Original Research
Medical Journal of the Islamic Republic of Iran. Vol. 24, No. 3, November, 2010. pp. 115-125

Comparison of Epstein Barr virus antibodies and T cell cytokines production in patients with multiple sclerosis and healthy individuals

Bahareh Laribi[1], Mehdi Shekarabi[2], Amir Hassan Zarnani[3], Majid Ghaffarpour[4], Mojdeh Ghabaee[5], Samileh Nourbakhsh[6], Masuomeh Bakhshayesh[7]

Research Center of Immunology, Iran University of Medical Sciences, Tehran, Iran

Received:11 August 2009 Revised:15 July 2010 Accepted:10 August 2010

Abstract

Background: Multiple sclerosis (MS) is the most common autoimmune disease of central nervous system with destruction of myelin sheath mediated by auto reactive CD4+ T Lymphocytes. Because of the possible role of Epstein-Barr virus in etiology of MS and T cells immune response, the aim of this study was to evaluate anti-Epstein Barr virus antibodies as a marker of reactivity and production of TH1 and TH2 cytokines in MS patients and healthy individuals.

Methods: Blood samples were taken from 68 MS patients at different stages of diseases and 20 apparently healthy individuals and plasma levels of anti- EBV nuclear antigen-1 (EBNA-1) and viral capsid antigen (VCA) antibodies determined and concentrations of IFN-γ, IL-12 and IL-4 in culture supernatants of PHA-activated peripheral blood mononuclear cells (PBMC) were measured by ELISA.

Results: The mean levels of anti EBNA-1 and VCA antibodies were significantly higher in patients compared to controls (p=0.04, p=0.001 respectively). Concentrations of IFN-γ, IL-4 & IL-12 were also significantly higher in MS patients than healthy individuals (p=0.001, p=0.005, p=0.002, respectively). Significant correlation was found between anti EBNA-1 and VCA antibodies and IL-12 production (p =0.02, r=0.27& p=0.04, r=0.25, respectively); whereas no significant correlation was found between these antibodies and production of IFN-γ or IL-4.

Conclusions: Due to elevated level of anti-EBV antibodies and T cell Cytokines in MS patients Rather than healthy individuals, Epstein Barr virus may play role in etiology of MS disease through activation of T cells immune response.

Keywords: Multiple sclerosis, Epstein Barr virus, Anti- EBNA-1, Anti-VCA, Cytokine, TH1.

Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disorder of the central nervous system characterized by destruction of myelin [1]. The etiology and pathogenesis of MS though have not been fully defined [2] but infectious agents may play a role in this regard [1]. A central mission in MS research has been to determine the sequence of events underlying

1.**Corresponding author**, Master of Science of Clinical Immunology, Research Center of Immunology, Iran University of Medical Sciences, Tehran, Iran, P.O.Box: 14155-5983 Tel: +982122011622, Bahare.laribi@Gmail.com.
2. Associate Professor of immunology, Research Center of Immunology, Iran University of Medical Sciences, Tehran, Iran. Email: m_shekarabi@yahoo.com
3. Assistant Professor of Immunology, Research Center of Immunology, Iran University of Medical Sciences, Tehran, Iran. Email: A.H.zarnani@iums.ac.ir
4. Professor of Neurology, Center of Neurological Research, Imam Khomeini Hospital, Tehran University of Medical Sciences, Tehran, Iran. Email: ghafarpour@tums.ac.ir
5. Professor of Neurology, Center of Neurological Research, Imam Khomeini Hospital, Tehran University of Medical Sciences, Tehran, Iran. Email: ghabaeem@tums.ac.ir
6. Associate Professor, Hazrate Rasoul Hospital Iran University of Medical Sciences, Tehran, Iran. Email: samileh@iums.ac.ir
7. Master of Science of Clinical Parasitology, Iran University of Medical Sciences, Tehran, Iran. Email: m.bakhshayesh@yahoo.com

