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# A Correlative Study on Epstein Barr Virus Load Determined by Real Time Pcr with Cytokine Profiles in Pediatric Post Transplant Patients

Microbiology

KEYWORDS : Past Transplant, Lympho Proliferative disorder, Infections, Monorilielosis, Cytokine, Real Time PCR

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## ABSTRACT

*OBJECTIVES: Present study was undertaken to determine the load of EBV and the expression of anti-viral cytokines in response to EBV infection among children after liver transplantation.*

*METHODS: Thirty blood samples were obtained from patients with suspected EBV infection and these patients were categorized into three groups as Group I: Liver transplant patients (n=10), group II: EBV induced Infectious mononucleosis (n=10), Group III: Other patients with no EBV (n=10). Peripheral blood specimens collected from renal transplant recipients and others were subjected to detection of antibodies to EBV, presence of EBV by reverse transcriptase PCR targeting Viral Capsid Antigen (VCA) gene and determination of viral load by Real Time PCR. Using a multi-analyte ELISA array, we determined the Plasma levels of 11 anti-viral cytokines and correlated with EBV infection and PTLD status.*

*RESULTS: Antibodies to EBV were detected by ELISA in 21 (70%) peripheral blood samples and genomic EBV was detected in 15 (50%) by RTPCR. In 6 (20%) patients, EBV antibodies only were detected and RTPCR did not reveal the presence of EBV in these specimens. Mean and peak EBV levels were significantly higher in liver transplant recipients than patients with EBV induced IM like syndrome. One patient with persistent EBV developed PTLD with high levels of IL-2, IL-1B, IL-17 and IL-10 in plasma. The IL-6 and IL-2 levels were significantly higher in post transplant patient with EBV infection than those in EBV induced IM patients and control population/ NEBV. Levels of IL-1b, IL-6, IL-2, IL-17A and IFN-G were also significantly higher in post transplant and IM patients than NEBV.*

*Conclusion: EBV DNA levels were significantly higher during post transplant patients than IM. Viral RNA or EBV viral load were not detected in control population. The levels of IL-1b, IL-6 and IL-17a were significantly high in post transplant patients than in IM patients and control population. Levels of IL-6 and IL-17a were significantly higher at high EBV DNA levels observed during PTLD and acute IM. These results demonstrate an important role of IL-6 and IL-17a during acute EBV infection and suggest that these cytokines together with anti-EBV antibody control EBV proliferation of during acute EBV infection in IM and post transplant patients and likely preventing PTLD.*

**Introduction:**

EBV infects over 95% of the United States population.(1) Most infections occur in childhood and are asymptomatic; infection of adolescents and young adults with EBV often results in infectious mononucleosis. EBV is associated with a spectrum of lymphoproliferative diseases in patients with congenital or acquired immunodeficiency. Post-transplant lymphoproliferative disorders (PTLD) comprise a range of lymphoid tumors divisible into several clinicopathological categories (2, 3). Most PTLDs are of B cell origin and contain the Epstein–Barr virus (EBV), which is considered to be a major cofactor for their development (4, 5).

EBV has evolved mechanisms utilizing its viral proteins to modulate host cytokine network in favor of its infection. First, it has been suggested that cytokines may serve to potentiate a host defense mechanism directed toward the eradication of virus. Infection Second, cytokines play a cardinal role in the pathophysiology of EBV-associated (6). The cytokine induction contributes to evade immune system and stimulates cell proliferation of specific immune cells.

DNA identification of genes transcribed late during the infectious cycle of herpesviruses was used to indicate herpesvirus active infection (7). EBV active infection leads to activation of mammalian cells and production of numerous cytokines and chemokines, which can induce further cell activation and cytokine production in a complex system of regulation and cross-regulation (8,9). Several researchers have developed Reverse Transcriptase based Real Time PCR [15-24], assays for detection and quantitation of EBV RNA (10-13). Information is still sparse on EBV in PTLD, and the cytokine production in such condition needs to be further elucidated. Therefore, this study the present study was undertaken to investigate the EBV load and its association with cytokine profile in post transplant pediatric patients.

**Materials and Methods**

**Patients and clinical specimens:**

The present study was approved by Ethics Committee of both the Research Institutes and informed consent was obtained from the patients who were enrolled in the study. The study subjects were categorized into three groups as Group I: Liver transplant patients, Group II: Patients with suspected Infectious mononucleosis, Group III: Other patients. Thirty peripheral blood specimens collected from liver transplant recipients (n = 10), Infectious mononucleosis patients, IMEBV (n=10) and others including healthy control individuals with no EBV infection NEBV (n = 10) were subjected to detection of antibodies to EBV, Real Time PCR and EBV induced innate immune response study consisting of estimation of Plasma levels of 11 anti-viral cytokines using multi analyte ELISA array.

