Epstein-Barr Virus and Multiple Sclerosis

Evidence of Association From a Prospective Study With Long-term Follow-up

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Objective: To determine whether serum titers of anti–Epstein-Barr virus (EBV) antibodies are elevated in blood specimens collected up to 30 years prior to onset of multiple sclerosis (MS).

Methods: Individuals with MS were identified among members of the Kaiser Permanente Northern California health plan who participated in the multiphasic examinations administered between 1965 and 1974. Stored serum samples were used to compare anti-EBV antibody titers in 42 individuals who developed MS with age-matched and sex-matched controls.

Results: The geometric mean titers of antibodies to the Epstein-Barr nuclear antigen (EBNA) complex and its component EBNA-1 were significantly higher in the MS cases when compared with matched controls. The relative risk of MS associated with a 4-fold increase in antibody titers was 2.1 (95% confidence interval, 1.1-3.8) for the EBNA complex and 1.8 (95% confidence interval, 1.1-2.9) for EBNA-1. Elevations of antibody titers to the EBNA complex and EBNA-1 among MS cases first occurred between 15 to 20 years before the onset of symptoms and persisted thereafter.

Conclusion: The elevation of anti-EBV titers is probably an early event in the pathogenesis of MS and is unlikely to be the result of an aspecific immune dysregulation.

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bers provided blood specimens that were processed and stored as sera frozen at −20°C. These sera were retained by KPNC until 1979, when they were transferred to the serum treasury of the Orentreich Foundation for the Advancement of Science (OFAS) in Cold Spring-on-Hudson, NY, for cataloging and indefinite frozen storage at −40°C.

Kaiser Permanente Northern California has maintained medical records of all members who provided serum specimens and who were KPNC members for all or some of the time between the administration of the multiphasic examination and 1999 (maximum time period, 1965-1999). Kaiser Permanente Northern California began computerizing inpatient hospitalization data in 1984 with the establishment of an admission, discharge, and transfer (ADT) hospitalization database. All outpatient encounters at KPNC hospitals, medical centers, and medical offices are stored electronically in a database called the outpatient summary clinical record (OSCR). The database, which was created in 1993 and implemented in all facilities by December 1994, uses more than 40 different optically scannable medical specialty-specific forms. The appropriate OSCR form is generated at the time of registration and contains a check-off list for the most commonly used diagnoses and procedures. The data in the OSCR contains, but is not limited to, medical record number, registration information, and procedure/diagnosis codes for each outpatient encounter. *International Classification of Diseases, Ninth Revision, Clinical Modification and Current Procedural Terminology, Fourth Revision* codes were used for diagnoses and procedures.

**CASE ASCERTAINMENT AND CONTROL SELECTION**

In the current study, during the years between 1995 and 1999, both the ADT and OSCR were searched for evidence of medical coding that would indicate a potential diagnosis of MS among active KPNC members. More than 2000 potential MS cases were identified and then linked to the OFAS serum treasury specimen database. The results of this linkage indicated that 93 potential MS cases had stored serum specimens. Manual review of medical records using an abstraction instrument designed for the Centers for Disease Control and Prevention’s Demyelinating Diseases Study was undertaken to confirm the MS diagnosis for the 93 potential cases. A medical records analyst recorded dates of onset, and duration of neurologic symptoms (eg, transient sensory, memory, or vision), as well as dates and duration of hospitalization and other medical procedures. The data in the OSCR contains, but is not limited to, medical record number, registration information, and procedure/diagnosis codes for each outpatient encounter. *International Classification of Diseases, Ninth Revision, Clinical Modification and Current Procedural Terminology, Fourth Revision* codes were used for diagnoses and procedures.

To account for matching, we used conditional logistic regression to estimate the relative risk of MS associated with a 4-fold increase in antibody titers. Under the design of our study, these relative risks estimate the corresponding incidence rate ratios. Unconditional logistic regression was also used in analyses including all controls (matched and unmatched) to increase power. These analyses also included controls originally matched to cases excluded from the analyses because of missing serum samples before baseline (n=132). All statistical tests were 2-sided, and P values of less than .05 were considered statistically significant. Point estimation was via maximum likelihood; statistical tests were based on the likelihood ratio sta-

**LABORATORY ANALYSES**

Serum samples for cases and controls were aliquotted into cryovials at OFAS for overnight shipment to Virolab Inc (Berkeley, Calif) in triplicates of 1 case and 2 matched controls in random order. Staff at OFAS and Virolab were blinded to case-control status and unidentified triplets were included as assay quality controls.

