Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disorder of the central nervous system characterized by 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 (r =0.2, r=0.27& r=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.
the development of the inflammatory plaque [3]. Multiple sclerosis are thought to be mediated by type 1 helper T cells (Th1) that produce interferon-γ (IFN-γ), while CD4+ type 2 helper T cells (Th2) 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 contribute 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 Th2 (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 3 days at 37°C CO₂ incubator (95% air, 5% CO₂, 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>
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<th>Table 1. Clinical summary of MS patients</th>
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<td>Clinical information</td>
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<td>Total individuals with MS (n)</td>
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<td>Mean disease duration (years ± SD)</td>
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¹RR: Relapsing-remitting MS, ²PP: Primary-progressive MS, ³SP: Secondary-progressive MS, ⁴PR: Progressive-relapsing MS, ⁵EDSS (Expanded disability status scale)
Cytokine analysis: For evaluation of Th1/Th2 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.1). 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 Th1 and Th2 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 Th1 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
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 TH1 immunity, elevated levels of TH2 cytokines could counter balance the effects of TH1 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 TH1 inflammatory response.

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Comparison of Epstein Barr virus

References