**RNA extraction:**

RNA was extracted from the peripheral blood specimens using RNA extraction kit (QIAGEN, Germany) according to the manufacturer's instructions. In brief, 280 µl of QIAGEN lysis buffer (AVL) was pipette into the bottom of a 1.5ml microfuge tube (DEPC treated) and 2.8 µl of carrier RNA was added. To this, 70 µl of the clinical specimen was added to the microfuge tube and mixed by pulse vortexing for 15 seconds. The specimen was incubated at room temperature for 15 minutes. 280 µl of ethanol (96-100%) was added to the sample and mixed by gentle pipetting for 15seconds. The mixture was carefully applied to the QIAamp mini spin column (in a 2ml collecting tube) without wetting the rim and centrifuged at 8000 rpm for 1 minute. The QIAamp mini spin column was placed in a clean 2 ml collect-

ing tube and the tube containing the filtrate was discarded. 500 µl of buffer AW1 was added and centrifuged at 8000 rpm for 1 minute. After discarding the filtrate, 500 µl of buffer AW2 was added without wetting the rim and centrifuged at 14,000 rpm for 3 minutes. For elution, 50 µl of buffer AVE was added and incubated at room temperature for 1 minute, followed by centrifugation at 8,000 rpm for 1minute the eluted RNA was used to determine the presence of EBV by reverse transcriptase PCR targeting viral capsid antigen (VCA) gene and determination of viral load by real time PCR.

**Determination of viral load:**

The viral load was estimated in the peripheral blood specimens using a commercial kit. The artus EBV RG RT-PCR kit was used for the detection of EBV RNA using polymerase chain reaction on Rotor Gene Q (QIAGEN, Hilden, Germany). The amplification reaction was carried out following the manufacturer's instructions. Reverse transcription was carried out 50°C f or 30 minutes followed by initial denaturation at 95°C for 15 minutes followed by 50 cycles of initial denaturation at 95°C for 30 seconds, annealing at 50°C for 60 seconds and extension at72°C for 30 seconds. The viral load was expressed in IU/ml.

**Cytokine measurement in Serum samples:**

The Multi-Analyte ELISArray analyzes a panel of 11 pro-inflammatory cytokines using a conventional ELISA protocol all at once under uniform conditions. The immune mediators detected were classified in 4 categories: (1) proinflammatory mediators (IL-A, IL-1B, IL-6, IL-8), (2) type 1 cytokines (IL-2, IL-12, interferon [IFN]-γ, and tumor necrosis factor [TNF]–α), (3) type 2 cytokines (IL-4, IL-10), (4) T helper (Th) 17 cytokine (IL-17). Multi-Analyte ELISArray simultaneously profiles multiple cytokines / chemokines using simple ELISA protocol. The Multi-Analyte ELISArray plate layout consisted of twelve different eight-well strips. Each strip is coated with one capture antibody all in one 96-well micro titer plate. The Multi-Analyte ELISArray consisted of 11 Antigen standards and detective antibodies & samples are diluted with sample dilution buffer provided in the kit. The Multi-Analyte ELISArray method was performed as recommended by the manufacturer.

**Statistical methods:** Standard deviation, mean and Logistic regression analysis comparing the correlation between viral load and serum bilirubin, SGPT, Alkaline phosphatase, total protein were calculated using SPSS software version 14.0.

**Results**

Antibodies to EBV were detected in 21 (70%) out of 30 peripheral blood samples. Genomic EBV was detected in 15 (50%) by Reverse Transcriptase and also by Real Time PCR. EBV antibodies were alone detected in 6 (20%) patients by ELISA but EBV RNA was not detected by RTPCR. The comparative analysis of detection of EBV RNA among different groups is provided in Table 1.

