78 (60.9)
Myeloma	15 (11.7)
Bone marrow failure	17 (13.3)
Autoimmune disease	1 (0.8)
Solid tumor	1 (0.8)

*BMT; Bone marrow transplantation.

as a heterophile antibody test (Hess, 2004). Serological tests for the early diagnose of EBV infection in the transplant recipients remain insufficient on its own due to delayed seroconversion (Sato et al., 2008).

Quantitative tests measuring the EB viral load should be repetitive, sensitive and appropriate for routine procedures (Clementi, 2000). Tests based on polymerase chain reaction (PCR) are valuable for the assessment of EB viral load in peripheral blood following BMT. These tests are also benefical for the prediction and the diagnosis of PTLD, in addition to following up the reaction to treatment (Wagner et al., 2002a). The aim of this study was to investigate the serological status of EBV and EBV DNA in the BMT recipients.

MATERIALS AND METHODS

In the present study, 128 patients with BMT at University of Erciyes, School of Medicine, Department of Hematology-Oncology, between June 2005 and October 2009, were investigated retrospectively. Ninety Six patients had allogeneic BMT and 32 patients were autologous BMT. EBV serological markers of the patients and allogeneic BMT donors (anti-Ebstein-Barr nuclear antigen (EBNA)-1 IgG, anti-viral capsid antigen (VCA) IgG and IgM) were studied with enzyme-linked immunosorbent assay (ELISA) (Euroimmun, Lubeck, Germany) according to manufacturer's instructions in the sera. While EBV serological markers were determined at all the patients before the transplantation, but in some patients after the transplantation. EBV DNA levels were investigated in the Department of Microbiology, Laboratory of Virology, University of Erciyes, School of Medicine.

Blood samples were taken with 2 ml ethylene diamine tetraacetic acid to search for EBV DNA from all the patients. The plasma were separated by a centrifuge and kept in -70°C until the time of study. DNA was extracted from plasma by using EZ1, DSP virus kit (Qiagen, Germany) according to manufacturer's instructions. EBV DNA was studied with real-time polymerase chain reaction (PCR) by using EBV RG PCR kit (Qiagen, Germany). Extracted 10 µl DNA was added to the media which had 15 µl reaction mixture. Then, about 0.25 µl internal control was supplemented to the reaction mixture. PCR was studied as follows: 1 cycle of 95°C for 10 min, and 45 cycles of 95°C for 15 s, 55°C for 30 s and 72°C for 20 s. The amplification procedure was performed with Rotor-gene 3000 (Germany). The quantitation range of real-time PCR test was 5×10^6 to 5×10^3 copy/ml and the analytical determination limit of the test was 1×10^2 copy/ml. Results were reported as $<1\times10^2$ copy/ml in the event of no amplification.

RESULTS

One hundred and twenty eight patients were included in this study. The characteristic features of all the subjects were showed in table 1. In 121 (94.5%) out of 128 patients, EBV antibodies in the period of pretransplantation were found as follows: anti-EBNA–1 IgG (+), VCA IgG (+) and VCA IgM (-). In 4 patients (3.1%), these antibodies were found as follows: EBNA–1 IgG (-), VCA IgG (+), VCA IgM (-); and in 1 patient (0.8%), they were found as follows: EBNA–1 IgG (+), VCA IgG (+), VCA IgM (weak positive). Euroline EBV profile 2 test (Euroimmun, Germany) was studied in the serum of the patient who had VCA IgM (weak positive) and the existence of a past infection was detected. EBV serological markers were found negative in 2 patients (1.6%) out of 128 patients pretransplantation. One of the patients had allogeneic BMT and the other one had autologous BMT. The serological markers of autologous BMT patient turned into positive following the transplantation, but EBV DNA remained negative. Both serological markers and EBV DNA of the other patient

Table 2. The EBV serology, DNA and clinical data of the patients.

	EBV Serology n (%)	EBV DNA n (%)	PTLD n (%)	Symptoms-linked to EBV n (%)
Positive	126 (98.4)	17 (13.3)	-	-
Negative	2 (1.6)	111 (86.7)	128 (100)	128 (100)
Total	128 (100)	128 (100)	128 (100)	128 (100)

*PTLD; Post-Transplant Lymphoproliferative Disease

who underwent allogeneic BMT from a seropositive donor were found negative in the period of post-transplantation. Out of the patients whose EBNA–1 IgG and VCA IgG were positive pretransplantation, 2 (1.6%) had EBNA–1 IgG (-) and other 2 (1.6%) had VCA IgG (-) post-transplantation.