the development of the inflammatory plaque [3]. Multiple sclerosis are thought to be mediated by type 1 helper T cells (T_H1) that produce interferon-γ(IFN-γ), while CD_4+ type 2 helper T cells (T_H2) represent an anti-inflammatory population of lymphocyte that produces large amounts of IL-4 and IL-5[3]. Moreover, intrathecal syntheses of immunoglobulins targeted at myelin antigens are increased in patients with multiple sclerosis [3]. In recent studies for identification of IgG specificity in CSF of MS patients, two high affinity epitopes derived from Epstein-Barr virus (EBV) were identified as important auto antigens. In these patients immunoreactivity to viral antigens EBNA-1(EBV Nuclear Antigen-1) and BRRF2, were significantly higher in serum and CSF of MS patients than those of control donors. These findings demonstrate an increased immune response to EBV in MS patients, suggesting a causative role for the virus in the pathogenesis of the disease [4]. More importantly, the levels of anti-viral antibodies in patients are elevated before onset of symptoms of disease. According to Levin et al [5] serum, levels of IgG antibodies to VCA (viral capsid antigen) or EBNA-1 (EBV Nuclear Antigen-1) are elevated on average of four years before onset of disease [5]. In addition, there is an increased titer of anti-EBNA-1 antibody in CSF of MS patients [6]. Interestingly, two-pentapeptide sequence identity were found between EBNA-1 and myelin basic protein (MBP) [6] and increased frequency of CD8+ T cells reacting with these immunodominant epitopes from EBV in patients with MS may be based on the cross-reactivity of this epitopes with MBP [7]. The risk for MS is significantly increased after infectious mononucleosis, and is rare among individuals without anti-EBV antibodies in serum [8]. In addition, individuals who will develop disease exhibit an altered immune response against the EBV virus characterized by a high IgG activity to EBNA-1 and absence of high activity to VCA. Study in samples collected 5 years before onset of relapsing-remitting MS has shown that high activity to EBNA-1 significantly increased, and high VCA activity significantly decreased risk of MS [9].These findings demonstrate an increased immune response to virus in patients and suggest the important role of this virus in pathogenesis of disease. Because of the possible role EBV virus contibute in etiology and pathogenesis of MS, the aim of this study was to compare serum levels of anti-Epstein Barr virus antibodies as the serological markers of EBV infection and production of TH1 (IFN-γ and IL-12) and T_H2(IL-4) cytokines by peripheral blood mononuclear cells of MS patients and healthy individuals in order to assess the putative role of EBV virus in immune responses of MS patients and healthy individuals.

Methods

Patients: 68 patients with MS who had been referred to the neurology clinics of Imam Khomeini hospital in Tehran from Oct 2006 to Jan 2007 and 20 age-and sex-matched apparently healthy individuals were enrolled in this study. The patients diagnosed and neurologists determined their clinical stages through expanded disability status scale (EDSS). The EDSS of patients was assumed from 0 to 10 based on Kurtz EDSS. Informed consent was obtained from all subjects before the study began.

Specimens: 10 ml of blood was taken by venipuncture from each individual on the day of admission and blood samples divided into two 5 ml aliquots. The first aliquot was let to clot and then centrifuged at 500g for 10 min for serum collection. The second aliquot was admixed with heparin for PBMC isolation. Sera were kept frozen (-70 °C) until use.

Measurement of anti-EBV antibodies: serum levels of IgG antibodies to EBNA-1 and VCA were determined by ELISA (IBL®,Germany) according to manufacturer's instruction. The sensitivity of anti-EBNA-1 and anti-VCA measurement kits was 9 U/ml and 18 U/ml, re-

spectively.

Cell culture: PBMC was isolated from whole blood by standard Ficoll (Biochrom®,Germany) density centrifugation. After two wash steps in phosphate buffered saline (PBS, pH 7.2), cells were resuspended in RPMI 1640 (Sigma®, Germany) supplemented with 10% heat inactivated fetal bovine serum (Gibco®, Germany), 10mM HEPES buffer, 100U/ml of penicillin, 100µg/ml of streptomycin at a concentration of 106/ml. 1ml of cell suspension was then added to each well of a 24-well tissue culture plate (Grinner®, Germany). Phytohemagglutinin (PHA-L) (Sigma®, Germany) was added at the final concentration of 5µg/ml to the wells at the next step except for negative control wells. Plates were incubated for 3days at 37°C CO_2 incubator (95% air, 5% CO_2, 100% humidity).

Cytokine assay: Cell culture supernatants were harvested on 48, 72 and 96h for cytokine analysis. The preliminary data revealed that production of IFN-γ, IL-12 and IL-4 reached to highest level on the third day and hence subsequent measurements were carried out on supernatants collected on 72h. The concentration of each cytokine was measured by commercial kits (Bender Med®, Austria). The lower limits of detection for IFN-γ, IL-12 and IL-4 were 1.56, 31.25 and 7.8 pg/ml, respectively.

Data analysis: statistical analysis was performed using SPSS version 16 (Chicago, USA). Mann-Whitney and t-test were used to compare the variables between MS patients and control groups. Correlation between anti-EBV antibodies and cytokines was analyzed by Pearson test, and the level of $p<0.05$ was considered statistically significant.

