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The antibody response to Epstein–Barr virions is altered in multiple sclerosis

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ABSTRACT

Infection with Epstein–Barr virus (EBV) is associated with multiple sclerosis (MS), and patients with MS have an increased antibody response to some EBV antigens. The major antigens of EBV are only partly defined. Our hypothesis is that the antibody response to EBV is altered in MS. With ELISA, we found that antibodies to EB virions were increased in both serum and CSF of MS patients. Western blots demonstrated that there are multiple different antigens recognized. The antibody response was generally higher in MS to all EBV antigens, with particularly significant increases for certain antigens. We conclude that the antibody response to EBV in MS is generally increased with altered specificity.

1. Introduction

The association between Epstein–Barr virus and MS has been recognized for decades (Sumaya et al., 1980; Bray et al., 1983). Serologic evidence of past EBV infection is consistently increased in MS patients (Ascherio and Munch, 2000); prior infectious mononucleosis increases risk of subsequent development of MS (Handel et al., 2010); and in large prospective studies, high titers of antibodies against EBV nuclear antigens (EBNA) increase risk of developing MS (Munger et al., 2011). Adults with MS are almost universally infected with EBV; and infection with EBV precedes the onset of MS symptoms (Levin et al., 2010).

EBV contains about 80 different proteins, most of which are expressed during lytic infection with only a restricted number expressed in latent infection. Over the decades that the association of EBV and MS has been recognized, the methods for detecting and measuring the anti-EBV antibody response have evolved from immunofluorescence assays with different types of EBV-infected cells to ELISA using single recombinant proteins as antigen. In clinical practice, one usually tests for antibodies against only a few selected EBV proteins, including the nuclear antigen 1 (EBNA-1), viral capsid antigen (VCA), and early antigen (EA). Research has tended to focus on these same proteins, with only occasional study of other antigens. The antibody responses to different antigens are not strongly correlated (Lindsey et al., 2010). Although measuring responses to EBNA, VCA, and EA is useful for the diagnosis and staging of EBV infection, it is not clear whether these are the main targets of the antibody response for either normal subjects or MS patients. A recent study using a protein microarray covering the entire EBV proteome suggests that healthy subjects have strong antibody responses to multiple EBV proteins (Zheng et al., 2011), including many which have received little attention in research or clinical use.

The hypothesis of this investigation was that MS patients have increased antibodies to specific EBV antigens, including antigens not usually tested in clinical practice. Identification of the target of an altered antibody response could lead to a cross-reactive antigen in myelin or could suggest some alteration in the response to EBV infection that might contribute to better understanding the pathogenesis of MS. We chose to study the antibody response against cell-free virus, since it contains the majority of virus proteins.

2. Methods

2.1. Specimens

Serum and CSF specimens were obtained from our collection of banked specimens from MS patients followed in our clinic. Control sera were from healthy subjects, and control CSF was from patients with a diagnosis other than MS. Additional CSF was obtained from the Human Brain and Spinal Fluid Resource Center, Los Angeles, CA. Paired serum and CSF samples from the same subject were residual specimens from the clinical laboratory at Memorial Hermann Hospital in the Texas Medical Center. Specimens were stored in aliquots at −80 °C. All specimen collection was approved by the Committee for Protection of Human Subjects at the University of Texas Health Science Center at Houston.

2.2. Epstein Barr virus

The EBV-infected cell line, B95.8, was grown in RPMI with 10% fetal calf serum at a starting concentration of 1×10^6 cells/ml with a

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total culture volume of 200 ml. Cultures were treated with PMA 40 ng/ml for 1 week to stimulate virus production, then cells were removed by centrifugation for 10 min at 200 *g*. The supernatant was centrifuged 60 min at 43,000 *g*. The pellet, which should contain EB virions along with some subcellular debris, was resuspended in 1 ml PBS with 1% Triton.

2.3. ELISA

The pelleted EBV was diluted with 0.1 M carbonate buffer, pH 9.2 to a protein concentration of 2 μg/50 μl. Nunc Medisorp ELISA plates were incubated overnight at 4 °C with 50 μl of diluted EBV per well. The plates were then washed and blocked with PBS containing 0.05% Tween 20 and 1% bovine serum albumin. After blocking, plates were incubated with either diluted sera or CSF in blocking buffer for 1 hour, followed by goat anti-human antibody conjugated to horseradish peroxidase (Southern Biotechnology, Birmingham, AL) diluted 1:4000 in blocking buffer. Plates were then developed with o-phenylenediamine (Sigma, St. Louis, MO) in phosphate–citrate buffer, and read on a plate reader. CSF was tested at 10 μl/well. Serum was tested at 0.05 μl/well. Between each step, plates were washed 5 times with PBS containing 0.005% Tween 20. After coating, each incubation step was 1 hour at room temperature. For CSF, each plate contained a standard curve with serial dilutions of known amounts of human IgG bound to the plate, and for serum, each plate had a standard curve of serial dilutions of a reference serum with a known concentration of EBV IgG. The anti-EBV concentration in the samples was calculated by linear interpolation between points of the standard curve.

