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Evaluation of Epstein-Barr virus-specific antibodies in Cypriot multiple sclerosis patients

Elie Deeba[a], Dana Koptides[a,c], Efthychia Gaglia[b], Astero Constantinou[c], Anastasia Lambrianides[a,b], Marios Pantzaris[a,b], Georges Krashias[a,c,*], Christina Christodoulou[a,c]

[a] Cyprus School of Molecular Medicine, The Cyprus Institute of Neurology and Genetics, Nicosia, Cyprus
[b] Neurology Clinic C, The Cyprus Institute of Neurology and Genetics, Nicosia, Cyprus
[c] Department of Molecular Virology, The Cyprus Institute of Neurology and Genetics, Nicosia, Cyprus

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ABSTRACT

Multiple Sclerosis (MS) is a chronic, demyelinating, inflammatory disease of the central nervous system (CNS) with a strong autoimmune component. Several genetic and environmental factors have been suggested to contribute in MS. The Epstein-Barr virus (EBV) is one pathogenic candidate proposed to be involved in the onset of MS and/or induction of subsequent exacerbations. The possible involvement of EBV in MS is highlighted by a number of national epidemiological studies showing a higher percentage of EBV seropositivity. This study aims to evaluate for the first time the seroprevalence of EBV in Cypriot MS patients. The serum of 133 MS patients and 101 healthy controls (HCs) was used to determine the positivity index of the EBV nuclear antigen-1 (EBNA-1) IgG, viral capsid antigen (VCA) IgG, and early antigen-D (EA-D) IgG, using ELISA. All MS patients were seropositive for both EBNA-1 IgG and VCA IgG as compared to 94.1% (Fisher's exact test, $p = 0.0059$) and 93.1% (Fisher's exact test, $p = 0.0025$) of HCs respectively. Furthermore, the positivity indexes of both antibodies were significantly higher in MS patients. There was no significant difference in the presence/absence of EA-D IgG between the two groups nor in the corresponding P.I. levels. The results obtained, revealing higher seropositivity of EBNA-1 IgG and VCA IgG in MS patients, seem to concur with previous findings of studies in other countries, thereby further asserting the theory of EBV involvement in MS.

1. Introduction

Multiple sclerosis (MS) is widely accepted as a demyelinating disease with conclusive aspects of autoimmunity (Frischer et al., 2009; Lassmann et al., 2007). Various genetic and environmental factors have been shown to play a role in disease pathogenesis either separately or in combinations; however, the exact mechanisms of how these might interact remain unknown (Ascherio and Munger, 2007a, 2007b; Olsson et al., 2017; Parnell and Booth, 2017).

The global prevalence of MS exhibits a latitude gradient, along with a few exceptions, whereby the highest is seen in North America, Canada, and Northern Europe (> 100/100,000), and the lowest is seen near the equator (< 5/100,000) (Browne et al., 2014). The prevalence of MS in Cyprus has been recently reported to be 198 per 100,000 (Charalambidou et al., 2016), strikingly higher than the prevalence estimated in 1991 (44.5/100,00) (Middleton and Dean, 1991), and higher than the official estimate of the 2013 Atlas of MS (178/100,000) (Browne et al., 2014). This change suggests a major role of the environmental factors in the causation of the disease (Lauer, 2010).

Microbial exposure is arguably one of the strongest environmental risk factors for the development and/or progression of the disease (Lauer, 2010). A list of pathogenic contributors has been put forth throughout the years, implicating bacteria (*Mycoplasma pneumoniae, Spirochetes, Campylobacter, Bartonella,* and others), viruses (Epstein-Barr virus (EBV), human herpesvirus 6, human endogenous retroviruses), protozoa, and parasites in the pathogenesis and/or progression of MS (Fierz, 2017; Libbey et al., 2014). Among this diverse group of infectious agents, EBV is considered to be the most promising etiological

