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# Antibodies against Epstein-Barr nuclear antigen (EBNA) in multiple sclerosis CSF, and two pentapeptide sequence identities between EBNA and myelin basic protein

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**Article abstract**—The Epstein-Barr virus (EBV) causes infectious mononucleosis and is linked to several disparate malignancies. Prior studies on patients with multiple sclerosis (MS) showed that 100% are EBV-seropositive and that their blood contains higher antibody titers than those of controls to both transformation and lytic cycle antigens. We performed three different assays for antibodies in CSF to three major EBV antigens from patients with MS and controls. Among 93 patients with MS, 79 (85%) had CSF that reacted with a 70 kD protein, shown to be the nuclear antigen, EBNA-1, whereas only 11 (13%) of 81 EBV-seropositive controls reacted,  $p < 0.001$ . The CSF of all 14 MS patients, unreactive on immunoblots, contained oligoclonal bands on agarose electrophoresis. Together, the two techniques exhibit 100% sensitivity in the confirmatory diagnosis of MS. We also performed amino acid searches of the Protein Identification Resource sequence database for protein homologies to EBNA. Two pentapeptide identities were found between EBNA-1 and myelin basic protein: QKRPS and PRHRD. None of more than 32,000 other proteins in the database contained both pentapeptides. In healthy EBV-seropositive persons, the EBV-specific, MHC-restricted T lymphocytes keep the EBV-containing B lymphocytes locked in the transformed state. However, in the host genetically susceptible to MS, the same population of lymphocytes might recognize and interact with either of the two identified pentapeptides, inadvertently damaging MBP.

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Burkitt's original epidemiologic observations suggested a role for an infectious agent in the pathogenesis of the jaw tumor, Burkitt's lymphoma (BL), which affects children living in the endemic malaria belt of central Africa.<sup>1</sup> Epstein et al<sup>2</sup> first observed the virus with the electron microscope in tumor cells cultured from patients with BL. Subsequently, Henle et al<sup>3</sup> and Niederman et al,<sup>4</sup> using vigilant clinical observations and extensive seroepidemiologic methods, showed that the Epstein-Barr virus (EBV) causes infectious mononucleosis. Worldwide studies have shown that all biopsies from undifferentiated nasopharyngeal carcinoma tumor cells contain EBV DNA in varying quantities.<sup>5</sup> The critical role of host immune mechanisms in responding to an EBV infection is dramatically exemplified by the EBV-

associated diseases in humans, including lymphoproliferative disorders in patients who are immunosuppressed therapeutically,<sup>6</sup> opportunistically,<sup>7</sup> or genetically.<sup>8</sup>

Multiple sclerosis (MS), the most common progressive demyelinating disease in the economically privileged western world, has been recognized as a clinicopathologic entity for more than 150 years. The cause and pathogenesis remain unclear, but one finds a consensus that abnormalities in both humoral and cellular immunity occur consistently.

Kurtzke<sup>9</sup> and Kurtzke and Hyllested<sup>10</sup> have carried out extensive epidemiologic studies, including (1) planetary incidence and prevalence surveys, (2) prevalence of MS in migrants, and (3) epidemics in the Faroe Islands and Iceland. They have suggested that one or more environmental factors may

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influence pathogenesis; Kurtzke<sup>9</sup> has stated, "If these findings (epidemics among the Faroese and Icelanders) are valid, both would indicate the definition of MS as not only an acquired disease but also a transmittable one." However, despite years of effort to incriminate a causal environmental factor, no agent has been recovered consistently from, or seen in, affected CNS tissue. Nor has the disease ever been transmitted to experimental animals.