Results

The study included blood samples from 68 patients (49 females and 19 males) referred to the neurology clinics of Imam Khomeini hospital and 20 healthy controls (10 females and 10 males). The average age of patients and control groups was 34.5±9.7 and 27±5 years, respectively. The clinical features of MS patients are summarized in Table 1. The MS patients group had average disease duration of 4.6±4.46 (range of lower 1 year to 25 years) and average baseline EDSS of 3.6±1.98 (0 to 8).

EBV seropositivity: VCA and EBNA-1 the markers of previous EBV infection, anti-VCA IgG and anti-EBNA-1 IgG were tested in all serum samples. The mean levels of anti-VCA and anti-EBNA-1 in MS patients (147 U/ml and 146 U/ml) were significantly higher than those of healthy individuals (89 U/ml and 113 U/ml)

Table 1. Clinical summary of MS patients

Clinical information	
Total individuals with MS (n)	68
Female / male ratio	2:1
Mean disease duration (years ± SD)	4.6±4
Disease course (n, %)	
[1]RR	4 (79.4)
[2]PP	4 (5.9)
[3]SP	(13.2)
[4]PR	1 (1.5)
[5]EDSS	
≤3	2 (61.8)
3.5-6	3 (26.5)
>6	(11.8)

RR: Relapsing-remitting MS, [2]PP: Primary-progressive MS, [3]SP: Secondary-progressive MS, [4]PR: Progressive-relapsing MS, [5](EDSS) expanded disability status scale.

(p=0.001, p=0.04 respectively, Fig.1).

Cytokine analysis: For evaluation of T_H1/T_H2 cytokine profiles in MS patients the IFN-γ, IL-4 and IL-12 productions by PHA-activated PBMC were measured by ELISA. The results showed that concentrations of IFN-γ, IL-4 and IL-12 were significantly higher in MS patients compared to healthy individuals (p=0.001, p=0.005 and p=0.002, respectively, Fig.2). Although ratios of IFN-γ/IL-4 and IL-12/IL-4 were higher in MS patients than healthy individuals, this difference was not statistically significant (p>0.05). Significant correlation was found between anti EBNA-1 and VCA antibodies and IL-12 production (p =0.02, r=0.27& p=0.04, r=0.25 respectively, Fig.3), whereas no significant correlation was found between these antibodies and production of IFN-γ and IL-4.

Discussion

This study confirms earlier reports that anti-EBV antibodies are positive in patients with multiple sclerosis [10]. Our results showed that mean levels of specific anti-EBNA-1 and VCA antibodies were significantly higher in MS patients than healthy individuals. Other groups [6-11, 12] reported similar results. In a prospective study, Levin et.al showed that serum levels of anti-EBNA-1 and VCA antibodies increased before onset of disease [5]. Therefore, it is likely that EBV has important role in pathogenesis of MS disease. To explore the causative role of cellular immune responses in pathogenesis of MS, we measured T_H1 and T_H2 cytokine production by mitogen-activated mononuclear cells. Our results showed that concentrations of IFN-γ, IL-4 & IL-12 released from PBMC of MS patients were significantly higher than these in control group (p=0.001, p=0.005 and p=0.002, respectively). Recent studies also indicated that production of IL-12 considerably increased in PBMC of MS patients. Ozenci et al. reported that even in the absence of high levels of IL-12, elevated expression of its receptor might be augmented in MS [15]. High levels of IL-12 in MS patients could be result of ongoing inflammatory immune responses and activation state of antigen presenting cells that produce this cytokine. This cytokine has the main role in differentiation of T lymphocytes to T_H1 phenotype and induces production of IFN-γ by these cells [15]. IFN-γ itself augments inflammatory responses. On the other hand, IFN-γ has synergic effect on IL-12-mediated TH1 profile differentiation and thus, increased levels of these cytokines in MS could explain predominance of TH1 immunity usually seen in this disease. Although our results showed a higher ratio of IFN-γ/IL-4 and IL-12/IL-4 in our MS blood samples compared to normal individuals, but

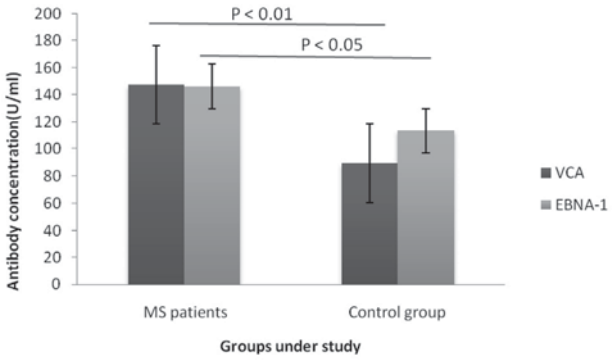

Fig.1. Mean serum level of anti-EBNA-1 and anti-VCA antibodies in MS patients and control groups. Bars indicate mean ± standard deviation.