Abbreviations: MS, multiple sclerosis; HCs, healthy controls; CNS, central nervous system; CIS, clinically isolated syndrome; EBV, Epstein-Barr virus; EBNA-1, Epstein-Barr nuclear antigen-1; VCA, viral capsid antigen; EA-D, early antigen-diffuse; P.I., positivity index; IgG, immunoglobulin G; PBMCs, peripheral blood mononuclear cells

* Corresponding author: Cyprus School of Molecular Medicine, The Cyprus Institute of Neurology and Genetics, 6 International Airport Avenue P.O.Box 23462, 2370, Nicosia, Cyprus.
E-mail address: georgek@cing.ac.cy (G. Krashias).

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agent of MS (Saberi et al., 2018).

EBV is a gamma-herpes virus, which is capable of infecting, activating, and latently persisting in B cell for life (Thorley-Lawson, 2015). Meta-analyses have agreed that there is an increased risk of MS after the development of infectious mononucleosis (IM), an atypical presentation of EBV infection, by a risk factor of 2.3, regardless of sex, age and time since IM (Thacker et al., 2006). In addition, the risk of developing MS in seronegative subjects is extremely low, but increases considerably after EBV infection (Ascherio et al., 2001). The possible involvement of EBV in MS is reiterated further by several studies showing that almost all individuals with MS (> 99%) are seropositive for EBV, as opposed to about 90% of healthy adults (Ascherio and Munch, 2000). The level of EBV-specific antibodies has been also shown to be different among MS patients and healthy individuals. An increased antibody response has been seen in MS patients versus healthy subjects towards different EBV antigens, including the EBV nuclear antigen-1 (EBNA-1), the small capsid protein BFRF3, and the tegument protein BRRF2 (Dooley et al., 2016; Levin et al., 2005; Sundström et al., 2004). Increased levels of EBNA-1-specific antibody responses may also predict conversion from clinically isolated syndrome (CIS) to MS (Lünemann et al., 2010).

Although the association of EBV-specific antibodies and MS risk is very robust, the association of the genetic material of EBV with the disease remains controversial. Several studies focusing on EBV DNA quantification in peripheral blood mononuclear cells (PBMCs) have failed to show any difference between MS patients and healthy controls (Alvarez-Lafuente et al., 2006; Alvarez et al., 2000; Cocuzza et al., 2014; Lindsey et al., 2009; Lünemann et al., 2006; Sotelo et al., 2007), although significantly higher EBV positivity in PBMCs of MS patients was reported in two studies (Ben Fredj et al., 2012; Nejati et al., 2016). In addition, an increased incidence of EBV DNA in serum during relapses compared to periods of remission has been reported (Wandinger et al., 2000). Increased risk of MS has been also associated with the presence of plasma EBV DNA (Wagner et al., 2004). The presence/absence of EBV has been also investigated in post-mortem MS brain tissue (Hassani et al., 2018; Hilton et al., 1994; Moreno et al., 2018; Opsahl and Kennedy, 2007; Sargsyan et al., 2010; Serafini et al., 2017, 2010, 2007; Willis et al., 2009), and CSF directly (Cocuzza et al., 2014; Sargsyan et al., 2010) with some (Hassani et al., 2018; Moreno et al., 2018; Serafini et al., 2017, 2010, 2007) but not all authors (Hilton et al., 1994; Opsahl and Kennedy, 2007; Sargsyan et al., 2010; Willis et al., 2009) reporting the presence of EBV in the affected MS brain or CSF.

So far, numerous studies have highlighted the possible association between EBV and MS through the analysis of EBV-specific antibody responses in different cohorts of MS patients (Almohmeed et al., 2013; Ascherio et al., 2001; Honarmand et al., 2015; Lünemann et al., 2010; Mameli et al., 2013; Myhr et al., 1998). However, such data are lacking for the Cypriot MS group. For this reason, given also the high prevalence of MS in Cyprus, we were prompted to obtain so-far-unavailable data related to EBV-specific antibody responses in Cypriot MS patients.