**STATISTICAL ANALYSIS**

Geometric mean antibody titers (reciprocal of the dilution) in cases and controls were compared using generalized linear models. Subjects with missing values for any of the covariates were excluded from the analyses.

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We excluded from analyses 4 seronegative (VCA <10) cases, 8 controls matched to these cases, and all seronegative controls. Table 1 lists the demographic characteristics of the remaining 42 MS cases and 79 controls. The median age at onset of MS was 45 years (mean ± SD, 46 ± 11 years; range, 24-69 years) and the median time between baseline blood collection and onset of MS was 15 years (mean ± SD, 15 ± 8.9 years; range, <1-32 years).

Geometric mean titers of EBV and CMV antibodies in cases and controls are presented in Table 2. Geometric mean titers to the EBNA complex and to EBNA-1 were significantly higher among MS cases when compared with matched controls (P = .007 and P = .01, respectively) and when compared with all controls (P = .001 and P = .004, respectively). Geometric mean titers to EBNA-2 were not significantly higher among MS cases when compared with matched controls, but were significantly elevated when compared with all controls. Geometric mean titers for IgG to EBV VCA, IgA to EBV VCA, IgG to anti–early antigen (diffuse and restricted), and CMV were not significantly elevated in MS cases. The relative risks of developing MS associated with a 4-fold increase in EBV and CMV antibody titers are presented in Table 3. Significantly elevated relative risks were observed for the EBNA complex and EBNA-1 antibodies in analyses including only matched controls or all controls. Excluding cases with onset at age 60 years or later (n = 4) did not materially change these results.

To examine temporal relationships between antibody responses to EBV and MS onset, we plotted the mean of anti-EBNA titers in MS cases as a percent of their matched controls’ means by the time between blood collection and MS onset. Elevations in both the anti-EBNA complex and anti–EBNA-1 titers among the cases became evident between 15 and 20 years before the first onset of neurological symptoms of MS and remained constant thereafter (Figure).

**RESULTS**

In each cohort, significant elevations in antibodies to the EBNA complex or EBNA-1 were found in serum or plasma collected several years before the onset of MS symptoms. Changes in antibody titers to other EBV antigens (VCA, EBNA-2, and early antigens) were more variable across the studies. The consistency of results for the EBNA complex and EBNA-1 is quite striking considering the markedly different age and ethnic composition of the study populations. Similar to our study, the Nurses’ Health Study mostly included individuals with late age at MS onset (median, 52 years; range, 39-66 years) and EBV antibody titers were measured in samples collected between several months before the onset of MS up to several years after MS onset; the mean age at blood collection was 49 years (range, 34-65 years). The anti-EBNA complex and anti–EBNA-1 titers were significantly elevated in plasma collected before the onset of MS and did not change significantly after MS onset. In the study of US Army personnel, which included a much younger population (mean age at MS onset, 27 years; range, 18-41 years; mean age at baseline blood collection, 24 years; range, 17-39 years), repeated samples (up to 3 per subject) were collected between 1 and 11 years before MS onset. Serum titers of antibody to the EBNA complex and EBNA-1 in individuals who later developed MS increased sharply in early adulthood—from means similar to those of controls at younger than 20 years to means 2- to 3-fold higher at age 25 years—and remained constant thereafter. Finally, in the Vasterbotten study, blood samples were collected at a median of 7 years before MS onset. The median age at blood collection was 28 years (range, 17-59 years) and the median age at MS onset was 34 years (range, 22-65 years). A significant association between anti–EBNA-1 titers and risk of MS was already present in samples collected more than 5 years before MS onset, but became more pronounced in the 5-year period preceding MS onset. This strengthening of the association could be explained by an increase in antibody titers in early adulthood, as seen in the US Army study, because individuals with MS with blood collected more than 5 years before onset were presumably younger than...
those with blood collected soon before onset. Although an increase in EBV antibody titers is observed in other EBV-related conditions, a specific increase in anti–EBNA-1 and the anti-EBNA complex without a concomitant increase in anti-VCA is unique to MS and is consistent with the hypothesis of T-cell hyperreactivity because anti–EBNA-1 titers are positively correlated with T-cell function.14

Overall, the results of these studies are consistent with the hypothesis that elevations in the anti-EBNA complex and anti–EBNA-1 titers in individuals who develop MS occur in the late teenage years or early 20s and may precede the time of onset of MS symptoms by 10 years or more. This hypothesis needs to be tested in further studies including teenagers and young adults. In the current study, only 2 individuals with MS had blood drawn before the age of 20 years; therefore, we could not examine changes in antibody titers in this age range. If confirmed, this finding would indirectly support the concept of an age of vulnerability for the acquisition of MS, which according to Kurtzke,15 would start at puberty and end in early adulthood.