Using seroepidemiologic data, Sumaya et al<sup>11</sup> were among the first to suggest an association between MS and EBV infection. Later, we assayed blood samples from MS subjects and controls, measuring seropositivity rates and antibody concentrations to seven common viruses.<sup>12</sup> Comparing MS and control populations, we found a highly significant difference only for EBV. MS patients showed 100% seropositivity compared with 89% for age- and sex-matched controls ( $p < 0.0001$ ).<sup>13</sup> Serum antibody titers against viral capsid antigen (VCA) were five to six times higher, and against the nuclear antigen (EBNA) they were three to four times higher, at the same level of statistical significance ( $p < 0.0001$ ).<sup>13,14</sup>

Recently, we observed five patients with a neurologically complicated primary EBV infection, in whom four (followed from 4 to 12 years) developed MS, and one had acute disseminated encephalomyelitis that left her, after a 2-year follow-up, with permanent neurologic deficits.<sup>15</sup>

The association between EBV blood seropositivity and MS, along with the aforementioned clinical association between primary EBV infection and demyelinating disease, prompted us to conduct studies on the CSF of MS and control patients. We performed assays of antibodies against the latency-associated antigen, EBNA, and against the lytic cycle antigens, VCA and early antigen-diffuse (EA-D), in MS and neurologic disease controls (NDC). We also carried out searches for amino acid sequence identities between myelin basic protein (MBP) and all other proteins listed in a national protein sequence database.

**Methods. Clinical patient selection.** The diagnosis and available laboratory findings were individually reviewed in a group of 246 patients. A neurologic or neuropsychiatric diagnosis was under consideration in all these patients, and so a CSF examination was performed. MS patients were included in the study only if two or more neurologists considered the diagnosis clinically definite. A clinical diagnosis of MS was made only if the neurologic disorder met standard criteria.<sup>16,17</sup> Confirmatory tests included the established methods for immunoglobulin analysis and brain imaging procedures. No patient was included in the study unless the blood-brain barrier (BBB) was intact, as determined by the ratio of CSF albumin/serum albumin.<sup>18</sup>

Each NDC patient received the same clinical and laboratory scrutiny. Subjects were included in this category only if their serum EBV antibody profile was positive, if they had an intact BBB, and if demyelinating disease was excluded. The diagnostic spectrum of this group ranged from patients with stroke, progressive nonde-

myelinating degenerative disorders, psychiatric disorders, tumor, and infectious-inflammatory diseases, excluding known primary EBV infections, to any patient who presented with complaints referable to the nervous system and in whom the neurologist considered a CSF examination advisable.

**Laboratory techniques. Quantitative and qualitative analyses of CSF IgG.** Approximately 95% of patients with the diagnosis of clinically definite MS exhibit an increased CNS synthesis of IgG or the presence of oligoclonal bands (OCB) in their CSF.<sup>19</sup> OCB were identified by agarose gel electrophoresis.<sup>20</sup>

**Cell lines.** We used several cell lines as sources for the different EBV viral antigens. The BL-derived, non-virus-producing Raji cell line carries the EBV genome and is known to express EBNA-1 constitutively.<sup>21</sup> The virus-producing line, P3HR1, was used as a source of VCA.<sup>22</sup> Raji cells superinfected with virus produced from the P3HR1 cell line were used as a source for expression of EA-D protein.<sup>22</sup> An EBV-genome-negative BL cell line, designated BJAB, was used as a negative control.

**Anticomplement immunofluorescence (ACIF).** Using P3HR1 cells, we employed serial dilutions of CSF to identify antibodies to VCA, as described previously.<sup>22</sup>

**Enzyme-linked immunosorbent assays (EIA).** EIAs were performed using purified EBV EBNA-IgG protein<sup>23</sup> and purified EBV VCA-IgG protein<sup>22-26</sup> (generously provided by Ortho Diagnostics Systems, Inc.) as target antigens and CSF as the antibody source. Methodologic details were performed according to the manufacturer's instructions. Preliminary experiments revealed that a 1:10 dilution distinguished best between background activity and specific antibody in both control and MS CSF samples.

