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Antibodies to the Epstein-Barr virus proteins BFRF3 and BRRF2 cross-react with human proteins

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ABSTRACT

We hypothesize that the immune response to Epstein-Barr virus (EBV) drives the autoimmune damage in multiple sclerosis (MS). We investigated whether antibodies to two EBV proteins targeted by MS patients cross-react with self proteins. Using affinity columns, immunoprecipitation, and mass spectrometry, we found that antibodies to the EBV protein BFRF3 cross-react with the cytoplasmic protein septin-9, and antibodies to BRRF2 also bind mitochondrial proteins. Using Western blots and ELISA, we demonstrated that MS patients were more likely to have high levels of antibodies to one or another of these self antigens.

1. Introduction

Epstein-Barr virus (EBV) is associated with multiple sclerosis (MS), but the role that EBV plays in MS is not clear (Ascherio and Munger, 2007; Lossius et al., 2012). Our hypothesis is that EBV antigens cross-react with brain antigens, so the robust immune response to the persistent EBV infection causes recurrent autoimmune damage in the central nervous system (CNS). In previous work, we have demonstrated that the antibody response to three EBV antigens is increased in MS (Dooley et al., 2016). These proteins are the nuclear antigen EBNA-1, the small capsid protein BFRF3, and the tegument protein BRRF2. We have previously shown that antibodies to EBNA-1 cross-react with heterogeneous nuclear ribonucleoprotein L (HNRNPL) (Lindsey et al., 2016). The objective of this work was to identify proteins present in human brain that cross-react with the EBV antigens BFRF3 and BRRF2.

2. Materials and methods

2.1. Specimens

Plasma samples were obtained from 80 relapsing-remitting MS patients and 80 controls matched for age, gender, and ethnicity. The mean ± standard deviation age for the MS patients was 41.9 ± 10.7 years, and for the controls, 41.5 ± 11.0 years. There were 55 females and 25 males, with 59 Caucasian, 13 African-American, 5 Hispanic, and 3 Asian. The majority of the MS patients were on disease modifying treatment; 26 on glatiramer, 26 on interferon, and 2 on dimethyl fumarate, with the remaining 26 untreated. Specimen collection was approved by the Committee for the Protection of Human Subjects of the University of Texas Health Science Center at Houston, and subjects signed an informed consent. Human brain tissue was obtained at autopsy.

2.2. Recombinant proteins

EBV and human proteins were produced as described (Dooley et al., 2016; Lindsey et al., 2016). We used the pET-45b(+) vector for all proteins, with NovaBlue cells used for vector amplification and Rosetta2(DE3)pLysS cells (EMD Millipore, Temecula, CA) for protein production. There are multiple variants of septin-9. We used the sequence for variant 1 (NM_001113491.1) to amplify a DNA sequence which was identical to the reference except for a single base change near the C terminal. This change was consistently present in multiple experiments. As a result, the protein used in this work substitutes valine for methionine at position 576. We selected isoform 1 because it included all the peptides identified in mass spectrometry. We amplified the DLST sequence for the predominant longer isoform (NM_001933.4). The DNA had two single base substitutions relative to the reference sequence, but neither affected the predicted protein sequence.

2.3. Affinity columns, immunoprecipitation, and mass spectrometry

We produced affinity columns with AffiGel 10 and recombinant EBV proteins as described (Lindsey et al., 2016). Use of affinity columns to select EBV-specific antibodies, use of the EBV-specific antibodies to immunoprecipitate human brain proteins, and mass spectrometry to identify the immunoprecipitated proteins was also as described (Lindsey et al., 2016). For each experiment, we used pooled plasma

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from 20 MS patients or 20 matched controls to isolate EBV-specific IgG. Specificity of the IgG was verified by ELISA and Western blot before use in immunoprecipitation. Each experiment included a control specimen to identify and exclude proteins that bound to the beads in the absence of specific antibody. In two of the experiments, the control specimen was pooled, unselected IgG from blood donors. The other two experiments included both anti-BFRF3 and anti-BRRF2 antibodies, and we assumed that any protein bound by both was non-specific.

2.4. ELISA

Quantitative ELISA was done essentially as described previously (Dooley et al., 2016; Lindsey et al., 2016). For DLST, each plate included an equal number of wells coated with DLST protein 0.6 μg/well or an equal volume of a bacteria negative control. The standard curve was wells coated with anti-IgG antibody and incubated with serial dilutions of a plasma with known IgG concentration. Plasma specimens were used at 1 μl/well. The secondary antibody was a goat anti-human IgG conjugated to horseradish peroxidase (Southern Biotech, Birmingham, AL) at 1:4000 dilution. The calculated IgG concentration in the bacteria negative control was subtracted from the value for the recombinant DLST protein to control for the presence of residual bacterial antigens in the recombinant protein. The intra-assay coefficient of variation (CV) was 7.2%, and the interassay CV was 47.2%.

For septin-9, coating concentration was 0.5 μg/well, plasma concentration was 0.1 μl/well, and the secondary antibody was rabbit anti-human IgG(H + L) at 1:4000 dilution. We used a plasma sample with a high level of anti-septin antibody for the reference, at concentrations of 0.1, 0.03, and 0.01 μl/well. For each sample, the absorbance of the bacteria negative control wells was subtracted from the absorbance of the septin wells, and concentration was expressed relative to the reference sample on that plate. The intra-assay CV was 3.9%, and the interassay CV was 13.0%.