A critical question is what causes the increase in the anti-EBNA complex and anti–EBNA-1 titers in early adulthood? Two possibilities deserve careful consideration. One possibility is that the triggering event is an infection with a separate microorganism that alters the host-EBV balance, perhaps by activating EBV-specific memory T-cells (heterologous T-cell immunity).16 The other possibility is reinfection with a strain of EBV different from that originally carried by the host. Two types of EBV circulate in human populations and they are identified as type 1 (or type A) and type 2 (or type B).17 Type 1 EBV predominates in the United States and Europe,18 but infection with type 2 is not uncommon. Other substantial variations exist in the EBV genotype, which are probably caused by geno-

### Table 2. Geometric Mean Titers of EBV/CMV Antibodies for MS Cases With Blood Collected Before Onset and Matched/All Controls*

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>MS Cases (n = 42)</th>
<th>Matched Controls (n = 79†)</th>
<th>P Value</th>
<th>All Controls (n = 132†‡)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG to EBV VCA</td>
<td>718</td>
<td>657</td>
<td>.54</td>
<td>633</td>
<td>.36</td>
</tr>
<tr>
<td>IgA to EBV VCA</td>
<td>4</td>
<td>4</td>
<td>.59</td>
<td>4</td>
<td>.49</td>
</tr>
<tr>
<td>EBNA complex§</td>
<td>320</td>
<td>186</td>
<td>.007</td>
<td>174</td>
<td>.001</td>
</tr>
<tr>
<td>EBNA-1§</td>
<td>299</td>
<td>162</td>
<td>.01</td>
<td>152</td>
<td>.004</td>
</tr>
<tr>
<td>EBNA-2§</td>
<td>13</td>
<td>10</td>
<td>.43</td>
<td>8</td>
<td>.04</td>
</tr>
<tr>
<td>Diffuse early antigen</td>
<td>4</td>
<td>4</td>
<td>.98</td>
<td>4</td>
<td>.75</td>
</tr>
<tr>
<td>Restricted early antigen</td>
<td>3</td>
<td>3</td>
<td>.48</td>
<td>3</td>
<td>.48</td>
</tr>
<tr>
<td>CMV</td>
<td>25</td>
<td>23</td>
<td>.73</td>
<td>22</td>
<td>.55</td>
</tr>
</tbody>
</table>

Abbreviations: CMV, cytomegalovirus; EBNA, Epstein-Barr nuclear antigen; EBV, Epstein-Barr virus; KPNC, Kaiser Permanente Northern California; MS, multiple sclerosis; VCA, viral capsid antigen.

*Individuals with MS were identified among members of the Kaiser Permanente Northern California health plan.
†Three controls were discordant for VCA IgG and the EBNA complex and therefore excluded.
‡Unconditional logistic regression adjusted for age at blood collection.
§For 2 cases and 1 control, IgG titers for the EBNA complex, EBNA-1, and EBNA-2 could not be determined because of aspecific binding.

### Table 3. Relative Risk Associated With a 4-fold Increase in Titers of Antibodies to EBV and CMV in MS Cases With Blood Collected Before Onset and Matched/All Controls*