**Western immunoblots.** P3HR1, Raji, and BJAB cells were washed in phosphate-buffered saline, which is 0.136 M NaCl, 2.68 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, and 1.76 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4. Cells were solubilized in 2% sodium dodecyl sulfate (SDS), and 15 µg of protein was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose sheets.<sup>27</sup> The sheets were cut into strips and incubated with individual CSF samples as the primary antibody in a final volume of 0.6 ml. The amount of IgG in each CSF sample was standardized to IgG 1.0 mg/dl in 0.5% bovine serum albumin/0.05% Tween-20 in phosphate-buffered saline. Control sera came from individuals with high-titer antibodies to EBNA-1, as assayed by ACIF. In some experiments, a monoclonal antibody specific for the EA-D antigen was used as the primary antibody in the immunoblots.<sup>22</sup>

**Analyses on the 70 kD protein.** Cell extracts for immunoblotting and peptide mapping were performed by sonicating  $2 \times 10^8$  Raji cells in a buffer containing 150 mM NaCl, 20 mM TRIS-HCl, pH 7.4, 10 mM NaF, 1 mM phenyl methyl sulfonyl fluoride, and 1 mM β-mercaptoethanol. The extract was clarified by centrifugation in a Sorvall centrifuge at  $20,000 \times g$  for 1 hour. The supernatant was removed from the pellet and was heat-treated at 70 °C for 10 minutes to precipitate heat-labile proteins. Then the heat-precipitated proteins were pelleted at  $10,000 \times g$  for 15 minutes. The supernatant was used in SDS-PAGE for immunoblotting, either without further treatment or after incubation with 10 µg/ml chymotrypsin (Sigma, Type VII-TLCK treated) at 37 °C for 12 hours.

**Protein sequence homologies between EBNA-1 and human myelin basic protein.** Using the FASTA program,

we searched the Protein Identification Resource (PIR) sequence database, version 29.0, for amino acid sequence identities between MBP and all other proteins of defined sequence.<sup>28</sup>

**Results** (figure 1). *Anti-EBNA-1 IgG CSF antibodies (EIA and immunoblots).* To identify possible anti-EBV antibodies in CSF samples, we performed EIAs, using purified EBNA-1 protein as the target antigen.

Using Ortho's high-titer positive and negative serum controls, we measured antibodies to EBNA-1 and VCA in the study and control populations.

