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Fine specificity of the antibody response to Epstein-Barr nuclear antigen-2 and other Epstein-Barr virus proteins in patients with clinically isolated syndrome: A peptide microarray-based case-control study

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ABSTRACT

We analyzed the fine specificity of antibodies to Epstein-Barr nuclear antigen-2 (EBNA-2) and other Epstein-Barr virus (EBV) proteins in 29 patients with clinically isolated syndrome (CIS, the first clinical manifestation of multiple sclerosis [MS]) and 29 controls with a peptide microarray containing 117 overlapping peptides representing the full-length EBNA-2 protein and 71 peptides from 8 further EBV proteins. While EBV peptide antibodies were elevated in CIS, suggesting that EBV contributes to MS early during disease development, they discriminated groups only slightly better than EBNA-1 antibodies. Thus, the additional value of EBV peptide antibodies as diagnostic biomarkers for CIS appears moderate.

1. Introduction

Several lines of evidence support a strong link between Epstein-Barr virus (EBV) infection and multiple sclerosis (MS) (Ascherio and Munger, 2007; Ascherio et al., 2012). Antibodies to EBV are nearly universally present in patients with MS, suggesting that MS risk of EBV-seronegative individuals is extremely low (Ascherio and Munger, 2007; Goodin, 2009; Pakpoor et al., 2013). Infectious mononucleosis (symptomatic primary EBV infection) is associated with an about twofold increased MS risk (Handel et al., 2010). Among healthy young adults, MS risk increases with increasing serum titers of antibodies to Epstein-Barr nuclear antigen-1 (EBNA-1) and the EBV nuclear antigen complex (EBNAc, consisting of EBNA-1, -2, -3, -4, and -6) (Ascherio et al., 2001; DeLorenze et al., 2006; Levin et al., 2005; Munger et al., 2011; Sundstrom et al., 2004). Accordingly, results of others and our group consistently show that, compared to EBV seropositive healthy controls, patients with MS or with a clinically isolated syndrome (CIS; i.e. the first clinical manifestation of MS) have elevated levels of serum antibodies against EBV (Larsen et al., 1985; Lindsey et al., 2010; Lucas et al., 2011; Lunemann et al., 2010; Ruprecht et al., 2014). While the mechanism underlying the association of EBV and MS is not clear, it was recently suggested that genomic variants in the EBNA-2 gene may be associated with MS (Mechelli et al., 2015).

Using a peptide microarray containing 1465 peptides representing a 15/11 scan through 8 different full-length EBV proteins, we previously characterized the fine specificity of the elevated antibody response to EBV in patients with MS and healthy controls (Ruprecht et al., 2014). This identified significantly elevated (unadjusted $p < 0.001$) antibody reactivities to 39 EBV-peptides in patients with MS. Remarkably, 17 of the 39 peptides were from EBNA-1 and 13 located within the glycine-alanine repeat of EBNA-1. Further different reactivities were observed for antibodies against EBNA-3, EBNA-4, EBNA-6, the viral capsid protein VP26, and the latent membrane protein LMP1. However, the microarray used in our previous study did not contain EBNA-2-derived peptides. Since, to the best of our knowledge, no peptide screen for antibodies to EBNA-2 has been performed in patients with MS or CIS so far, the

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fine specificity of the antibody response to EBNA-2 in patients with MS or CIS is currently unknown.

Our previous data also suggested a potential usefulness of antibody responses against EBV peptides as diagnostic biomarkers for MS with an area under the curve (AUC) value for discrimination of patients with MS and healthy controls of 0.9 for the three most significantly different peptide antibody reactivities combined (Ruprecht et al., 2014). Nevertheless, in clinical practice, biomarkers for discrimination of patients with CIS from healthy controls would be even more desirable.

Thus, this study addresses two points: Firstly, we systematically studied the fine specificity of the antibody response against overlapping EBNA-2 peptides, representing the entire EBNA-2 protein, in patients with CIS and healthy controls. Secondly, we analyzed whether serum antibody reactivities to a set of previously identified EBV peptides, to which patients with MS had elevated antibody reactivities, may likewise discriminate between patients with CIS and healthy controls.

2. Patients and methods

2.1. Patients and healthy controls

The study was approved by the institutional review board, Charité — Universitätsmedizin Berlin (EA1/182/10), and all participants provided written informed consent. All patients included in this study participate in an on-going prospective observational study of patients with CIS/early MS (Berlin CIS Cohort, NCT01371071). Serum samples were collected within 6 months after symptom onset by peripheral venipuncture from 29 patients with a first clinical event suggestive of inflammatory demyelination (CIS) (Montalban, 2014) and at least two T2-weighted hyperintense lesions typical of inflammatory demyelination on 3 Tesla brain magnetic resonance imaging (MRI). While some of the patients therefore met the current MRI criteria for dissemination in space, none of the patients met the current MRI criteria for dissemination in time (Polman et al., 2011). Thus, the patients included in this work did not (yet) meet the current diagnostic criteria for relapsing-remitting MS (Polman et al., 2011). 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. Sera were likewise collected from 31 age- and sex-matched healthy controls. Serum samples were stored at −80 °C before further analyses. Serum collection and storage conditions were exactly identical for patients with CIS and healthy controls.

