ORIGINAL CONTRIBUTION

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.

Arch Neurol. 2006;63:(doi:10.1001/archneur.63.6.noc50328)

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EPIDEMIOLOGIC EVIDENCE OF changes in risk among migrants clearly implicates exogenous factors in the etiology of multiple sclerosis (MS),[1] but the nature of these factors remains uncertain. During the past 2 decades, results of numerous studies have suggested a possible role for the Epstein-Barr virus (EBV). In a meta-analysis, EBV-infected individuals were found to have greater than a 10-fold higher risk of developing MS than EBV-seronegative individuals.[2] A similar result has recently been found in children, which virtually rules out genetic susceptibility to EBV infection as a sufficient explanation of these serologic findings.[3] Further, increased anti-EBV antibody titers have been reported as long as 10 years prior to the first symptoms of MS.[4,5] To confirm and extend these findings, we undertook a prospective serologic study of EBV infection from serum samples collected up to 30 years before the clinical diagnosis of MS among patients and matched controls in a large integrated health plan. This is the first MS-EBV investigation to use a prospective study design with a very long period of follow-up.

METHODS

This study has been approved by the Human Subjects Committees of the Harvard School of Public Health, Boston, Mass, and the Kaiser Foundation Research Institute, Oakland, Calif. The sponsors of the study had no role in the study design, in the collection, analysis, and interpretation of the data, or in the writing of the article and the decision to submit it for publication.

STUDY POPULATION

The study population was composed of patients who were members of the Kaiser Permanente Northern California (KPNC) health plan and participated in the multiphasic examinations administered between 1965 and 1974. As a fully integrated health plan, KPNC currently provides inpatient and outpatient medical care to more than 3 million members in northern California. During the multiphasic examinations, new members were asked to provide a blood specimen and answer a questionnaire on health background and medical risk behaviors. More than 100 000 new KPNC mem-

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[6] was undertaken to confirm the MS diagnosis for the 93 potential cases. A medical records analyst recorded dates of MS diagnosis (including optic neuritis or transverse myelitis), dates and duration of neurologic symptoms (eg, transient scanning speech, Bell palsy, Lhermitte sign, tic douloureux, tonic seizures), dates and interpretation of brain magnetic resonance images, cerebrospinal fluid IgG concentrations, cerebrospinal fluid oligoclonal bands, and other criteria used to make a diagnosis. Of the 93 potential MS cases, 72 received diagnostic confirmation of MS made by a neurologist and were considered eligible for the study. Onset date was set as the earliest date of any symptom attributable to MS as described by Poser,[7] or a diagnosis of optic neuritis. Onset date was determined for 58 cases, 46 of whom had a serum sample collected before onset. Cases with unknown date of MS onset were excluded from all analyses. The clinical course of MS at the time of the latest clinical assessment was progressive for 26 members (57%), relapsing-remitting for 7 (15%), and other or unknown for 13 (28%). Most individuals with progressive MS were probably in the secondary progressive phase of relapsing-remitting MS, but the information on the medical records was often insufficient to distinguish a primary (typically accounting for only 10% of all cases) from secondary progressive course, and therefore this distinction was not made in the analyses.

For each case, 3 controls were selected from the serum treasury database and matched by age at the time of blood collection (±1 year), by sex, and by date of blood collection. All controls were active members of KPNC on the date of onset of their matched case and between 1995 and 1999, and showed no evidence of receiving a MS diagnosis in the ADT and OSCR databases between 1995 and 1999. For laboratory analyses, 2 of 3 control specimens matched to a MS case were randomly selected. If 1 of 2 control specimens proved to be inadequate (ie, insufficient or desiccated sample), the third specimen was substituted.