2.5. Western blots

Western blots were done as described (Lindsey et al., 2016). Each experiment included two 12 lane gels, with one lane on each gel used for a reference sample, one lane for molecular weight markers, and ten lanes for unknowns. For DLST, we loaded 0.4 μg protein in each lane, used plasma at 10 μl/lane, and used mouse anti-human IgG Fc conjugated to alkaline phosphatase (Southern Biotech) at 1:1000 dilution for secondary antibody. Blots were developed for 15 min to achieve good visualization of the bands. For septin-9, we used 0.4 μg protein per lane, 3 μl/lane plasma, and mouse anti-human IgG Fc 1:2000 plus rabbit anti-human IgG(H + L) at 1:2000 for secondary antibody. Blots were developed for 4 min. Representative examples of Western blots are presented in Fig. 1.

We photographed the blots from each experiment with a Sony α6000 digital camera and used ImageJ version 1.50i software (http://imagej.nih.gove/ij) to measure the density of the antigen bands. The band density for each subject was divided by the band density for the reference sample from that experiment. The intra-assay CV was 4.5% for DLST and 11.2% for septin-9, and the inter-assay CV was 42.4% for DLST and 19.6% for septin-9.

2.6. Data analysis

Data were analyzed using the statistical software in SigmaPlot version 11.0, using either rank sum test or Chi square test.

3. Results

3.1. Immunoprecipitation

We performed 3 independent experiments with antibody specific for each EBV antigen. We selected antibody specific for BFRF3 or BRRF2 from pooled plasma specimens from 20 MS or control subjects. We used the EBV-specific antibodies to immunoprecipitate brain proteins and identified potential cross-reactive proteins (Table 1, full list of immunoprecipitated proteins in supplementary files). Antibodies to BFRF3 from MS patients immunoprecipitated several different septin proteins, with septin-9 (SEPT9) being the strongest and most consistent. One of the three samples of antibodies to BFRF3 from controls also weakly immunoprecipitated septins. Antibodies to BRRF2 immunoprecipitated several mitochondrial proteins in both MS and controls.

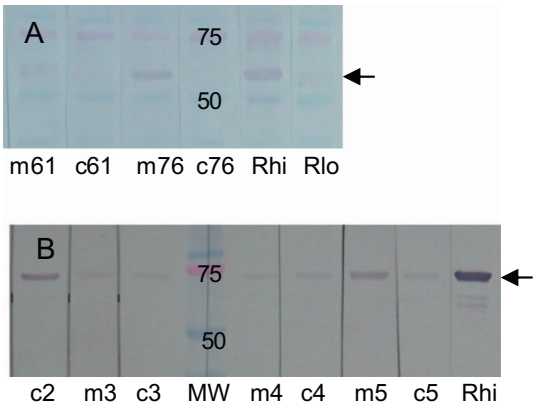

Fig. 1. Representative Western blots. A. Western blot for DLST. Each lane contains antigen mixed with colored molecular weight markers, the arrow indicates the location of DLST, numbers indicate the location of the 75 kDa and 50 kDa markers. Each lane incubated with a different subject plasma, labeled m61, c61, m76, and c76. Rhi and Rlo are the high and low concentrations of the reference sample. B. Western blot for septin-9. Arrow indicates the location of septin-9 and numbers indicate molecular weight markers, each lane contains plasma from a different subject, indicated as c2, m3, c3, etc. MW is the lane with molecular weight markers and Rhi is the high concentration of the reference sample.

Table 1
Mass spectrometry identification of brain proteins cross-reactive with BFRF3 or BRRF2.

Antibody	Gene name	Sample and experiment					
		CP1	MP1	CP2	MP2	CP3	MP3
Anti-BFRF3	SEPT9	0	1	0	14	1	5
	SEPT8	0	0	0	9	0	2
	SEPT7	0	0	0	8	0	0
	SEPT6	0	0	0	6	1	0
	SEPT5	0	0	0	7	1	1
Anti-BRRF2	DLST	0	1	6	9	7	0
	DLAT	0	0	7	10	3	0
	PDHA1	0	0	6	10	2	0
	PDHB	0	0	4	6	1	1
	PDHX	0	0	4	10	1	1
	OGDH	0	0	3	5	0	0
	OGDHL	0	0	0	4	1	0

CP1, CP2, and CP3 denote the three pools of control plasma from the first, second, and third experiment. MP1, MP2, and MP3 are the corresponding pooled MS samples. Numbers in cells are the number of peptides from that protein identified in the experiment. Proteins are identified by gene name.
SEPT9 = septin-9, etc., DLST = Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, DLAT = Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, PDHA1 = Pyruvate dehydrogenase E1 component subunit alpha, PDHB = Pyruvate dehydrogenase E1 component subunit beta, PDHX = Pyruvate dehydrogenase protein X component, OGDH = 2-oxoglutarate dehydrogenase, OGDHL = 2-oxoglutarate dehydrogenase-like.

3.2. Assessment of cross-reactivity

We tested two of the specimens of antibody specific for BFRF3 extracted from pooled plasma (MP2 and MP3 from Table 1) for binding to