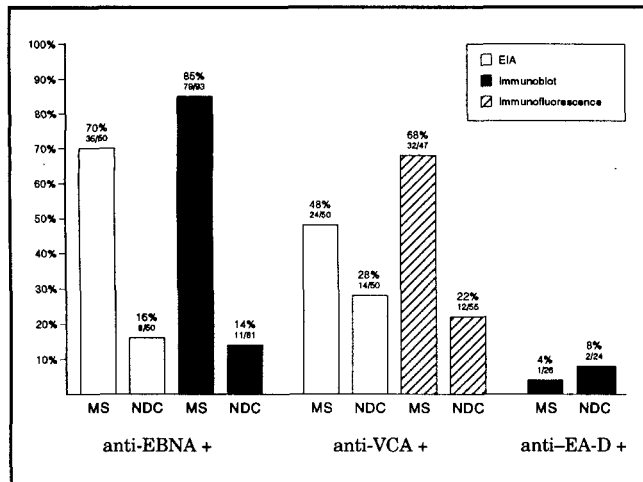


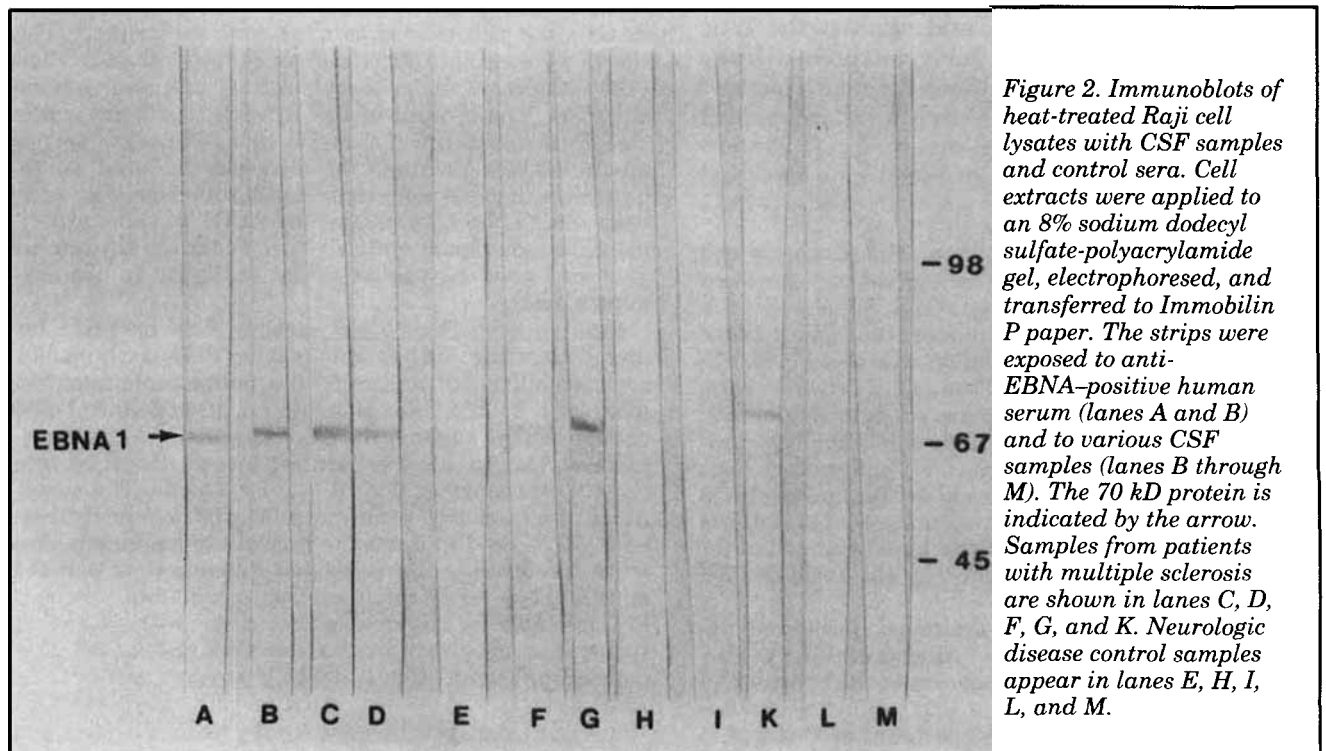
Figure 1. Summary of Epstein-Barr virus CSF antibody data in patients with multiple sclerosis and neurologic disease controls.

Among the MS patients, 35 of 50 (70%) of the CSF samples were EIA-positive compared with eight of 50 (16%) NDC, results that differed significantly:  $\chi^2 = 29.74$ ,  $df 1$ ,  $p < 0.001$  (figure 1). These results suggested the presence of a protein in the CSF of patients with MS that reacted with the EBNA-1 protein in this assay.

To rule out the possibility of a contaminating immunoreactive, non-EBNA-1 antigen in the EIAs, we then performed Western immunoblots on both untreated and heat-treated Raji cell extracts using whole CSF as the primary source of antibody. An additional group of 174 MS and NDC patients was studied. The results of the CSF immunoblot analyses from these patients are also summarized in figure 1. Among the CSF samples from patients with MS, 79 of 93 (85%) exhibited a 70 kD protein, and 14 of 93 (15%) were negative. Seventy of 81 (87%) NDC samples were unreactive, and 11 of 81 (13%) did react.

A typical sampling of these results is seen in figure 2. Lanes A and B contain sera from healthy controls, which reacted with a 70 kD protein. Five of the 10 CSF samples, all from patients with the clinical diagnosis of MS, also reacted with a co-migrating 70 kD protein (lanes C, D, F, G, and K). The CSF from five NDC (lanes E, H, I, L, and M) did not react. These results support the findings of the EIA, indicating that this antibody in the CSF reacts with a heat-stable protein. EBNA-1 is known to migrate with a Mr~70 kD protein in SDS-PAGE.