Demographic data for cases and controls (age, sex, race/ethnicity) were obtained from the KPNC patient demographics computerized database, the ADT, and the multiphasic examination. During the multiphasic examination, clinicians classified individuals based on their perception of a new member's race; this classification was related to the subgroups white, black, Asian, and other race. Although some new members may have mentioned their race/ethnicity during the examination, KPNC routine practice during 1965 to 1974 did not include asking new patients to identify their race/ethnicity. However, this classification has high concordance among a subgroup of individuals in our study who were asked to self-report their race/ethnicity at a later visit, usually at the time of a hospital admission (whites, 93%; blacks, 83%; Asians, 100%). Although clinicians may have misclassified some members based on personal perception, we felt that the information was preferable to having no race/ethnicity information for a given patient.

LABORATORY ANALYSES

Serum samples for cases and controls were aliquotted into cryovials at OFAS for overnight shipment to Virolab Inc (Berkeley, Calif) in triplets 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.

Immunoglobulin G and IgA antibodies to EBV viral capsid antigen (VCA) and IgG to anti–early antigen complex (diffuse and restricted) were determined by indirect immunofluorescence.[8] Immunoglobulin G antibodies against the Epstein-Barr nuclear antigen (EBNA) complex and 2 of its individual components, EBNA-1 and EBNA-2, were determined using anticomplement immunofluorescence.[9] Immunoglobulin G antibody titers against cytomegalovirus (CMV) were also determined to assess the specificity of any association found between MS and EBV serology.[10] The intra-assay coefficients of variation were less than 5% for VCA IgG and IgA, diffuse early antigen, restricted early antigen, and EBNA-1; the coefficients of variation were 17.8% for the EBNA complex, 8.3% for EBNA-2, and 12% for CMV.

STATISTICAL ANALYSIS

Geometric mean antibody titers (reciprocal of the dilution) in cases and controls were compared using generalized linear models.[11] Subjects with missing values for any of the covariables were excluded from the analyses.

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.[4] 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-

Table 1. Selected Demographic and Clinical Characteristics of MS Cases and Controls*

Characteristic	Cases (n = 42)	Matched Controls (n = 79)	All Controls (n = 132)
Sex, No. (%)			
Female	36 (86)	68 (86)	105 (80)
Male	6 (14)	11 (14)	27 (20)
Race, No. (%)			
White	31 (74)	54 (68)	89 (68)
Black	9 (21)	14 (18)	25 (19)
Other/unknown	2 (5)	11 (14)	17 (13)
Age at time of blood collection, y			
Mean ± SD	32 ± 7.4	32 ± 7.1	32 ± 8.3
Range	19-49	19-50	17-50

Abbreviation: MS, multiple sclerosis.
*Individuals with MS were identified among members of the Kaiser Permanente Northern California health plan.

tistic and Wald confidence intervals.[12] Statistical analyses were conducted using SAS statistical software (version 8.0; SAS Institute Inc, Cary, NC).

RESULTS

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**).

COMMENT

The main finding of our study is that serum antibody titers to the EBNA complex and EBNA-1 in individuals with MS were increased up to 20 years before the first symptoms of the disease and then remained constant over time. It is important to note that this finding was obtained in a population with an unusually late age at MS onset, and as discussed in the following section, may not apply to individuals with MS onset at younger ages. The temporal relationship between changes in anti-EBV antibody titers and the onset of MS can be better understood by considering jointly the results of our study and those of the following 3 previous prospective investigations: the Nurses' Health Study, the study conducted among US Army personnel,[5] and the study in Vasterbotten County, northern Sweden.

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.[4] 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.[5] 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.[5] 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).[13] 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

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

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

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.
†3 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*

