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Multiple sclerosis: The elevated antibody response to Epstein–Barr virus primarily targets, but is not confined to, the glycine–alanine repeat of Epstein–Barr nuclear antigen-1

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ABSTRACT

Patients with multiple sclerosis (MS) have elevated antibodies against Epstein–Barr virus (EBV), but data on the epitope-resolved specificity of these antibodies are scarce. Using a peptide microarray containing 1465 peptides representing 8 full-length EBV proteins, we identified higher ($p < 0.001$) antibody reactivities to 39 EBV-peptides in MS patients (n = 29) compared to healthy controls (n = 22). Seventeen of the 39 peptides were from EBNA-1 and 13 located within the glycine–alanine repeat of EBNA-1. Further reactivities were directed against EBNA-3, EBNA-4, EBNA-6, VP26, and LMP1. Thus, antibodies against EBV in MS patients primarily target, but are not confined to, the glycine–alanine repeat of EBNA-1.

1. Introduction

Strong and consistent evidence suggests that infection with Epstein–Barr virus (EBV) is a risk factor for multiple sclerosis (MS) (Ascherio and Munger, 2007; Ascherio et al., 2012). EBV seroprevalence in patients with MS is nearly 100%, indicating that MS risk is extremely low in EBV seronegative persons (Ascherio and Munger, 2007; Goodin, 2009; Pakpoor et al., 2012). Symptomatic primary EBV infection manifesting as infectious mononucleosis is associated with an about twofold increased MS risk (Handel et al., 2010). Moreover, when compared to EBV seropositive healthy controls, EBV seropositive patients with MS or with a clinically isolated syndrome suggestive of MS have elevated levels of antibodies against EBV, especially against the Epstein–Barr nuclear antigen-1 (EBNA-1) (Larsen et al., 1985; Lindsey et al., 2010; Lunemann et al., 2010; Lucas et al., 2011). Furthermore, among apparently healthy young adults MS risk increases with increasing serum titres of antibodies to EBNA-1 and the EBV nuclear antigen complex (EBNAc) (Ascherio et al., 2001; Sundstrom et al., 2004; Levin et al., 2005; Delorenze et al., 2006; Munger et al., 2011), rendering the level of anti-EBNA antibodies the strongest non-genetic risk factor for MS (Ascherio et al., 2012).

Previous studies that analysed the epitopes targeted by EBV-specific antibodies in patients with MS by peptide arrays or using EBV protein fragments exclusively investigated reactivities to EBNA-1 (Lunemann et al., 2008; Sundstrom et al., 2009; Jafari et al., 2010; Mechelli et al., 2011) and could not scan the complete EBNA-1 protein sequence in all instances (Sundstrom et al., 2009). To systematically analyse the fine specificity of the altered antibody response against EBV proteins in patients with MS, we herein screened sera from patients with MS

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and healthy controls with a large peptide microarray containing 1465 peptides representing a 15/11 scan through 8 different full-length EBV proteins, i.e. EBNA-1, EBNA-3, EBNA-4, and EBNA-6, the tegument protein BLRF2, the trans-activator protein BZLF1, the latent membrane protein LMP1, and the viral capsid protein VP26.

2. Patients and methods

The study was approved by the institutional review board of Charité — Universitätsmedizin Berlin (EA1/182/10). All participants provided written informed consent.

2.1. Patients and healthy controls

Serum samples were collected by peripheral venipuncture from 29 patients (21 female, 8 male) with a diagnosis of relapsing–remitting MS (RRMS) according to the revised McDonald criteria (Polman et al., 2011) as well as from 24 healthy controls (15 female, 9 male). The sex distribution was not different between the groups of patients with MS and healthy controls ($p = 0.56$; Fisher's exact test). With a median (range) age of 34 (19–53) years patients were older than controls (28 [22–62] years, $p = 0.02$; Mann–Whitney U test). Patients had not been treated with glucocorticosteroids for at least 8 weeks before blood withdrawal. Pregnancy or intercurrent infections at the time of blood withdrawal were exclusion criteria. Ten of the patients with MS were untreated, 12 received treatment with interferon-β, and 7 with glatiramer acetate. Serum samples were stored at −20 °C before further analysis.

2.2. Laboratory analysis

Serum immunoglobulin (Ig)G antibodies to EBNA-1 and the EBV viral capsid antigen (VCA) were measured by a commercial enzyme-linked immunosorbent assay (ELISA; medac, Germany) or western blot (recomBlot EBV IgG, Mikrogen, Germany). Persons without detectable antibodies to EBNA-1 and VCA were considered to be EBV seronegative. A peptide library covering full-length EBV proteins EBNA-1, EBNA-3, EBNA-4, EBNA-6, BLRF2, BZLF1, LMP1, and VP26 was generated using EBV (strain B95-8) protein sequences obtained from the Uniprot Knowledge base (www.uniprot.org). The proteins were represented as peptide scans of 15-mer peptides with an offset of 4 and an overlap of 11 amino acids. Table 1 summarizes data on the protein length and the number of synthesized peptides per protein. A total of 1465 peptides were synthesized using SPOT synthesis (Wenschuh et al., 2000). Peptides were processed and chemoselectively immobilized in triplicates onto functionalized glass slides as described earlier (Panse et al., 2004). Serum samples were diluted 1:200 in Protein-Free T20 (TBS) Blocking Buffer (Pierce) and incubated using the automated incubation workstation HS4800 with 24 parallel incubations per run. A secondary Cy5-conjugated AffiniPure mouse anti-human IgG antibody (Jackson Immuno Labs) was used for detection. Readout was performed using a Genepix 4200A scanner equipped with an autoloader. GenePix software (Version 7.0) was used for image processing, and the R statistical programming system (Version 2.11.1, www.r-project.org) was used for data analysis and calculation of mean values for statistical evaluation. Quantile normalization was applied to the data set as implemented in the R-package preprocessCore (version 1.20.0). Data are reported as arbitrary fluorescence units (AFU).