EBV DNA levels were studied on total 357 samples (1 to 12 sample(s) from each patient, mean 2.8±2.5 samples) between 15th days and 22nd months post-transplantation. The samples were taken from lung tissue (1), pleural fluid (1), bronchoalveolar lavage (3) and blood (123). The data concerning to the clinical status of the patients were gained from patient's files and by the clinics being followed up. EBV DNA was found in at least one sample of 17 (13.3%) out of 128 patients who were included in the study. All the patients that EBV DNA was found positive were also seropositive. EBV DNA was found positive in 22 (33.8%) out of the total 65 samples taken from 17 patients (1 to 9 sample(s) from each patient, mean 4 samples). Three samples were BAL and 19 samples were plasma. The patients were classified in 2 groups according to their viral loads. Group 1: The patients who had a low viral load: those in which their plasma DNA were permanently below $1x10^4$ copy/ml. Group 2: The patients who had a high viral load: those their plasma DNA were $1x10^4$ copy/ml and over for at least one time. EB viral load was found high in only one patient (5.9%). Others (94.1%) had a low viral load. The first detection time for EBV DNA following the transplantation ranged from 15 days to 1 year in 16 patients. EB viral load positivity was first seen after 22 months only in one patient. In this study, none of the patients developed PTLD and the symptoms in terms of EBV (infectious mononucleosis, malign lymphoma and etc.) were not detected. The EBV serology, DNA and clinical data of the patients were showed in table 2.

DISCUSSION

EBV serology in transplant recipients should be gained before the transplantation. This is because, seronegativity is a risk factor for this infection (Linde and Falk, 2007; Suzuki et al., 2007). EBV seroprevalence in western societies can be as high as 95% among adult people (Rickinson and Kieff, 2001). Ozkan et al. (2003) reported that seropositivity in Turkey was 99.4%. In our study, seropositivity was found to be 98.4%. Since EBV maintains lifelong latent following primary infection, it can be reactive in seropositive patients from time to time. Lim et al. (2006) announced that intense immunosuppression could reactivate EBV. Juvonen et al. (2007) reported that EBV reactivation was common in allogeneic BMT, but no need to treat for viremia in the majority of the individuals. They also emphasized that the development of acute graft-versus-host disease and anti-thymocyte globulin therapy were risk factors for EBV viremia. In the other study, it was reported that the reactivation following allogeneic BMT was an indicator of the cellular defensive immune response of the host (van Esser et al., 2001).

In the studies, it was revealed that the serological tests used for indicating EBV reactivation could be affected from various factors such as hypogammaglobulinemia and impairment of immunoglobulin synthesis which has been seen in the patients who had solid organ transplantation (Goldfarb et al., 2001; Coralesv et al., 2000). Wagner et al. (2002b) pointed out that the defect of immunoglobulin synthesis would lead to lower EBV reactivation than expected in the patients who had a high EB viral load. Gärtner et al. (2000) stressed that even gold standart methods in the serological diagnosis of reactivation remained insufficient in terms of correlation with EBV load in immunosuppressed individuals. In another study, it was explained that other tests except serology such as EB viral load measurement would be needed in order to evaluate EBV reactivation (Gärtner et al., 2001). Wagner et al. (2002b) reported that the measurement of the EB viral load was advisable instead of serological tests in order to follow up immunosuppressed patients who were under the risk of PTLD development. In a study, it was showed that subclinic EBV reactivation was seen frequently after allogeneic BMT and it was emphasized that EBV DNA quantitation seemed useful in order to detect the patients who were under the risk of EBV-lymphoproliferative disease development (van Esser et al., 2001). Seropositivity was found high in the current patients and EBV reactivation was important.

Many patients might have abnormal cell subpopulation for a long time following BMT. The increase of EBV antibodies can depend on immunoglobulin therapy. It can lead to changes in serological results (Wagner et al., 2002a). Collins et al. (2001) stressed that seroconversion was an indicator of a good immune system with regard to recovery from PTLD. In this study,

EBV seroconversion was seen only in one seronegative

patient after 45 days following transplantation. But EBV DNA positivity and the symptom-linked to EBV were not detected at all. EBNA–1 IgG antibodies occur in late period in the EBV infection (Lennette, 1995; Bauer, 2001). Under normal circumstances, they remain in body for lifespan, but sometimes the positivity of EBNA–1 IgG antibody cannot be seen through a whole life after the primary infection. Some people cannot form obviously the antibody or the antibodies, previously being formed, might be lost due to the various factors such as an immunosuppression (Bauer, 2001; Bauer, 1995). It is suggested to test more advanced tests such as avidity, Western Blot analysis or PCR in the case of EBNA–1 IgG (-), VCA IgG (+), VCA IgM (-) (Hess, 2004). In this report, VCA IgG was positive, VCA IgM was negative and no EBNA–1 IgG antibody was detected at the serological profiles of 4 patients.