2. Methods

2.1. Ethical approval

This study was approved by the Cyprus National Bioethics Committee (EEBK/ΕΠ/2016/51). MS patients and healthy controls (HCs) completed and signed an informed consent form.

2.2. Study population and sample collection

A total of 133 MS patients and 101 gender- and age-matched HCs were recruited for this study. Patients with MS were diagnosed using the widely accepted McDonald criteria (Polman et al., 2011). Blood samples were collected from MS patients during their routine, follow-up visits at clinic C of The Cyprus Institute of Neurology and Genetics. The inclusion criteria were: 1) patients with clinically definite multiple sclerosis (CDMS) with clear clinical course (RRMS, SPMS, PPMS), 2) patients not experiencing any relapse symptoms during blood collection, 3) availability of a detailed clinical history (age of onset, disease duration, Expanded Disability Status Scale (EDSS) score, and treatments received), 4) being above 18 years of age, and born and have resided in Cyprus from birth to at least early adult life. Exclusion criteria were: 1) presence of relapse in the 30 days before enrollment in the study, 2) inability or unwillingness to provide informed consent, 3) a history of alcohol or drug abuse, and 4) pregnancy. The female/male ratio in the MS group was 1.6, matching the reported female/male ratio of Cypriot MS patients (Charalambidou et al., 2016). Table 1 shows the demographic details and clinical characteristics (EDSS, diseases duration, treatment at time of blood collection) of the MS patients and HCs.

Table 1
Demographic and clinical characteristics of the study groups used for antibody detection and analysis. The Mann-Whitney U test was used for age matching, and the Fisher's exact test was used for gender matching.

Features	MS patients (n = 133)	HCs (n = 101)	*p*-value
Age (mean[SD])	48.26 ± 13.76	46.62 ± 11.93	0.2922
Gender (male/female)	51/82	40/61	0.8926
Disease course (RR/SP/PP)	111/14/8	N/A	
Duration of disease (years) [median (interquartile range)]	33 (28–42)	N/A	
EDSS [median (interquartile range)]	3.5 (2.5–5)	N/A	
Type of treatment [n (%)]		N/A	
Fingolimod	15 (11.3)		
Glatiramer Acetate	6 (4.5)		
IFNβ (IFNβ-1a or IFNβ-1b)	36 (27.1)		
Natalizumab	14 (10.5)		
Other*	8 (6.0)		
None	54 (40.6)		

MS: Multiple Sclerosis; HCs: Healthy Controls; RR: Relapsing Remitting MS; SP: Secondary Progressive MS; PP: Primary Progressive MS; SD: Standard Deviation; N/A: Not Applicable; *Alemtuzumab, Azathiprine, Dimethyl fumarate, Methotrexate, Mycophenolate, Rituximab, Teriflunomide.

Blood samples were collected in tubes containing clotting activators at the Neurology clinic C of The Cyprus Institute of Neurology and Genetics. Following blood drawing, samples were centrifuged for 10 min at 500xg at 20 °C to obtain cell-free serum. Serum was stored at -20 °C until analysis.

The serum obtained from the two groups of the study was used to determine the positivity index (P.I.) of EBNA-1 IgG, viral capsid antigen (VCA) IgG, and early antigen-D (EA-D) IgG, using commercial ELISA kits (all from VIDIA, Czech Republic). All kits are *in vitro* diagnostic medical device (IVD) approved. Each kit was run with a negative control, a positive control, and a calibrator in order to calculate the P.I. of the samples. The P.I. was calculated as the ratio of the absorbance of the sample acquired to the cutoff absorbance value obtained from the standard provided by the kit. According to the manufacturer, values above 1.1 were considered positive for the respective antibody tested among groups.

2.3. Statistical analysis

The non-parametric Mann-Whitney U test was used to evaluate significance of the P.I. levels of the different antibodies tested among the MS patients and HC. The Fisher's exact test was used to evaluate the significance of antibody presence among the study groups. The GraphPad Prism v7.00 for Windows software program was used to perform the statistical analyses (GraphPad Software, La Jolla, California, USA, www.graphpad.com).