Table 1
Name of EBV protein, UniProt ID number, protein length, and number of 15-mer peptides represented on the peptide microarray used in this study.

EBV protein	UniProt ID	Protein length (amino acids)	Number of 15-mer peptides on array
EBNA-1	EBNA1_EBVB9	641	210
EBNA-3	EBNA3_EBVB9	944	311
EBNA-4	EBNA4_EBVB9	938	309
EBNA-6	EBNA6_EBVB9	992	327
BLRF2	BLRF2_EBVB9	162	50
BZLF1	BZLF1_EBVB9	245	78
LMP1	LMP1_EBVB9	386	125
VP26	VP26_EBVB9	176	55

2.3. Bioinformatic analysis

Statistical significance of different antibody responses between patients with MS and healthy controls was assessed for each screened peptide by the non-parametric Mann–Whitney U test considering p-values $<10^{-3}$ as significant. Adjustment of p-values of antibody reactivities against EBNA-1 peptides for overall anti-EBNA-1 titres was performed using a generalized linear model. In view of the large number of tested peptides we also analysed data with correction for multiple testing using the Bonferroni method (Proschan and Waclawiw, 2000). Thus, the global significance level ($p < 0.05$) was divided by the total number of tests performed ($n = 1465$), resulting in a new local significance level of $p < 3.41 \times 10^{-5}$. Subgroup analyses of untreated, interferon-β-, and glatiramer acetate-treated patients were carried out using the non-parametric Kruskal–Wallis test. All statistical analyses were performed with IBM SPSS Statistics 21.

3. Results

To confirm that only EBV seropositive persons were included in this work we first measured antibodies to EBNA-1 by ELISA in the groups of patients with MS and healthy controls. Consistent with previous studies (Ascherio and Munger, 2007; Goodin, 2009; Pakpoor et al., 2012), all 29 patients with MS (100%) but only 22 of the 24 (91.7%) healthy controls had serum antibodies to EBNA-1. The 2 controls without antibodies to EBNA-1 neither had antibodies to VCA. They were therefore considered EBV seronegative and excluded from further evaluations. In the following analyses we thus compared antibody reactivities to each of the 1465 EBV peptides represented on the EBV peptide microarray between 29 EBV seropositive patients with MS and 22 EBV seropositive healthy controls.

Evaluation by Mann–Whitney U tests with the significance threshold set to $p < 10^{-3}$ demonstrated differences of antibody reactivities between patients with MS and healthy controls for a total of 39 peptides (Table 2). Antibody reactivities against all 39 peptides were higher in patients with MS than in healthy controls. While the majority of those peptides (17 of 39; 44%) were derived from EBNA-1, significant differences were also observed for peptides derived from EBNA-3, EBNA-4, EBNA-6, VP26, and LMP1, but not for peptides from BLRF2 and BZLF1 (Fig. 1). Remarkably, 13 of the 17 (76%) EBNA-1 peptides were located in the glycine–alanine repeat of EBNA-1 (Fig. 2). The remaining 4 peptides were located in the C-terminal region (between amino acids 355 and 588) of EBNA-1. Several of the significantly different antibody reactivities were directed against overlapping peptides, e.g. EBNA-1_151–165, EBNA-1_154–168, and EBNA-1_157–171. Furthermore, antibody reactivities to the EBNA-1_382–396 peptide, which is flanked by two peptides (EBNA-1_379–393 and EBNA-1_385–399) to which patients with MS had significantly higher ($p < 10^{-3}$) antibody reactivities, were also higher ($p = 6 \times 10^{-3}$) in patients with MS than in healthy controls, although not reaching the significance threshold of $p < 10^{-3}$ (Fig. 2). We also analysed the antibody responses to the 17 EBNA-1 peptides that differed between the groups of patients with MS and healthy controls with a p-value of $<10^{-3}$ after adjustment for overall anti-EBNA-1 titres (as measured by ELISA). Following the adjustment, antibody reactivity differences to 9 of the 17 EBNA-1 peptides (including the two top-ranking EBNA-1 peptides EBNA-1_109–123 and EBNA-1_214–228, as well as the C-terminal peptide EBNA-1_385–399; Table 2) remained significant with a p-value $<10^{-3}$. This indicates that also after adjustment for overall anti-EBNA-1 titres antibody