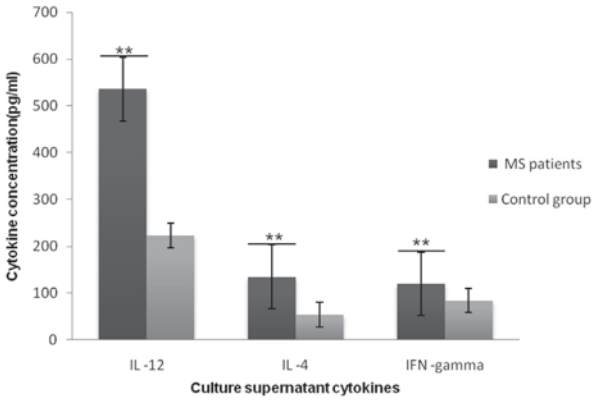

Fig.2. Mean levels of IL-12, IL-4 and IFN-γ productions by PHA-activated PBMC of MS patients and control groups. (Bars indicate mean ± standard deviation. (* P < 0.05, ** P < 0.01).

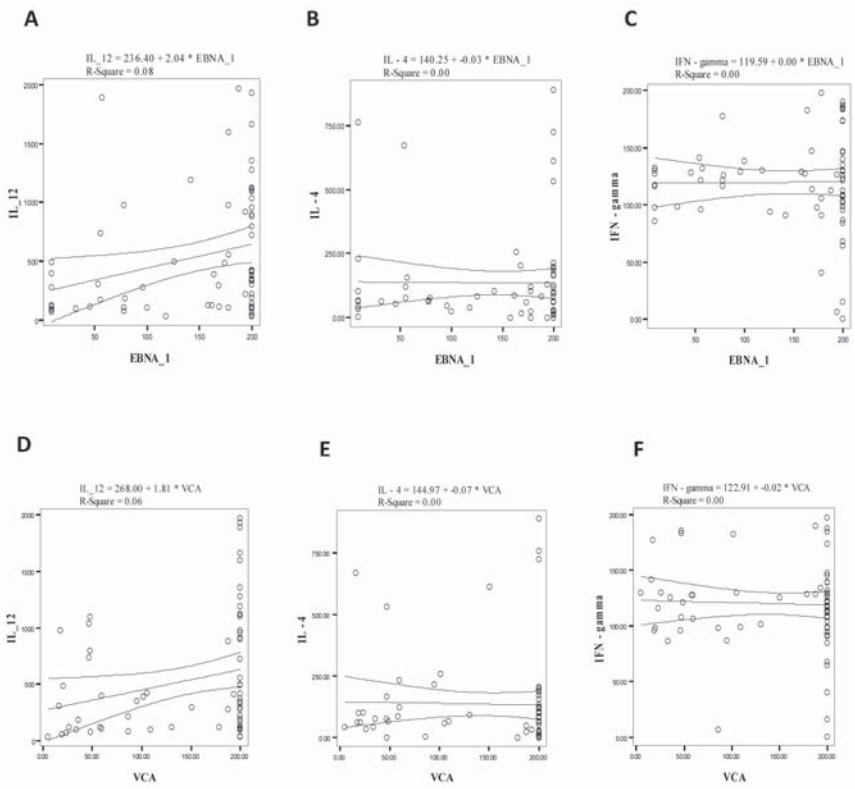

Fig. 3. Relationship between serum levels of anti-EBV antibodies and production of IFN-γ, IL-4 & IL-12 by PBMCs in MS patients. Statistically significant correlation was found between anti-EBNA-1 and VCA antibodies and IL-12 production (p =0.02; r=0.27 and p=0.04; r=0.25, respectively) (scatter plots of A and D), whereas no significant correlation found between antibodies and production of IFN-γ or IL-4.

this increase was not statistically significant ($p>0.05$). Kip et al showed that the percent of T cells producing IL-4 are significantly reduced in MS patients than control groups [16] which is in contradict with what presented here. Such increase in IL-4 production by mononuclear cells of MS patients is expected, since during inflammatory process of T_H1 immunity, elevated levels of T_H2 cytokines could counter balance the effects of T_H1 cells, which could be potentially harmful. Indeed, it is plausible that high levels of IL-4 may hinder B-lymphocytes from production of complement fixing IgG auto antibodies. We also showed that there was a significant correlation between increased concentrations of anti EBNA-1 and VCA specific antibodies and production of IL-12 by mononuclear cells ($p= 0.02$, $p= 0.04$ respectively). This correlation indicate that prior infection with EBV in MS patients could be a triggering factor for skewing immune response toward TH1 inflammatory immunity.