To rule out the possibility of a coincidentally co-migrating protein, we sought supporting evidence that the 70 kD protein was, in fact, EBNA-1. We



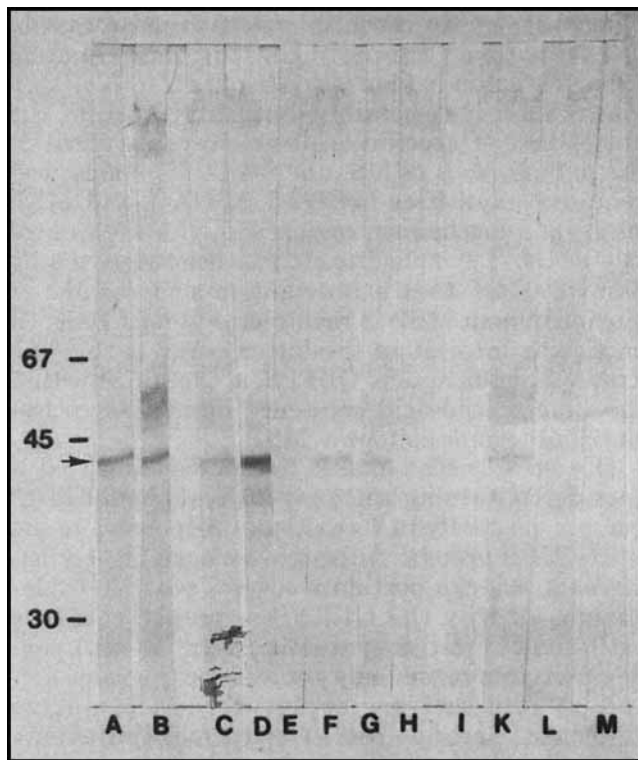


Figure 3. Immunoblots of heat-treated and chymotrypsin-digested Raji cell extracts. Treated extracts were applied to a 10% sodium dodecyl sulfate polyacrylamide gel, electrophoresed, and transferred to Immobilon P paper. The 40 kD peptide is indicated by the arrow. The serums and CSF samples are identical with and appear in the same alphabetized lane sequence as those shown in figure 2.

incubated the Raji cell extracts with chymotrypsin to determine a limited peptide map of the 70 kD protein. Figure 3 shows the results obtained when CSF was reacted with these digested Raji cell extracts. As in figure 2, the same two positive sera and five MS CSF samples reacted, but with a smaller peptide of Mr~40 kD. The NDC, as in figure 2, did not identify this fragment.

**Anti-VCA CSF IgG antibody (ACIF and EIA).** Previous work by us, using the Raji cell line, has shown that the VCA antigen (160 kD) cannot be detected by Western blotting (unpublished observations). In the standard serum assay for identification of anti-VCA antibodies, ACIF is performed on P3HR1 cells. We modified this procedure by using CSF as the antibody source. Antibodies to VCA were present in 32 of 47 (68%) MS CSF samples and in 12 of 55 (22%) NDC (figure 1). Geometric mean titers of anti-VCA from 77 patients with MS and 41 NDC, respectively, were 2.79 compared with 1.41 in CSF, and 292 compared with 218 in serum. The two groups differed statistically but the absolute differences were small: CSF  $\chi^2 = 22$ ,  $df$  3,  $p < 0.001$ ; serum  $\chi^2 = 18.5$ ,  $df$  44,  $p < 0.001$ . The BBB had no influence on the statistically higher concentrations in the MS population:  $r = 0.00$  (Pearson's correlation coefficient).<sup>29</sup>

Figure 1 shows that on EIA, 24 of 50 (48%) MS CSF samples contained anti-VCA antibodies compared with 14 of 50 (28%) NDC that exhibited seropositivity, results that differed slightly from one another:  $\chi^2 = 4.24$ , 1  $df$ ,  $p < 0.05$ .