VCA IgM antibodies can remain positive even after the formation of EBNA–1 antibodies in some individuals (Linde and Falk, 2007; Bauer, 2001). In this study, only a patient had seropositive EBNA–1 IgG and VCA IgG and weak positive VCA IgM. Confirmation test showed the patient to have had a past infection of EBV. Antibody production and maintenance can show impairments in immunosuppressed patients (Gärtner et al., 2000). In this study, before transplantation, two patients had EBNA–1 IgG antibody and the other two patients had VCA IgG antibody. However, after transplantation all the patients had negative in aspect of VCA IgG and EBNA–1 IgG antibodies. The patients who had BMT have a prolonged humoral failure following transplantation (Wagner et al., 2002a). Immunoglobulins used for the therapy may change the antibody results. Even quantitative EBV serological patterns have a limited usage due to their highly variable features. The same serological profile can be seen in the event of primary and past infection (Gärtner et al., 2000; Bauer, 2001; Lennette, 1995). Due to the reasons stated earlier, it is not safe and it is highly difficult to interpret EBV serology in transplantation recipients (Wagner et al., 2002a). EBV DNA can be a useful test for reaching a diagnosis in the event of serological uncertainty (Bauer et al., 2005).

Although, EBV is detected in the serum or plasma of the patients with immune deficiency, the symptoms which are associated with EBV cannot be seen (Linde and Falk, 2007). In a study, it was revealed that elevated viral load more than 4×10^3 EBV-copy/µg peripheral blood mononuclear cells DNA, but no PTLD was seen in some recipients following the transplantation (Wagner et al., 2001). Wagner et al. (2002a) stressed that these results possibly reflected immune response to EBV; therefore, no treatment was needed and this situation would greatly help PTLD management. In one study, it was reported that immunosuppressive treatment could elevate the number of B cells which was induced by EBV in peripheral blood. It was also reported that no PTLD was developed in the patients, although the highest viral load was found 2×10^4 copy/10^6 peripheral blood leukocyte (Zawilinska et al., 2008).

In other study, it was reported that although the mechanisms inducing persistant EBV DNA elevation were not clear yet, immunosuppression could be related to this situation (Suzuki et al., 2007). Tysarowski et al. (2007) stressed that elavated viral load in transplantation recipients did not indicate PTLD all the time and the intepretation of high EB viral load was important in the immunosuppressive patients who have no symptom. In our study, it was detected that EB viral load was found 10^4 copy/ml only in one (5.9%) out of 17 patients who were EBV DNA positive. PTLD and the symptoms linked to EBV did not develop in spite of the elevated viral load.

In one study carried out for renal transplantation recipients, it was emphasized that the cause of low EB viral load in peripheral blood was unclear. It was also reported that prophylactic antiviral treatment would be responsible from this situation or there could be some inconsistencies between virus load in peripheral blood and local lesions such as lymph nodes enabling EBV to live (Sato et al., 2008). Aalto et al. (2007) underlined that low viral load frequently occurred in the serum samples after the BMT and this situation could improve spontaneously without managing a specific treatment. But at the same time, they emphasized that high viral load (that is, $>5\times10^4$ copy/ml) was diagnostic for PTLD and it could not improve spontaneously, so it should be treated promptly. In other study, it was showed that EBV DNA which was detected in the plasma of some patients could originate from the replication of EBV-like virus particules or it could release from EBV infected cells which were exposed to apoptosis or necrosis in peripheral blood or lymphoid tissue (Wagner et al., 2002b). Suzuki et al. (2007) pointed out that a strong immunosuppression could be an important factor in the elevation of EBV DNA load. In this study, it was detected that 16 (94.1%) out of 17 EBV DNA positive patient had low viral load posttransplantation. But no clinical finding was associated with this situation. This result is also similar to the result of the study Wagner et al. (2002b) carried out.

In conclusion, for the diagnosis and follow up of EBV infections in patients with BMT, detection of EBV DNA by PCR method is also beneficial in addition to serologic tests regarding EBV.

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