2.2. Laboratory analyses

Serum immunoglobulin (Ig) G antibodies to EBNA-1 and EBV viral capsid antigen (VCA) were measured by Liaison® (DiaSorin, Saluggia, Italy) automated chemiluminescent immunoassays (CLIA). According to the manufacturer's information, the antigens used in these assays are a synthetic EBNA-1 peptide of about 50 amino acids and the VCA major component p18 antigen. EBNA-1 IgG levels <5 U/ml were considered negative, levels between 5 and 20 U/ml were considered equivocal, and levels ≥20 U/ml were considered positive. VCA IgG levels <20 U/ml were considered negative and VCA IgG levels ≥20 U/ml were considered positive. Persons with EBNA-1 IgG antibodies >5 U/ml and/or VCA IgG antibodies ≥20 U/ml were considered EBV seropositive. Persons with both EBNA-1 antibodies <5 U/ml and VCA antibodies <20 U/ml were considered EBV seronegative.

Microarray-based analysis of peptide-specific antibody reactivities was carried out by JPT Peptide Technologies, Berlin, Germany, as described previously (Ruprecht et al., 2014). Briefly, 117 15-mer peptides with an offset of 4 and an overlap of 11 amino acids representing the full length EBNA-2 protein of EBV strain B95-8 (Uniprot P12978) were synthesized using SPOT synthesis (Wenschuh et al., 2000). A set of 71 EBV peptides from a total of 8 EBV proteins (i.e. the nuclear antigens EBNA-1, EBNA-3, EBNA-4, EBNA-6, the tegument protein BLRF2, the transactivator protein BZLF1, the latent membrane protein LMP1, and the viral capsid protein VP26) to which patients with MS had significantly elevated antibody responses in a previous study (Ruprecht et al., 2014) was synthesized as well. Thus, a total of 188 peptides were chemoselectively and covalently immobilized to the glass surface of the array together with human and mouse IgG as assay controls. An optimized hydrophilic linker moiety was inserted between the glass surface and the antigen derived peptide sequence to avoid false negatives caused by sterical hindrance. Serum samples were diluted 1:200 with 50 mM TBS-buffer for the incubation on microarray slides in a multiwell incubation chamber. All samples were incubated in one parallel experiment on the same day. 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 pixel intensities measured by the Genepix 4200A scanner (reported in arbitrary fluorescence units [AFU]) were used as raw data. The R statistical programming system (Version 2.11.1, www.r-project.org) was used for raw data analysis and calculation of mean values for statistical evaluation. On each microarray, each peptide is represented by 3 identical spots as technical replicates. The measured intensities for these three spots were processed to calculate the final value. Normally, the mean of the three signals was used. If the coefficient of variation for an individual peptide was >50% the mean of the two closest signals was taken and the remaining signal discarded as an outlier. The average coefficient of variation for these technical replicates was 10.5% including the outliers and 9.2% neglecting the outliers. We also measured 12 of samples on two occasions on different arrays. Signals of 6 of these samples spanned the dynamic range of the measurements and the coefficients of determination (R^2) for these 6 samples were all >0.9, indicating a good reproducibility of signal intensity values obtained on two different occasions. The other 6 samples all had signal intensities below the dynamic range and the R^2 values of these samples were somewhat lower (0.88, 0.69, 0.7, 0.89, 0.86 and 0.69), due to expected less precise measurements below the dynamic range of the assay.

2.3. Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics 21. Normally distributed variables are displayed as means with 95% confidence interval and not-normally distributed variables as medians with minimum and maximum (range) in brackets. Comparisons between two groups of normally distributed variables were performed by Student's *t*-test. Categorical data were compared using a Chi-square-test or Fisher's exact test. Comparison of distributions of AFU-values between groups was performed using the non-parametric Mann-Whitney *U* test. To increase robustness, we also normalized the AFU-values via a log-transformation and repeated the comparisons using univariate analysis of covariance with and without adjustment for individual protein-specific reactivities (AFU-values averaged over all peptides of the relevant protein for each individual; normality of the log-transformed data was confirmed with one-sample Kolmogorov-Smirnov test). We also performed receiver-operator-characteristic (ROC) curve analyses to evaluate the discriminatory power of individual peptides and proteins and their combinations to accurately predict patient or control status. Areas under the ROC curve (AUC) with asymptotic 95% confidence intervals were calculated. Assessing combinations of peptides and/or proteins for ROC-analysis was preceded by creating dummy variables as linear combinations of relevant peptides/proteins with the coefficients and intercepts derived from binary logistic regression analyses (McIntosh and Pepe, 2002). We accounted for multiple testing using the methods of Bonferroni and Benjamini-Hochberg. If not indicated otherwise, adjusted *p*-values < 0.05 were considered significant.