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>All MS Cases (n = 42)</th>
<th>Matched Controls (n = 79†)</th>
<th>P Value</th>
<th>All Controls (n = 132†‡)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG to EBV VCA</td>
<td>1.2 (0.66-2.4)</td>
<td>.50</td>
<td></td>
<td>1.3 (0.75-2.3)</td>
<td>.36</td>
</tr>
<tr>
<td>IgA to EBV VCA</td>
<td>1.1 (0.68-1.9)</td>
<td>.63</td>
<td></td>
<td>1.2 (0.75-1.8)</td>
<td>.49</td>
</tr>
<tr>
<td>EBNA complex§</td>
<td>2.1 (1.1-3.8)</td>
<td>.02</td>
<td></td>
<td>2.5 (1.4-4.5)</td>
<td>.002</td>
</tr>
<tr>
<td>EBNA-1§</td>
<td>1.9 (1.1-2.9)</td>
<td>.03</td>
<td></td>
<td>2.0 (1.2-3.4)</td>
<td>.006</td>
</tr>
<tr>
<td>EBNA-2§</td>
<td>1.3 (0.81-2.1)</td>
<td>.28</td>
<td></td>
<td>1.3 (0.99-1.7)</td>
<td>.75</td>
</tr>
<tr>
<td>Diffuse early antigen</td>
<td>1.0 (0.64-1.6)</td>
<td>.95</td>
<td></td>
<td>1.2 (0.69-2.2)</td>
<td>.48</td>
</tr>
<tr>
<td>Restricted early antigen</td>
<td>1.2 (0.63-2.2)</td>
<td>.62</td>
<td></td>
<td>1.2 (0.69-1.5)</td>
<td>.55</td>
</tr>
<tr>
<td>CMV</td>
<td>1.1 (0.78-1.5)</td>
<td>.66</td>
<td></td>
<td>1.1 (0.80-1.5)</td>
<td>.55</td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; CMV, cytomegalovirus; EBNA, Epstein-Barr nuclear antigen; EBV, Epstein-Barr virus; KPNC, Kaiser Permanente Northern California; MS, multiple sclerosis; RR, relative risk; VCA, viral capsid antigen.

*Individuals with MS were identified among members of the Kaiser Permanente Northern California health plan.
†Three controls were discordant for VCA IgG and the EBNA complex and therefore excluded.
‡Unconditional logistic regression adjusted for age at blood collection.
§For 2 cases and 1 control, IgG titers for the EBNA complex, EBNA-1, and EBNA-2 could not be determined because of aspecific binding.
netic evolution influenced by HLA-restricted EBV-specific cellular immunity. Munch et al have recently reported that individuals with MS in a small Danish cluster (n=8) were infected with a single subtype of EBV. No other investigations have addressed the possible role of EBV genetic variations in MS. Several variations may influence functions that are critical for the viral life cycle and hence are likely to be relevant for the pathogenesis of EBV-associated diseases, or they may result in mutations in highly immunogenic peptide epitopes and thus influence the T-cell mediated immune response to EBV infection. There is growing evidence that coinfection with multiple EBV strains, either acquired sequentially or simultaneously, is common even in healthy subjects, but little is known about the serologic response or other consequences of reinfection/coinfection.

Although the epidemiologic evidence indicating that people with elevated titers of antibodies directed against the EBNA complex and EBNA-1 are at greater risk of developing MS is compelling, the mechanisms underlying this association are uncertain. A hypothesis that is gaining increasing support is that EBV infection in genetically susceptible individuals activates T-lymphocytes that cross-react with myelin antigens, but other mechanisms have been proposed, such as cross-reacting antibodies, infection of autoreactive B lymphocytes, the activation of superantigens, and an increased expression of αB-crystallin. Recently, 2 EBV peptides, one of which is from EBNA-1, have been identified as targets of the immune response in the cerebrospinal fluid of patients with MS. The mounting evidence that relates EBV infection to other autoimmune diseases, particularly systemic lupus erythematosus, suggests that EBV may have a broad role in predisposing to autoimmunity. A comparative investigation of individuals with systemic lupus erythematosus and MS may provide new clues to their possible commonalities. A fine understanding of the mechanisms that connect EBV infection to MS is important because it will provide the basis for the translation of this epidemiologic finding into new ways to treat and prevent MS.

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Figure. Mean Epstein-Barr virus nuclear antigen (EBNA) complex and its component EBNA-1 IgG titers among multiple sclerosis (MS) cases as percentage of mean control titers by time of blood collection in years, excluding Epstein-Barr virus–negative cases and controls. Asterisks indicate that P value is less than or equal to .05 (paired t test comparing mean levels of EBNA-1 in cases and matched controls). Daggers indicate that 2 cases and 1 control were excluded because EBNA titers could not be determined (an aspecific reaction in indirect immunofluorescence assay). Controls matched to the 2 cases with missing EBNA titers were also excluded.

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determined nuclear antigen (EBNA)-1 and EBNA-2 in acute and chronic Epstein-Barr virus infection. Proc Natl Acad Sci U S A. 1987;84:570-574.

Archives Express

The ARCHIVES launched a new ARCHIVES Express section in the September 2000 issue. This section will enable the editors to publish highly selected papers within approximately 2 months of acceptance. We will consider only the most significant research, the top 1% of accepted papers, on new important insights into the pathogenesis of disease, brain function, and therapy. We encourage authors to send their most exceptional clinical or basic research, designating in the cover letter a request for expedited ARCHIVES Express review. We look forward to publishing your important new research in this accelerated manner.

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