**Table 1: Comparative analysis of detection of EBV RNA among different groups:**

| Groups | Genomic EBV Detected N=15 | Genomic EBV not detected N = 15 |
|---|---|---|
| Group I Liver transplant N= 10 | 7 (46.6%) | 3# (20%) |
| Group II IM patients N = `10 | 8 (53.3%) | 2@ (15.3%) |
| Group III Control samples N = `10 | 0 | 10* (66.6%) |

**Note: Antibodies to EBV was detected by third generation ELISA kit in 2l samples in which viral load was also detected**

**# EBV antibodies were not detected by ELISA in all 3 serum sample obtained from a liver transplant patients.**

**@ EBV antibodies alone were not detected by ELISA in 2 serum samples obtained from patients with infectious mononucleosis.**

*** EBV antibodies alone were detected by ELISA in six samples obtained from Healthy control population**

The distribution of viral load in Group I: Liver transplant, Group II: Patients with suspected IM is provided in Figure 1and 2 respectively. Mean and peak EBV levels were significantly higher in liver transplant recipients than patients with EBV induced IM like syndrome.

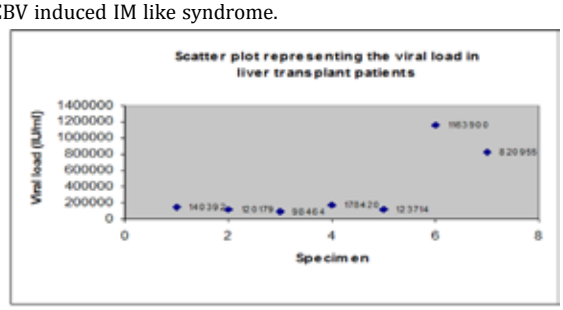


**Figure 1:** Scatter plot representing the viral load (IU/ml) in Liver transplant patients (Group I: n = 7).

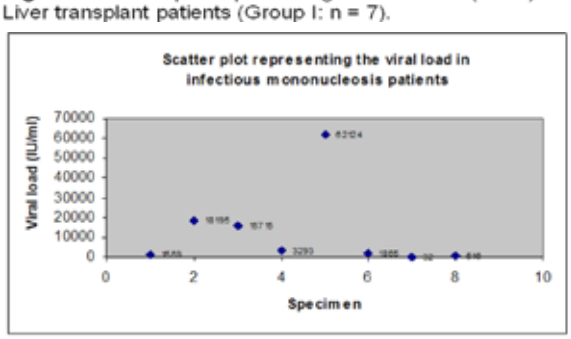


**Figure 2:** Scatter plot representing the viral load (IU/ml) in IM patients (Group II: n = 8).

The statistical analysis performed on of cytokine parameters with respect to diseased and control groups and Logistic regression analysis - Hosmer and Lemehow test performed on Cytokine expression in both EBV RNA positive and EBV RNA negative group using
T test is provided in Table 2.

**Table 2: Comparative analysis of the mean and standard deviation of cytokine parameters with respect to diseased and control groups and Logistic regression analysis - Hosmer and Lemehow test performed on Cytokine expression.**

| (A) category | (B) category | Mean Difference (A-B) | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound |
| Group 1 | Group 2 | .4373019 | .2248616 | .144 (NS) | -.117043 | .991647 |
| | Group 3 | 1.9448455* | .2248616 | .000 (S) | 1.390501 | 2.499190 |

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| | | | | | | |
|---|---|---|---|---|---|---|
| Group 2 | Group 1 | -.4373019 | .2248616 | .144 (NS) | -.991647 | .117043 |
| | Group 3 | 1.5075436* | .2248616 | .000 (S) | .953199 | 2.061889 |
| Group 3 | Group 1 | -1.9448455* | .2248616 | .000(S) | -2.499190 | -1.390501 |
| | Group 2 | -1.5075436* | .2248616 | .000(S) | -2.061889 | -.953199 |

*** *P* value < 0.05 statistically significant, NS: Not significant**

The IL-1b, IL-6 and IL-17A levels were significantly higher in post transplant patients than IM patients and NEBV. IFNg showed a similar trend, but not statistically significant. The IL-1b, IL-6 and IL-17A levels were significantly higher in EBV induced IM patients than NEBV but were significantly lower than those in transplant patients (Figure 3).

A logistic regression analysis was performed on cytokine elevation with EBV viral load. The inflammatory cytokines levels IL-6 and IL-17A were found to have a correlation with EBV viral load **(***P* value < 0.05 statistically significant) and all the other cytokines were found to have no correlation or statistical significance. Among 7 post transplant patients one patient with persistent EBV developed PTLD with high levels of IL-2, IL-1B, IL- 6, IL-17A, IL-10 and TNFA in serum.

