Epstein–Barr virus nuclear antigen-1
B-cell epitopes in multiple sclerosis twins

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Abstract
Background: Compared with quantitative observations, the search for qualitative changes that may characterize the immune response to Epstein–Barr virus (EBV) in multiple sclerosis (MS) has been less intense.
Objective: To examine the B-cell epitopes of antibodies against the Epstein–Barr nuclear antigen-1 (EBNA-1) and their relevance for MS, through a study in disease-discordant identical twins.
Methods: We evaluated the antibodies to all unique, maximally overlapping octapeptides of EBNA-1 in 12 pairs of monozygotic (MZ) twins (9 MS-discordant, 3 healthy), 3 non-twin patients and 2 healthy subjects. All except one of the patients were untreated. The EBV serology of these individuals had been assessed in advance using commercially available and in-house enzyme-linked immunosorbent assay (ELISA) kits, including assays for antibodies against select peptides of EBNA-1: EBNA-72 (GAGGGAGAGG) and EBNA-206 (EADYFYHQEIGDGE).
Results: The glycine–alanine rich domain of EBNA-1 was immunodominant in all subjects. Compared with healthy individuals, and similarly to what has been described in infectious mononucleosis (IM) patients, affected co-twins and non-twin patients had a significantly increased response to another EBNA-1 epitope (aa. 401–411).
Conclusion: In a study that controls for confounders, our data focus an EBNA-1 specificity that may be associated with MS pathogenesis.

Keywords
antibodies, B-cell epitope, Epstein–Barr virus nuclear antigen-1, infectious mononucleosis, monozygotic twins, multiple sclerosis

Introduction
In spite of the robustness of sero-epidemiological data, explanations for the association between Epstein–Barr virus (EBV) and multiple sclerosis (MS) are lacking. The presence of EBV infection in the MS brain is controversial1 and the search for qualitative changes that may characterize the immune response to EBV in MS has been less intense than quantitative observations.2 Important clues are emerging from studies that are beginning to delineate specificities of the humoral immune response to Epstein–Barr nuclear antigen-1 (EBNA-1).3,4 The combination of DRB1*1501 haplotype with high-titer responses to the whole EBNA-1 protein5 or to a particular sequence domain3 markedly increased the risk of MS. To pursue these findings we analyzed the fine specificity of the humoral immune response to EBNA-1 in MS-discordant monozygotic (MZ) twins, a setting that considerably decreases the influence of heritable and non-heritable confounders.6,7

Methods
Nine pairs of MS-discordant MZ twins (F/M = 7/2; mean age 32.56 ± 6.82) were selected as described previously.7 Three healthy MZ twin pairs (F/M = 2/1; mean age 34 ± 2.64), 3 non-twin MS patients (F/M = 2/1; mean age 32.67 ± 5.68) and 2 healthy subjects (F/M = 2/0; mean age 30.5 ± 2.12) were also included in

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this study. Only one patient (a co-twin) was under interferon-β therapy at the time of sampling. All samples were obtained at least 3 months after the last steroid therapy. The local ethics committee approved the study and all participating subjects gave written informed consent. The following commercially available enzyme-linked immunosorbent assay (ELISA) kits were used to detect antibodies against select EBV proteins: Captia™ EBNA-1 IgG, VCA(P-18) IgG and Captia™ EA-D IgG; Wampole Laboratories™ EBV VCA IgG; and EBNA-1 IgG by a standardized in-house ELISA. In addition, IgG antibodies against two select peptides of EBNA-1, EBNA-72 [aa: GAGGGAGAGG] and EBNA-206 [aa: EADYFEYHQEPPGPDGE] constructed on a branching poly-lysine backbone, were detected through ELISA using a method adapted from that published previously. Sera were evaluated for antibodies to all unique, maximally overlapping octapeptides of EBNA-1 by solid phase ELISA.

All commercial ELISAs were normalized based upon the use of duplicate calibrators per plate using the instructions provided by the manufacturer. In addition, all assays had to meet pre-determined quality measures based upon the optical density readings of the positive, negative and blank controls. EBNA-1 and EBNA-1 peptide ELISAs were normalized to a positive control of known concentration that was allowed to develop to a specific optical density (OD) on each plate. The average OD of each sample (run in duplicate) was then multiplied by the inverse of the mean of the positive control OD. Samples were considered positive if they had a normalized OD of greater than 0.3. Samples tested on solid phase epitope mapping for EBNA-1 were normalized with a set standard positive control which is run in at least triplicate wells on each plate as described above for EBNA-1 and EBNA-1 peptide ELISAs.

Statistical analysis was performed using Graph Pad Prism 5. Statistical significance was expressed as a p-value and was generated using an F-test without corrections.

Results

Out of the 12 pairs of MZ twins (9 MS-discordant and 3 healthy), non-twin patients, and healthy subjects, all individuals tested positive for IgG toward EBV-VCA (as measured against a historical total VCA lysate; Wampole Laboratories) and EBNA-1, with the exception of two EBNA-1 negative subjects from a pair of healthy twins. There were no significant differences in seropositive frequencies for EBNA-72, and EBNA-206 between affected and healthy co-twins and between non-twin patients and non-twin healthy controls. MS-discordant twin sets did not exhibit higher within-pair variability in their antibody responses against EBV-VCA, EBNA-1, EBNA-72, or EBNA-206 compared with healthy twin sets.

Complete analysis of the unique maximally overlapping octapeptides of the full-length EBNA-1 protein showed that, as expected, the glycine-alanine rich domain harbored the epitopes that were recognized more frequently by sera from EBNA-1 seropositive individuals, irrespective from their MS or healthy status (Figure 1). However, the level of response to an epitope in the C-terminal third of the protein (aa. 401–411, GRRPFFHPVGE) differed between affected and healthy identical co-twins, \( p = 0.006 \) (Figure 2A). The two EBNA-1-positive healthy MZ twin pairs showed a profile comparable to the healthy co-twins of disease discordant pairs. The overall comparison between the EBNA-1-positive patients and controls confirmed an increased response to the 401–411 epitope in MS \( p = 0.002 \) (Figure 2B).