**Anti-EA-D antibody (immunoblots and ACIF).** To detect antibodies against the EA-D antigen, we probed superinfected Raji cells by using CSF on Western blots, as above. A monoclonal antibody specific for EA-D was used to identify its position on the filter. We studied 50 additional CSF specimens, 26 samples from MS and 24 from NDC patients. The CSF from one patient with MS and two NDC patients reacted with a peptide that comigrated with the protein identified by the monoclonal anti-EA-D antibody (figure 1), findings that appear insignificantly different.

ACIF has been performed on 177 CSF specimens, mostly from MS and NDC patients. EA-D was measurable in only one sample, from a patient with a primary T-cell brain lymphoma (unpublished observations).

**CSF OCB and immunoblot data.** Of note is that all 14 MS patients who lacked antibodies in their CSF to EBNA-1 on immunoblot assays demonstrated CSF IgG OCB on agarose electrophoresis. By contrast, the 11 NDC patients who were anti-EBNA antibody-positive had normal CSF quantitative and qualitative IgG. No obvious clinical differences in the 14 MS patients whose CSF was anti-EBNA-negative were found when compared with the MS population as a whole. We conclude that, in this study, by applying a combination of agarose electrophoresis and immunoblotting, we have been able to identify 100% of MS patients.

**Protein sequence identities between EBNA-1 and MBP.** The finding of an anti-EBNA-1 antibody in the CSF of 85% of our patients with MS led us to ask whether any protein similarities existed between EBNA-1 and MBP in view of the fact that the latter is conspicuously lost or damaged in the CNS of patients with MS. We used the FASTA program to search the PIR sequence database for identities (see Methods). Short identical matches were found for two separate five-amino-acid sequences between EBNA-1, a protein containing 641 amino acids, and human MBP, a protein containing 169 amino acids. Using the conventional one-letter amino acid code,<sup>30</sup> we found two separate pentapeptides, QKRPS and PRHRD. QKRPS corresponds to published amino acid positions 74 to 78 in EBNA-1 and 3 to 7 in MBP. PRHRD corresponds to published amino acid positions 64 to 68 in EBNA-1 and 30 to 34 in MBP.<sup>31,32</sup>

The FASTA program was then used to search the PIR database for amino acid sequences containing both QKRPS and PRHRD. Among approximately 32,000 proteins in the database, only EBNA-1 and MBP contained both sequences.

**Discussion.** Our data show that the CSF of patients with MS, when compared with controls,

contains excessive amounts of antibodies directed against EBNA-1. Several lines of evidence indicate that these antibodies are specific for EBNA-1. First, elevated anti-EBNA-1 titers in the CSF were detected in an EIA using purified EBNA-1 as the target antigen. Second, CSF reacts with a 70 kD protein on Western blots of Raji cells, which are known to express EBNA-1, and 70 kD is the expected Mr of EBNA-1.<sup>22</sup> Unlike over 80% of the cellular proteins that are heat-aggregated at 70°C, EBNA-1 remains soluble at this temperature,<sup>33</sup> as does the peptide we have identified on Western immunoblots. Third, Hennessy and Kieff<sup>34</sup> have shown that EBNA-1 contains a domain that includes a glycine-alanine copolymer. Although this region cannot be cut or digested by many proteases (personal observations, J. Luka), digestion by chymotrypsin yields a 40 kD immunoreactive fragment containing the glycine-alanine copolymer typical of EBNA-1 in Raji cells. The unique glycine-alanine repeating sequence migrates as a ~40 kD peptide on SDS-PAGE and will react with anti-EBNA-1 positive sera. It has been shown in the general population that nearly 100% of the anti-EBNA antibody-positive human sera recognize this domain, so this proteolytic cleavage fragment can be used for identification of anti-EBNA-1 antibodies.<sup>35</sup> When chymotrypsin was used to digest the Raji cell extracts, the CSF samples from patients with MS reacted with a 40 kD peptide. Taken together, these data strongly support the presence of excessive anti-EBNA-1 antibody in the CSF from patients with MS.

The results presented in this report can be viewed arbitrarily from two vantage points—one with a view toward possible mechanisms involved in the pathogenesis of MS and one with minor diagnostic implications.