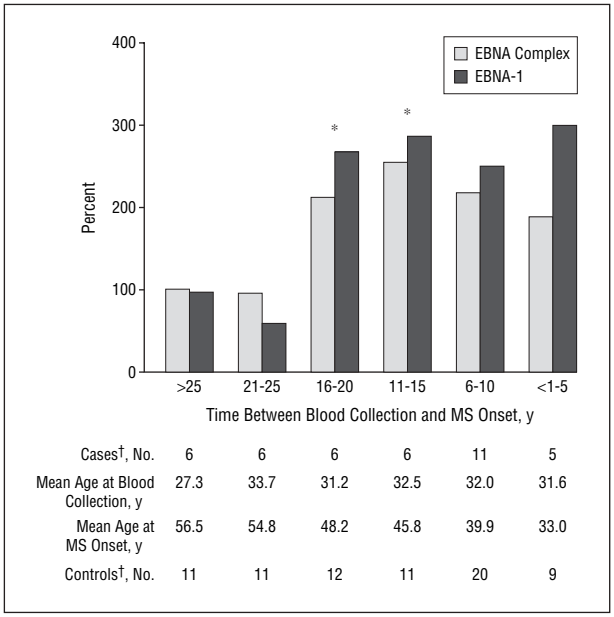
	All MS Cases (n = 42)			
	Matched Controls (n = 79†)		All Controls (n = 132†)‡	
Antibodies	RR (95% CI)	*P* Value	RR (95% CI)	*P* Value
IgG to EBV VCA	1.2 (0.66-2.4)	.50	1.3 (0.74-2.3)	.36
IgA to EBV VCA	1.1 (0.68-1.9)	.63	1.2 (0.75-1.8)	.49
EBNA complex§	2.1 (1.1-3.8)	.02	2.5 (1.4-4.5)	.002
EBNA-1§	1.8 (1.1-2.9)	.03	2.0 (1.2-3.4)	.006
EBNA-2§	1.3 (0.81-2.1)	.28	1.5 (1.0-2.3)	.04
Diffuse early antigen	1.0 (0.64-1.6)	.95	1.1 (0.69-1.7)	.75
Restricted early antigen	1.2 (0.63-2.2)	.62	1.2 (0.69-2.2)	.48
CMV	1.1 (0.78-1.5)	.66	1.1 (0.80-1.5)	.55

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.
†3 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.

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 ex-



	>25	21-25	16-20	11-15	6-10	<1-5
Cases†, No.	6	6	6	6	11	5
Mean Age at Blood Collection, y	27.3	33.7	31.2	32.5	32.0	31.6
Mean Age at MS Onset, y	56.5	54.8	48.2	45.8	39.9	33.0
Controls†, No.	11	11	12	11	20	9

Figure. Mean Epstein-Barr nuclear antigen (EBNA) complex and its component EBNA-1 IgG titers among multiple sclerosis (MS) cases as percent 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.

ist in the EBV genotype, which are probably caused by genetic evolution influenced by HLA-restricted EBV-specific cellular immunity.[19] Munch et al[20] 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,[21] 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.[4] A hypothesis that is gaining increasing support is that EBV infection in genetically susceptible individuals activates T-lymphocytes that cross-react with myelin antigens,[22-24] but other mechanisms have been proposed, such as cross-reacting antibodies,[25] infection of autoreactive B lymphocytes,[26] the activation of superantigens, and an increased expression of αB-crystallin.[27] 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.[28] The mounting evidence that relates EBV infection to other autoimmune diseases, particularly systemic lupus erythematosus,[29] 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.

Published Online: April 10, 2006 (doi:10.1001/archneur.63.6.noc50328).
Accepted for Publication: October 7, 2005.
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Funding/Support: This study was supported by grant NS43604 from the National Institute of Neurological Disorders and Stroke and by a pilot grant from the National Multiple Sclerosis Society.
Acknowledgment: We thank Allen Bernstein, MD, chief of neurology at Kaiser Permanente Santa Rosa Medical Center, Santa Rosa, Calif, for providing consultation and advice during the initial MS case confirmation reviews. We also thank Steven Black, MD, for providing the MS chart abstraction forms, and Alice Jacobson (Kaiser Permanente Division of Research) for her data processing efforts in matching OFAS serum treasury specimens to Kaiser Permanente patients and in the selection of study controls. We thank Elsa Jiménez and Eilis O'Reilly for their technical assistance and Nancy P. Durr at OFAS for her editorial critique.

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