Table 2
Distribution of MS patients (n = 133) and HCs (n = 101) who tested positive for IgG antibodies targeting EBV antigens EBNA-1, VCA and EA-D. The Fisher's exact test was used for statistical analysis ($p < 0.05$).

Positivity for target antigen	MS No. (%)	HCs No. (%)	P value
EBNA-1 IgG	133 (100)	95 (94.1)	0.0059
VCA IgG	133 (100)	94 (93.1)	0.0025
EA-D IgG	39 (29.3)	26 (25.7)	0.5596

3. Results

EBV seroprevalence and EBNA-1, VCA, and EA-D IgG antibody levels in MS patients and HCs

All EBV-specific antibodies were measured in serum. IgG antibodies to EBNA-1 were detectable in 133/133 (100%) of MS patients, compared to HCs where only 94.1% of the latter (95/101) showed a history of EBV exposure ($p = 0.0059$). In a similar manner, all MS patients (100%) compared to 94/101 HCs (93.1%) were positive for VCA IgG ($p = 0.0025$). In contrast to the EBNA-1 and VCA antibody responses, IgG antibodies targeting EA-D were detected in only a minority of MS cases (39/133; 29.3%) and HCs (26/101; 25.7%). This difference, however, did not reach statistical significance ($p = 0.5596$) (Table 2).

The results of P.I. levels for each antibody tested in the study groups can be seen in Fig. 1. The median EBNA-1 IgG index among the subjects was significantly higher ($p < 0.0001$) in the MS group (median [interquartile range]: 5.681 [4.717–6.356]) than in the HC group (5.098 [4.104–5.706]) (Fig. 1A). Similarly, the median VCA IgG index was significantly higher ($p = 0.0007$) in MS patients (6.938 [5.649–8.429]) than in HCs (6.061 [4.721–7.394]) (Fig. 1B). However, the median EA-D IgG index was not significantly different between the two groups ($p = 0.737$), with MS patients showing a P.I. median of 1.788 [1.361–3.373] compared to 1.774 [1.322–2.853] for the HC group (Fig. 1C).

4. Discussion

Recent epidemiological data have highlighted a trend of increasing MS prevalence in the Cypriot population over the last 30 years (Charalambidou et al., 2016; Middleton and Dean, 1991). Given that genetic risk factors are unlikely to be solely responsible for this observation (Ascherio and Munger, 2007a, 2007b), changes in environmental risk factors might also be contributing to the documented increased MS risk in Cyprus. Current scientific literature provides considerable evidence that EBV infection is indeed a strong environmental risk factor the development of MS (Ascherio and Munch, 2000). So far, several studies have highlighted the association of MS with EBV infection in different population cohorts (Almohmeed et al., 2013). However, an association between EBV infection with the Cypriot MS group remains unclear. This study was designed to investigate the seroprevalence of EBV in MS patients and HCs for the first time, using a validated, IVD-approved, serological method for IgG antibody detection against EBNA-1, VCA, and EA-D.

In agreement with other studies (Almohmeed et al., 2013; Ascherio and Munch, 2000), our results show that all Cypriot MS patients (100%) had been exposed to EBV in the past compared to around 92.9% of HCs, as concluded by the presence of IgG antibodies directed towards EBNA-1 in their sera. Moreover, it appears that titer levels of EBNA-1 IgG antibodies are significantly higher in MS patients than in HCs. This is in accordance with other studies throughout the world, where higher EBNA-1 antibody titers were detected in MS patients (Abdelrahman et al., 2014; Almohmeed et al., 2013; Ascherio et al., 2001; Ascherio and Munch, 2000; Dooley et al., 2016; Honarmand et al., 2015; Lindsey et al., 2010; Lünemann et al., 2010; Mameli et al., 2013; Mouhieddine et al., 2015; Nociti et al., 2010; Sundström et al., 2004). Previously reported elevated EBNA-1 immune responses were believed to predict the conversion from CIS to MS (Lünemann et al., 2010), although recent studies contradict this finding, i.e. no relation between EBNA-1 antibody levels and CIS to MS conversion (Gieß et al., 2017; Kuhle et al., 2015; Munger et al., 2015). In addition, EBNA-1 titer levels were shown