2.4. Western blot

EBV was treated with DNAse, and then dissolved in 0.5 volume Laemmli sample buffer, heated to 70 °C for 5 min and briefly centrifuged. Then 5 μl was run in each lane of a 4–12% gradient bis/tris polyacrylamide gel (Invitrogen, Carlsbad, CA) in MES buffer with colored molecular weight markers (Bio-Rad, Hercules, CA) in every third lane, and then blotted onto nitrocellulose membrane (Bio-Rad). Membranes were blocked with TBS containing 2.5% non-fat dried milk and 0.05% Tween 20. After blocking, membranes were cut into strips containing a single lane of EBV and a half lane of molecular weight marker and incubated with 15 μl serum in 3 ml of blocking buffer. On each blot, one lane of EBV was used for the reference serum, human pooled AB serum (Valley Biomedical, Winchester, VA). The remaining 6 lanes of EBV were used for 3 pairs of serum samples from MS patients and matched controls, with the MS specimen and the corresponding control always run in the same experiment. The secondary antibody was mouse anti-human IgG Fc conjugated to alkaline phosphatase (Southern Biotechnology) diluted 1:2000 in blocking buffer. After the secondary antibody, the blot was washed extensively in water, and then bound antibody was visualized with NBT/BCIP. The procedure for CSF was similar, but we used 100 μl CSF per lane, and rabbit anti-human IgG(H + L) as the secondary antibody. The reference sample for CSF was 0.3 μl of the reference serum. For the serum-CSF paired samples, we used 3 μg of IgG per lane for both serum and CSF, with the volume calculated using the values obtained by the clinical laboratory for the IgG concentrations.

After color development, the blots were reassembled and band densities were measured by densitometry with a Hewlett Packard LaserJet 3030 scanner and Kodak 1D software. The amount of antibody was calculated as the band density relative to the same band in the reference serum on that blot. This procedure was semi-quantitative, with an intra-assay coefficient of variation of 5 to 11% and an interassay coefficient of variation of 8 to 25%. The less dense bands tended to have more variability.

Additional Western blots were performed with the same antigen, using mouse monoclonal antibodies against various EBV antigens as primary antibodies. The defined antigens for these antibodies were the gp350 envelope protein (72A1, prepared as previously described, (Lindsey et al., 2010)), EA-D (Chemicon, Temecula, CA; MAB818), latent membrane protein 1 (LMP1) (Abcam, Cambridge, MA; ab78113), and EBNA-1 (two different monoclonal antibodies, Santa Cruz Biotechnology, Santa Cruz, CA; sc-81584 and Millipore, Temecula, CA; MAB8173). A goat anti-mouse IgG-alkaline phosphatase (Southern Biotechnology) was the secondary antibody.

2.5. Immunoprecipitation

Pooled human immunoglobulin G was bound to 3 ml of AffiGel 10 (BioRad), put in a column, and washed extensively with PBS. Six aliquots of cell free EBV were homogenized with a final concentration of 1% octyl glucoside and 1× PBS in a teflon-glass Dounce homogenizer. The solubilized virus was centrifuged for 10 min at 30,000 *g*, passed through a 0.2 μm filter, and loaded on the IgG column. The column was washed with PBS and then with 0.5 M NaCl. Bound proteins were then eluted with 0.1 M glycine/pH 2.7. The eluted fraction with the highest concentration of protein was run on the same type of gel used in the Western procedure, stained with Coomassie, and bands at the areas of interest excised. Identification of the proteins present in the excised bands was done by mass spectroscopy of trypsin digests at the Proteomics Facility at the M. D. Anderson Cancer Center.

2.6. Statistics

None of the ELISA or Western data was normally distributed. Groups were compared using the Mann–Whitney rank sum test in the statistical functions of SigmaPlot 11.0. Correlation coefficients were calculated using the Analysis ToolPak in Microsoft Excel 2007.

3. Results

3.1. ELISA

Antibodies against cell free EBV as measured by ELISA were increased in MS serum (Fig. 1A). The median concentration for MS patients was 7.98 μg IgG/ml, compared to 4.94 for controls ($p<0.001$, rank sum test, $n=60$ for each group). Antibodies against EBV were also increased in MS CSF (Fig. 1B). The median concentration of EBV-specific IgG in the CSF was 33 ng/ml for MS patients, versus 19 ng/ml for controls ($p=0.006$, $n=37$ for MS and 43 for controls).

We previously tested these same serum samples for antibodies to EBNA-1 and EBV early antigen and for EBV neutralizing antibodies (Lindsey et al., 2010). The amount of EBV IgG correlated modestly with neutralizing antibodies ($r=0.35$), but was less correlated with EBNA-1 ($r=0.18$) or EA ($r=0.16$).

3.2. Serum Western blot

The above results suggested that MS patients have increased antibodies against EBV antigens. To assess the specificity of the anti-EBV IgG response in MS, we performed Western blots on whole virus. With this technique, a number of bands were visible (Fig. 2). We selected 12 prominent bands which could consistently be identified for detailed analysis. There was substantial variation between individuals, and the data were not normally distributed. But when band density was expressed relative to the reference sample included on each blot, the median band densities for the MS group were consistently higher for all antigens (Fig. 3). Differences were greatest for two low molecular weight bands at about 19 and 12 kilodaltons (kD). The median density of the 12 kD band in MS patients was more than 4 times that in controls. The most significant differences were for the 77 kD band and the 12 kD band ($p=0.007$ for 77 kD