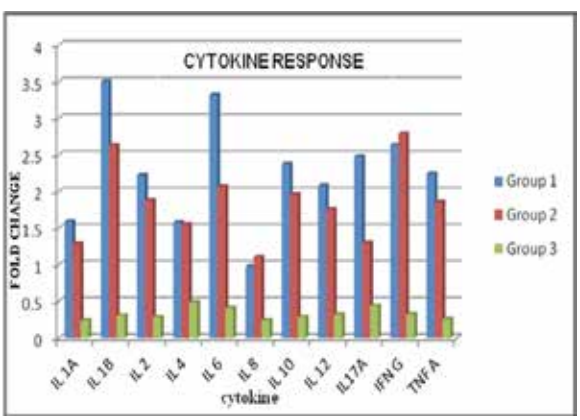


**Figure 3: Quatification of cytokines in serum samples collected from post transplant, infectious mononucleosis and healthy control population.**

**Discussion:**

The present study provides the evidence that EBV participate in the pathogenesis of PTLD. Assuming that the amount of virus and cytokine production is an important parameter when evaluating a role in pathogenesis, quantitative real-time PCRs were performed in the present study.

The difference in occurrence of EBV and cytokines between patients and control group was statistically significant. No EBV Luas detected in the control group, which is in agreement with previous studies reported by Sabeti et al (14 – 17).

Semiquantitative PCR analysis of EBV DNA with use of mononuclear cells is useful for the diagnosis of EBV-associated diseases (18, 19). Kimura et al. have quantified EBV DNA in mononuclear cells of patients with symptomatic EBV infections by real-time PCR and have indicated the usefulness of the method for monitoring virus load (20). EBV genome can be detected in cell-free samples of patients with posttransplantation lymphoproliferative disease or human immunodeficiency virus infection (21, 22). The amount of viral DNA seems to be a prognostic marker for chronic active EBV infection (23, 24).

The presence of viral RNA implies that active transcription occurs. This study clearly demonstrates the significance of active EBV infection (RNA expression) in key cytokine production. In this context, the comparative analysis in the result section exhibited a parallel marked increase in the cytokines and EBV expression in post transplant patients when compared with control population.

The elevation of IL-2 and IFN-g levels in serum samples of patients indicates the characteristic T-lymphocyte activation during active EBV infection in post transplant patients and patients with EBV induced infectious mononucleosis when compared to control population (25). In addition to the T-cell activation, we observed significantly larger amounts of TNF-a, IL-1b, and IL-6 in the serum sample patients compared with healthy controls. We interpret these findings as signs of increased activity of monocytes during primary EBV infection, although other leukocytes could, at least partly, be responsible for the secretion of these cytokines; e.g., IL-6 is also produced by activated TH2 cells. Interestingly, this elevated activity was not accompanied by an elevation of the absolute number of monocytes in the blood of the examined patients.

General cytokine effects of IL-6 and IL-1b are the induction of B-cell proliferation and differentiation (26). Furthermore, IL-6 enhances the differentiation of cytotoxic CD81 T lymphocytes in the presence of IL-2 or IFN-g (27, 28) and regulates Ig gene transcription in B lymphocytes (29). Both effects could be demonstrated during the immune response to EBV. Moreover, TNF-a, IL-1b, and IL-6 induce hypothalamic prostaglandin E2 synthesis and fever, and IL-1 and TNF-a initiate the release of acute-phase proteins. Therefore, these cytokines may be responsible for clinical symptoms during EBV infection. Gene transcription of all three cytokines was shown to be strongly enhanced in lymphoid tissue during acute EBV infection in transplant patients and infectious mononucleosis (30).

Collectively, our results confirm monocyte-derived factors such as TNF-a, IL-1b, and IL-6 may play an important role in the pathogenesis of primary EBV infection. Increased levels of IL-1b and IL-6 may further explain B-lymphocyte proliferation and differentiation, and the synthesis of unspecific antibodies. Selective regulation of cytokine synthesis by EBV may, on the other hand, favor different pathological proceedings, such as reactivation of other infectious agents. Knowledge about the pathogenic factors of PTLD and IM may help in the development prognostic markers and therapeutic strategies for treating chronic infectious mononucleosis EBV induced PTLD in immunocompromised post transplant patients.

**Conclusion:** EBV DNA levels were significantly higher during post transplant patients than IM. Viral RNA or EBV viral load were not detected in control population. The levels of IL-1b, IL-6 and IL-17a were significantly high in post transplant patients than in IM patients and control population. Levels of IL-6 and IL-17a were significantly higher at high EBV DNA levels observed during PTLD of acute IM. These results demonstrate an important role oif IL-6 and IL-17a during acute EBV infection and suggest that these cytokines together with anti-EBV antibody control EBV proliferation during acute EBV infection in IM and post transplant patients and likely preventing PTLD.

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