Patients and healthy individuals of the present study are part of a cohort that was thoroughly investigated by means of an 80-item questionnaire on risk factors for MS. A review of these records in each twin pair did not disclose associations with the response to the 401–411 epitope other than the MS status.

Figure 1. Average reactivity of sera from 12 multiple sclerosis (MS) patients and 15 healthy subjects against the maximally overlapping octapeptides of Epstein-Barr virus nuclear antigen-1 (EBNA-1). MS sera recognize two major areas of EBNA-1. One of these regions (arrow) is not identified in the healthy individuals.
In particular, history of infectious mononucleosis (IM) or smoking did not correlate with the pattern of humoral response to EBNA-1. The same was true for the serology to human herpesvirus-6, varicella zoster, herpes simplex 1-2, human cytomegalovirus, measles virus and Bordetella pertussis.11

Discussion

In a fine specificity study that hinges on a series of MS-discordant MZ twins, hence controlling for major founders, we show that the humoral immune response to EBNA-1 in MS frequently comprises an epitope that overlaps with a portion of EBNA-1 which is targeted during IM.8 This specificity wanes over time in asymptomatic virus carriers. The epitope that we identified narrows down other EBNA-1 specificity domains (amino acids 385–420; amino acids 394–451)3,4 that had been described as associated with an increased risk of MS. These findings may provide an interpretative key for two consistent epidemiological observations: the association of MS with EBV serology and with a history of IM. IM is an immunopathologic disease ‘per se’ since its symptoms correlate, also temporally, with the immunologic alterations and not necessarily with virologic changes. It has a profound impact on the immune system: during the acute phase, up to 10% of the B cells are infected and 1–40% of the CD8+ T cells are specific for EBV antigens. To cut short an immune-mediated disease that may become self-perpetuating, and to restore a repertoire that is not monopolized by specificities against a single pathogen, the immune system is deeply reset in the aftermath of the disease. The enduring presence of a B-cell response that normally wanes after IM may increase and prolong the risk of immunopathology. During IM, IgM are produced that recognize the glycine–alanine portion of EBNA-1. These IgM can cross-react with similar epitopes on host proteins since DNA sequences encoding glycine–alanine regions are present on all human chromosomes except the Y chromosome. During convalescence from IM the response is shifted to IgG that have lost affinity for host epitopes.12 Similarly, the persistence of a high B-cell response to the GRRPFFHPVGE sequence may be a potentially harmful circumstance since this motif encompasses the RRPFF epitope that has homology with zB-crystallin, a candidate autoantigen in MS.13 Notably, EBV infection can induce expression of zB-crystallin in B cells and its HLA-DR restricted presentation to T cells.14 Importantly, there are antibodies against the RRPFF sequence in the MS cerebrospinal fluid (CSF).15 Such scenarios are plausible after both subclinical infection and IM since the latter ‘magnifies but does not distort’ the events of the former16 although, in accordance with epidemiological data in MS, the potent immune activation that occurs during IM may increase the risk of subsequent immunopathology. Future studies on T-cell response and CSF reactivity to EBNA-1 immunodominant regions, including the GRRPFFHPVGE sequence, are planned in MS and IM patients as well as in control subjects.

Figure 2. Humoral response to the octapeptides within the 401–411 (GRRPFFHPVGE) sequence of Epstein–Barr nuclear antigen-1 (EBNA-1) in multiple sclerosis (MS)-discordant monozygotic (MZ) twins (A) and in sera from all EBNA-1 positive individuals (B). In (A), the twins of each pair are linked by a line. In (B), horizontal lines represent mean absorbance values of the octapeptides within the 401–411 sequence. An F-test was used to analyze the data. MS = multiple sclerosis; H = healthy.
They will help to confirm and refine the role of immune responses to EBNA-1 in MS development.

At variance with our results and previous studies, the antibody response in pediatric MS primarily targets the glycine–alanine portion of EBNA-1 in both patients and controls, with patients recognizing a broader number of epitopes. This result suggests that the immune response to EBNA-1 may vary according to the age/developmental state of the immune system upon primary infection. In this context the recognition of specific EBNA domains in susceptible individuals may influence the onset of MS during childhood.

Our result derives from the analysis of a series that prevalently includes MS discordant MZ twins, implying that the observed difference can be, at least in part, independent of host genetic and epigenetic influences. Variations in viral genotypes may be an alternative explanation. Nonetheless, data from studies that investigated possible associations between polymorphisms of different EBV genes did not yield clear-cut results to date. A third possibility could be a more stochastic risk depending on how previous infections have shaped the immunologic memory of an individual at the time when a new pathogen intersects their immune system. This process, termed ‘heterologous immunity’, can have an influence on the outcome of an infection and may be particularly relevant in the case of EBV, given the propensity of EBV-specific T cells to cross-react with various self and non-self epitopes, including cross-activities at the level of CD4+ central memory T cells, once again between EBNA-1 and myelin antigens. It may also help explain why epidemiological and immunological studies on twins have often associated multiple infectious events and pathogens to MS but have eluded the identification of a single microbial culprit.

This study in MS-discordant MZ twins refines an EBNA-1 epitope specificity associated with MS and supports its pathogenetic relevance. Be it a failure of the affinity maturation process or a defect of other homeostatic responses after EBV infection, the persistence of an immune response that is generally confined to the acute phase of IM may foster a virus-induced immunopathology of the CNS.

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Conflict of interest statement
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