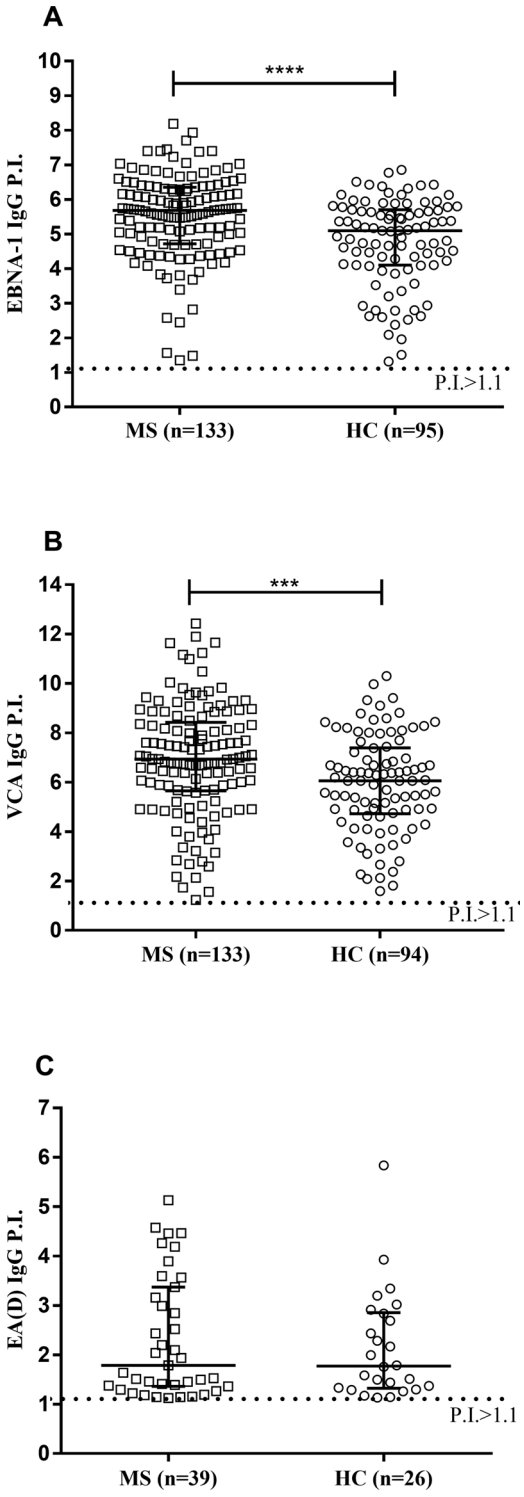

Fig. 1. Distribution of positivity index (P.I.) of EBNA-1 IgG (A), VCA IgG (B), and EA-D IgG (C) antibodies in seropositive MS patients and HCs. The horizontal dotted line represents the threshold value (1.1) above which the P.I. is considered positive. Bars represent the median and the interquartile range for each group. The Mann-Whitney U test was used for analysis (***$p < 0.001$; ****$p < 0.0001$).

to affect disease risk in combination with other genetic and/or environmental MS risk factors. Studies showed an additive interaction between EBNA-1 antibody levels and the presence of the HLA-DRB1*1501 (De Jager et al., 2008; Sundqvist et al., 2012). Whether this holds true for the elevated EBNA-1 antibody levels observed in our MS group remains to be seen. In agreement with previous published studies (Abdelrahman et al., 2014; Ascherio et al., 2001; Mouhieddine et al., 2015; Nociti et al., 2010; Sundström et al., 2004), significantly higher titer levels of VCA IgG antibodies were detected in MS patients compared to HCs. Nevertheless, the relationship between the increase in EBNA-1 IgG and/or VCA IgG titers and MS severity remains to be studied in our case, as previously reported in a British MS cohort (Farrell et al., 2009).