Conclusion

Because of elevated level of anti-EBV antibodies and significant correlation of these with production of IL-12 in MS patients rather than healthy individuals, it seems that Epstein Barr virus may play a role in etiology of Multiple sclerosis through stimulation of T_H1 inflammatory response.

Acknowledgement

The authors wish to express their deep gratitude to the neurologists who provided essential clinical information for the case analysis and for valuable support of the staff of immunology research center, IUMS. This work was supported in part by a grant from the Iran University of Medical Sciences.

References

1. Poser CM. The environment and the nervous system. J Neurol Sci. 2007; 15(262):98-9.

2. Laplaud DA, Confavreux C. Etiology of multiple sclerosis 2006;56:1306-12.

3. Elliot MF, Michael KR, Cedric SR. Multiple sclerosis-The plaque and its pathogenesis. N Engl J Med 2006; 354: 942-5.

4. Markus R, Michael K, Thomas B. Antibodies as biological markers for pathophysiological processes in MS. J Neuro Immunol 2006;180(1-2): 50-62.

5. Levin LI, Munger KL, Rubertone MV, et al. Multiple sclerosis and Epstein-Barr virus JAMA 2003; 289:1533-6.

6. Patrick F. Bray JL, Paul F, Bray KW, Clup BS, Schlight JP. Antibodies against Epstein-Barr nuclear antigen (EBNA) in multiple sclerosis CSF and two pentapeptide sequence identities between EBNA and myelin basic protein. Neurology 1992; 42:1798-1804.

7. Hollsberg P, Hansen H J, Haahr S. Altered CD8+ T cell responses to selected Epstein-Barr virus immunodominant epitopes in patients with multiple sclerosis. Clin Exp Immunol 2003; 132:137-143.

8. Holmoy T, Vartdal F. Cerebrospinal fluid T cells from multiple sclerosis patients recognize autologous Epstein-Barr virus-transformed B cells. J Neurovirol 2004; 10: 52-56.

9. Sundstrom P, Juto P, Wadell G, Hallmans G, Svenningsson A, Nystrom L, et al. An altered immune response to Epstein-Barr virus in multiple sclerosis: a prospective study. Neurology 2004; 62 (12): 2277-82.

10. Buljevac D, van Doornum GJ, Flach HZ, Groen J, Osterhaus AD, Hop W, van Doorn PA, van der Meche FG, Hintzen RQ Epstein-Barr virus and disease activity in multiple sclerosis. J Neurol Neurosurg Psychiatry 2005; 76(10): 1377-81.

11. Larsen PD, Bloomer LC, Bray PF. Epstein-Barr nuclear antigen and viral capsid antigen antibody titers in multiple sclerosis. Neurology 1985; 35: 435-438.

12. Wagner HJ, Hennig H, Jabs WJ, Siekhaus A, Wessel K, Wandinger Kp.Altered prevalence and reactivity of Epstein-Barr virus Antibodies in patients with multiple sclerosis. Viral immunol 2000; 13(4): 497-502.

13. Makhlouf K, Weiner HL, Khoury SJ.Increased percentage of IL-12+ Monocytes in the blood correlates with the presence of active MRI lesions in MS. J Neuroimmunol 2001; 119(1): 145-9.

14. Van Boxel-Dezaire AH, Hoff SC, Oosten BW, Verweij CL, Drager AM, van Houwelingen JC, et al. Decreased Interleukin (IL)-10 and increased IL-12 p40 mRNA are associated with disease activity and characterize different disease stages in multiple sclerosis. Ann Neural 1999; 45: 695-703.

15. Onzenci V, Pashenkov M, Kouwenhoven M, Rinaldi L, Soderstrom M. Link H. IL-12/IL-12R System in multiple sclerosis. Journal of Neuro immunol 2001; 242-252.

16. Kipp B, Bar-Or A, Gausling R, Oliveira EM, Fruhan SA, Stuart WH, Hafler DA. A novel population of B7-1+ T cells producing intracellular IL-4 is decreased in patients with multiple sclerosis. Eur J Immunol 2000; 30: 2092-2100.