Journal of Neuroimmunology 285 (2015) 57–61

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

Gender influence in EBV antibody response in multiple sclerosis patients from Kuwait

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ARTICLE INFO

Article history:
Received 2 March 2015
Received in revised form 18 May 2015
Accepted 20 May 2015

Keywords:
Multiple sclerosis
Kuwait
Epstein–Barr virus
EBNA1
VCA
HLA-DRB1*1501

ABSTRACT

Background: Epstein–Barr virus (EBV) infection is implicated with multiple sclerosis (MS) risk, exacerbation, and progression. The HLA-DRB1*1501 haplotype is a strong MS risk factor consistently documented in MS populations. There are no studies of EBV infections and HLA-DRB1*1501 haplotype associating with MS from Kuwait where MS prevalence has increased significantly.
Objectives: To determine the association of EBV infection with MS incidence, and to investigate HLA-DRB1*1501 as a potential genetic risk factor for MS in Kuwait.
Methods: This is a case–control study involving 141 MS patients and 40 healthy controls. Antibody titers against EBV antigens' viral capsid antigen (VCA) and Epstein–Barr nuclear antigen 1 (EBNA1) were measured using enzyme-linked immunosorbent assays. HLA-DRB1*1501 haplotype assessment was done using rs3135005 TaqMan genotyping assay.
Results: Antibody titers against EBV were significantly elevated in MS patients compared to healthy controls (anti-EBNA1, $p = 0.008$; anti-VCA, $p = 0.028$). MS males had higher antibody titers to EBNA1 than healthy male controls ($p = 0.005$) and female MS patients ($p = 0.03$). HLA-DRB1*1501 haplotype genotypes failed to generate a risk association with MS or EBV antibody titers ($p = 0.6$).
Conclusion: An increased immune response to EBV infection is associated with MS incidence influenced by the type of antigen and sex. HLA-DRB1*1501 haplotype is not associated with MS risk in our Kuwaiti MS cohort.

1. Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disorder resulting from an autoimmune reaction against myelin and myelin associated antigens in the central nervous system (CNS), however the exact etiology remains unknown. Repeated demyelination events in the white matter result in subsequent neuro-axonal degeneration and oligodendrocyte cell death in the CNS manifesting primarily as lesions in the white matter of the brain and spinal cord causing perturbed central sensory and motor nerve conduction. MS incidence is 2:1 female:male ratio at a typical age of onset between 20–40 years of age (Liguori et al., 2000). MS is considered a multi-factorial complex disorder where genetic and environmental factors play a role in MS pathogenesis and relapse risk (Milo and Kahana, 2010). Kuwait has become a high-risk area for MS, its prevalence has increased from 4.4 in 1990 to 85 cases per 100,000 individuals in 2014 (Al-Din et al., 1990; Alroughani et al., 2014). One of the most consistently reported association with MS risk is the association of viral infections, specifically Epstein–Barr virus (EBV) infection (Owens and Bennett, 2012; Lucas and Taylor, 2012). EBV is a human gamma-herpes virus that specifically infects nasopharyngeal epithelial cells and resting B-lymphocytes. EBV has the ability to activate or persist in a latent phase within the cells of infected individuals throughout their lives. EBV can mimic the stimuli of antigens and T-cell receptors in activating naïve B-lymphocytes into antigen specific memory B-cells. As infected memory B-cells differentiate into plasma cells EBV switches to lytic reproductive phase to produce new EBV particles (Laichalk and Thorley-Lawson, 2005). Human immune response against flourishing EBV production is to eliminate production houses; mainly EBV-infected plasma cells, effectively via the action of cytotoxic $CD8^+$ T-cells (Hislop et al., 2007). EBV infection specificity to immune cells has resulted in its association with the incidence of several autoimmune disorders and malignancies in individuals predisposed to such disorders. Systemic lupus erythematosus, rheumatoid arthritis, Burkitt's lymphoma, and Hodgkin's lymphoma are examples of such disorders (Toussirot and Roudier, 2008; Saha and Robertson, 2011).

In recent years, the role of EBV infections as an MS risk factor preluding MS incidence in susceptible individuals has become a field of

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http://dx.doi.org/10.1016/j.jneuroim.2015.05.021
0165-5728/

interest in MS research (Owens and Bennett, 2012; Pender, 2011). Although EBV association with MS has become common knowledge in the field of MS research, no study has been reported from Kuwait investigating its association among Kuwaiti MS patients. Moreover, the most common known genetic risk factor associated with MS is the human leukocyte antigen (HLA) DRB1*1501 haplotype. HLA-DRB1*1501 has consistently proven its association in many case–control and familial genetic studies of MS risk and inheritance. (Hillert et al., 1994; Barcellos et al., 2006; Schmidt et al., 2007). However, the exact mechanism of how this haplotype increases susceptibility to MS is still unknown, but some evidence suggests its association in antigen detection, specifically EBV antigens. (Sundqvist et al., 2012; Kumar et al., 2014). Here we investigated the association of EBV antibody titers in Kuwaiti MS case–control study in relation to gender, antibody titers, HLA-DRB1*1501 genotype, infection stage, and other clinical variables pertinent to MS disease course.