EA IgG production is considered a marker of acute primary or reactivated EBV infections, and the titers are reduced or disappear during latent infection (Middeldorp, 2015). In the present study, EA-specific IgG responses were detected in 23.7% of MS patients. This observation is consistent with previous studies of similar nature conducted in other MS cohorts that reported EA IgG responses in 0%–68.7% of MS patients (Almohmeed et al., 2013; Santiago et al., 2010). A direct association between EA IgG responses and MS is yet to be determined, with some studies reporting a significantly higher percentage of seropositivity among MS patients (Buljevac et al., 2005; Myhr et al., 1998; Riverol et al., 2007), while others were unable to observe such a difference (Almohmeed et al., 2013). In fact, a number of studies have documented a lower percentage of EA seropositivity among MS patients (Almohmeed et al., 2013; Alotaibi et al., 2004; Munch et al., 1998; Wandinger et al., 2000). In agreement with the former observation, the proportion of EA-positive cases in our MS group was greater than that observed in HCs, though it did not reach statistical significance. Overall, these discrepancies might be attributed to the differences in study populations and designs as well as due to the different methodologies used for sample analysis. Additionally, we could not detect any significant difference in the level of EA IgG antibodies between MS patients and HCs. There is some indication that high levels of anti-EA IgG correlate with disease activity (Buljevac et al., 2005; Wandinger et al., 2000). It might therefore be interesting to characterize EA IgG responses during relapses in our MS group.

Several Hypotheses are currently under investigation to understand the mechanism of EBV involvement in MS pathogenesis. (A) The molecular mimicry hypothesis, whereby the pathogenic antigen is structurally similar to self-proteins. This results in the inability of the immune cells to distinguish the differences between foreign and self-antigens. One example comes from a study showing EBNA-1-specific T cells from MS patients are cross reactive to myelin antigen (Lünemann et al., 2008). (B) The bystander damage hypothesis, whereby the damage caused to the central nervous system is considered as collateral damage due to the immune system's attempt to control the EBV infection. This theory suggests the presence of EBV-directed immune cells in the CNS. One study was able to consistently show the presence of an active EBV infection in CNS-infiltrating B cells in MS patients (Serafini et al., 2007). (C) EBV-infected autoreactive B cells hypothesis, whereby individuals who are genetically predisposed to autoimmune diseases have EBV-infected autoreactive B cells that migrate to target organs and subsequently produce pathogenic auto-antibodies (Pender, 2003). This activates autoreactive $CD4^+$ T cells that are maintained in the target organ and ultimately result in organ damage through autoreactive $CD8^+$ T cell recruitment. This hypothesis effectively combines the two aforementioned hypotheses. It was also shown that MS patients fail to regulate EBV as efficiently as healthy individuals, as seen by the reduced EBV-reactive $CD8^+$ T cells (Pender et al., 2017). This dysregulation further compliments the EBV-infected autoreactive B-cells hypothesis due to the inability to control and/or remove the EBV infection. Additionally, a recently proposed hypothesis suggests that the B cell lineage invasion and intrathecal antiviral antibody production in the CNS occurs predominantly at the time of acute EBV infection and/or is triggered by it due to an excessive unspecific polyclonal activation of B cells (Otto et al., 2016; Ruprecht et al., 2017).

In summary, our results provide so far unavailable data related to EBV seroprevalence in Cypriot MS patients. Of equal importance, our results further assert the association between MS and EBV exposure. Our study hopes to further enrich the serological data conducted worldwide across genetically and environmentally diverse populations. This may allow us to better understand the interplay between the environment, genetics and MS onset and/or pathogenesis.

Disclosure statement

The authors have no conflicts of interest to declare.

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