For more than a generation, many investigators studying MS have been devoted to the idea that demyelination results from an autoimmune process. Several lines of evidence support this belief. First, the classic animal model for T-cell-mediated autoimmune disease, experimental allergic encephalomyelitis (EAE), has an almost identical histopathologic resemblance to MS. However, there are crucial differences between EAE and MS. The former is an artificially induced paralytic disorder that develops in animals that have been injected with a major protein constituent of myelin, whereas MS is a naturally occurring disease of humans in which genetic and environmental factors are incompletely understood. Second, at least 13 different viruses share limited amino acid sequence homology with human MBP.<sup>36,37</sup> Third, within the past few years a series of reports<sup>38-41</sup> has shown that T cells isolated from MS patients react with restricted synthetic peptides derived from the MBP sequence. These observations, along with the ability of T cells taken from animals with EAE to prevent or reverse EAE by vaccination of unaffected animals, have reinforced the suspicion that T lymphocytes

play an essential role in the process of demyelination.<sup>42</sup> Precisely how the demyelination process is set in motion is still unclear.

Although it is possible, but unlikely, that the anti-EBNA-1 specific antibodies are involved in the pathogenesis of MS, our finding of amino acid sequence identities between EBNA-1 and MBP suggests a mechanism compatible with the central role of the T lymphocyte. It has been reported by Zamvil et al<sup>43</sup> that a nine-amino-acid residue of murine/human MBP is required to induce EAE. Of particular interest in the latter study is that the EBNA-1 pentapeptide, QKRPS, is contained within the nine-amino-acid sequence identified in the encephalitogenic epitope of MBP.

Ota et al<sup>41</sup> were unable to demonstrate that a peptide containing the first 20 residues of MBP induced reactivity in T-cell clones responsive to the entire MBP protein. Although we have no conflicting data, we can postulate several possible explanations of why the QKRPS sequence could be pathogenic. First, a synthetic 20-amino-acid peptide from this region may not assume the same secondary and tertiary structure as the native molecule. Second, the *in vitro* manipulations required to generate T-cell clones reactive to MBP may not reflect an accurate representation of the *in vivo* response. Third, as pointed out by Zamvil et al,<sup>43</sup> production of EAE in the mouse requires the acetylation of the amino terminus of the peptide containing residues 1 to 9 of MBP. Ota et al did not describe this acetylation modification in the first 20-amino-acid residue they synthesized from MBP.<sup>41</sup> Fourth, the highly basic region of EBNA-1 could conceivably induce charge-dependent cross-reaction to MBP. Finally, it is not unreasonable to speculate that a heterogeneous group of molecular defects causes the MS phenotype.

Our observation of modest elevations in both blood and CSF anti-VCA antibodies may be relevant in the light of Rickinson's *in vitro* studies<sup>44</sup> of EBV-transformed B cells. He and his coworkers have shown that although most B cells remain permanently locked in the transformation cycle, a small percentage of cells enters the lytic cycle, releasing virus particles. If this mechanism occurs *in vivo*, one would expect the production of anti-VCA antibody.

The diagnosis of MS remains clinical because no specific laboratory test has yet been developed. Nevertheless, it is now known that approximately 95% of patients with MS exhibit an increased quantity or an abnormal quality (OCB) of IgG in their CSF on agarose gel electrophoresis, the simplest technique for the routine diagnostic laboratory. The authors readily acknowledge that qualitative IgG changes may be demonstrable in almost 100% of MS CSF if SDS-PAGE or sensitive immunofixation techniques, rather than agarose, are used. However, our experience at this time convinces us that these more sensitive methods, as well as Western blots, require interpretation by an expert

devoted to the study of CSF immunoglobulins.

As a rule, in healthy EBV-seropositive persons, the EBV-specific, class II, MHC-restricted T lymphocytes keep the EBV-genome-containing B cells locked in the transformed state. However, in the host genetically susceptible to MS, the same population of T cells could recognize and interact with either of the highly charged epitopes, QKRPS or PRHRD, inadvertently damaging MBP. Further studies will be required to address this hypothesis.

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