2. Materials and methods

2.1. Patient and healthy control recruitment

Blood samples from 141 Kuwaiti MS patients and 40 healthy control individuals were collected. Patients were recruited at the MS clinic in the Dasman Diabetes Institute (DDI). Information of procedures to be performed was fully explained to patients prior to procurement of their informed consent in agreement with the Joint Committee for the Protection of Human Subjects at Kuwait's Health Sciences Center (HSC), and in agreement with the Ethical Review Committee (ERC) of DDI. The inclusion criteria were as follows: 1) Patients aged 14–60 years, 2) patients with clinically definite multiple sclerosis (CDMS) with clear clinical course (RRMS, SPMS, PPMS, Benign), 3) availability of a detailed clinical history (demographics, age of onset, disease duration, Expanded Disability Status Scale (EDSS) score, and treatments received), 4) being born in Kuwait and have resided in Kuwait from birth to at least early adult life, and 5) willingness to provide a blood sample. Exclusion criteria included patients who have Clinically Isolated Syndrome (CIS), possible MS (not yet confirmed) or other demyelinating disorders such as acute disseminated encephalomyelitis/neuromyelitis optica, and patients with incomplete data or those who are unable to provide informed consents. Progression index (PI) was computed for each patient to determine the rate of disease progression by dividing EDSS score over disease duration since diagnosis.

2.2. EBV ELISA

Blood samples were centrifuged at 2500 ×*g* for 5 min in a swinging bucket centrifuge at room temperature. Blood phases were isolated into plasma, buffy coat, and compact red blood cell pellet fractions. Plasma fractions were subjected to three ELISA assays for the presence of antibodies against specific viral antigens associated with EBV infection stage. Immunoglobulins against viral coat antigens (VCA), and Epstein–Barr nuclear antigen 1 (EBNA1) titers were assayed using GenWay's EBV VCA IgG, and EBNA1 IgG kits (GenWay Biotech Inc., CA, USA). The manufacturer's protocols were followed throughout the procedure. In brief, diluted plasma samples and ready-to-use standards were pipetted into the wells of microtiter plates pre-coated with respective EBV antigens. Plasma antibodies binding to immobilized EBV antigens were facilitated by incubation for 1 h at room temperature. The plate was rinsed with diluted wash solution to remove unbound material. The ready-to-use anti-human-IgG peroxidase conjugate was added and incubated at room temperature. After a second washing step, the substrate TMB solution was added and incubated at room temperature in the dark. The color development was terminated by the addition of a stop solution. The resulting dye was measured spectrophotometrically at 450 nm wavelength. The concentration of antibodies is directly proportional to the intensity of the color, and can be inferred in arbitrary units (U/mL) from the standard curve plotted using ready-to-use standards. Positive EBV EBNA1 and VCA antibody titers were recorded as positive if titers were >20 U/mL, whereas ≤20 U/mL titers were recorded as negative. Segregation of the antibody titers into high (>50 U/mL) and low (50–20 U/mL) was performed to reach a grouped view of the non-normally distributed variation. Viral infection stage was determined according to De Paschale and Clerici, by categorization of EBV infection stage according to comparative assessment of antibody titers of both antigens into: acute infection, reactivation, past infection, and negative (De Paschale and Clerici, 2012).

2.3. HLA-DRB*1501 genotyping

Blood samples were collected following patient and control consent. An additional 7 patients and 15 healthy controls provided frozen blood samples that were omitted from plasma antibody analyses. Approximately 3–4 mL of blood was collected from each individual and an aliquot of 200 μL of blood was retained for DNA extraction prior to blood fractionation. Genomic DNA was extracted using QIAamp DNA blood mini kit (Qiagen, CA, USA). The manufacturer's protocol was followed with minor modifications. In brief, whole blood samples were added to 25 μL of proteinase K and 200 μL of lysis buffer. Incubation at 56 °C was increased to 30 min to maximize lysis. DNA was precipitated in 200 μL absolute ethanol and bound to a filtered spin column. The column was washed twice in two different concentrations of wash buffer, followed by a dry spin at maximum speed for 1 min. DNA was eluted with nuclease free water. Genomic DNA yield and quality were ascertained using a NanoDrop spectrophotometer. HLA-DRB1*1501 genotyping was performed using tagSNP rs3135005 shown previously to correlate with the HLA-DRB1*1501 polymorphism ($r^2 = 0.93$) and is in complete linkage disequilibrium with it (De Jager et al., 2008). TaqMan® rs3135005 genotyping assay (Life Technologies, CA, USA) was used for genotyping according to standard protocols and analyzed using the ABI 7500 Fast Real-time PCR system (Life technologies, CA, USA).

2.4. Statistical analysis

All statistical analyses were performed using GraphPad prism 6.0 software. None of the ELISA results was normally distributed. Mann–Whitney rank sum test was used to compare groups with a two-tailed significance of $p < 0.05$. Hodges–Lehmann relative risk (RR) was used at 95% confidence interval (CI) for differences between median comparisons, and $p < 0.05$ was considered significant. In addition, Fisher exact test and Chi-square test with Yate's corrections and Student t-test were used when appropriate for the effect of HLA-DRB1*1501 locus on EBV antibody titers, infection stage, interferon treatment, and MS PI index. Positive HLA-DRB1*1501 genotype was assigned to homozygous/heterozygous allele A, while ancestral allele G homozygosity was assigned negative.

3. Results

3.1. EBV related antibody titers in MS patients

A total of 141 MS patients were compared to 40 healthy controls' plasma EBV related antibody titers, patient and control demographics and clinical characteristics are depicted in Table 1. Most MS patients had RRMS course. Female to male ratio were at ~2:1 in both MS patients and healthy controls to account for gender difference in disease incidence. Five healthy controls and five MS patients were negative (no immunity) for antibodies against both VCA and EBNA1, whereas the remaining samples had detectable titers. Total anti-EBNA1 titers in MS patients differed significantly from healthy controls with higher positive response in MS patients than that of healthy individuals ($p = 0.008$, RR 22.59, 95% CI: 5.07–40.45). Grouping of the anti-EBNA1 titer into high