Temporal Relationship Between Elevation of Epstein-Barr Virus Antibody Titers and Initial Onset of Neurological Symptoms in Multiple Sclerosis

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Context Infection with Epstein-Barr virus (EBV) has been associated with an increased risk of multiple sclerosis (MS), but the temporal relationship remains unclear.

Objective To determine whether antibodies to EBV are elevated before the onset of MS.

Design, Setting, and Participants Nested case-control study conducted among more than 3 million US military personnel with blood samples collected between 1988 and 2000 and stored in the Department of Defense Serum Repository. Cases were identified as individuals granted temporary or permanent disability because of MS. For each case (n=83), 2 controls matched by age, sex, race/ethnicity, and dates of blood sample collection were selected. Serial samples collected before the onset of symptoms were available for 69 matched case-control sets.

Main Outcome Measures Antibodies including IgA against EBV viral capsid antigen (VCA), and IgG against VCA, nuclear antigens (EBNA complex, EBNA-1, and EBNA-2), diffuse and restricted early antigens, and cytomegalovirus.

Results The average time between blood collection and MS onset was 4 years (range, <1-11 years). The strongest predictors of MS were serum levels of IgG antibodies to EBNA complex or EBNA-1. Among individuals who developed MS, serum antibody titers to EBNA complex were similar to those of controls before the age of 20 years (geometric mean titers: cases=245, controls=265), but 2- to 3-fold higher at age 25 years and older (cases=684, controls=282; $P<.001$). The risk of MS increased with these antibody titers; the relative risk (RR) in persons with EBNA complex titers of at least 1280 compared with those with titers less than 80 was 9.4 (95% confidence interval [CI], 2.5-35.4; P for trend <.001). In longitudinal analyses, a 4-fold increase in anti-EBNA complex or anti–EBNA-1 titers during the follow-up was associated with a 3-fold increase in MS risk (EBNA complex: RR,3.0; 95% CI, 1.3-6.5; EBNA-1: RR, 3.0; 95% CI, 1.2-7.3). No association was found between cytomegalovirus antibodies and MS.

Conclusion These results suggest an age-dependent relationship between EBV infection and development of MS.

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ELEVATIONS OF LEVELS OF SERUM antibodies to Epstein-Barr virus (EBV) occurring several years before diagnosis have been characteristically found in diseases probably caused by EBV, such as Burkitt lymphoma[1] and nasopharyngeal carcinoma,[2] and in Hodgkin disease.[3] Anti-EBV antibodies are elevated in individuals with multiple sclerosis (MS),[4,5] and a premorbid increase has been reported in 2 studies,[6,7] but both relied on a single blood sample from each study participant. We therefore conducted a larger prospective investigation using serial blood samples collected several years before onset of MS.

METHODS

Study Population

The source population for the current study is more than 3 million US military personnel whose blood samples are stored at –30°C in the Department of Defense Serum Repository.[8] This repository contains more than 30 million serum specimens from active-

See also pp 2466 and 2536.

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duty and reserve personnel of the US military collected at entry and, on average, every 2 years thereafter since 1988. The research protocol was approved by the institutional review boards of Harvard School of Public Health and Walter Reed Army Institute of Research, which waived the need for informed consent to use archived blood products or medical records.

Case Ascertainment and Selection of Controls

Cases were identified by searching the computerized database of the US Army Physical Disability Agency for active-duty personnel granted temporary or permanent disability because of MS and by reviewing medical records. We classified cases as "confirmed MS" if there was a history of 2 or more attacks (occurrence of symptoms of neurological dysfunction lasting more than 24 hours), a diagnosis of MS made by a neurologist, and a positive magnetic resonance imaging (MRI) result or if the final diagnosis in the record was specified as definite MS, clinical definite MS, or laboratory-supported definite MS.[9] Cases were classified as "probable MS" if they did not meet the criteria for confirmed MS but had at least 2 of the following: history of 2 or more attacks, positive MRI result, and diagnosis of MS made by a neurologist. These criteria (confirmed or probable MS) were met by 118 cases, 83 of whom had at least 1 serum sample collected before onset of MS symptoms (defined as the earliest neurological symptom ever reported) and were included in the study. For each of these 83 cases, we identified the earliest available serum sample (baseline sample) plus up to 2 additional samples collected before onset of MS and the first sample collected after onset of MS. For each of the 83 cases, we randomly selected 2 controls matched on age (±1 year), sex, race/ethnicity (white, black, Hispanic, or other), and dates of blood collection (±30 days). For serial samples, each blood sampling date was matched to within 30 days. Serial serum samples before MS onset were obtained for 69 case-control sets, including 40 with 2 samples and 29 with 3 samples.

Race/ethnicity was provided by the Army Medical Surveillance Activity, based on categories defined by the Department of Defense, independently from the investigators. We included this variable as a matching factor because of its association with risk of MS, and possible relationship with age at infection and antibody response.

Laboratory Analyses

Serum samples from MS cases and controls were sent to the laboratory in triplets containing the case and the 2 matched controls in random order without identification of case-control status. Immunoglobulin G and IgA antibodies to EBV viral capsid antigen (VCA) and anti–early antigen complex (diffuse [EA-D] and restricted [EA-R]) were determined by indirect immunofluorescence[10,11]; IgG antibodies against the EBV nuclear antigen (EBNA) complex and 2 of its individual members, EBNA-1 and EBNA-2, were determined by anti-complement immunofluorescence.[12] Immunoglobulin G antibody titers against cytomegalovirus (CMV) were also determined to assess the specificity of any association that may be found between MS and EBV serology.[13]

Statistical Analyses

Geometric mean antibody titers (reciprocal of the dilution) in serum samples collected at baseline were compared between cases and controls using generalized linear models.[14] Conditional logistic regression was used to estimate the relative risk (RR) of MS associated with mean serum levels of specific antibody titers. To reduce the within-person random variation, in these analyses we used for each MS case the geometric mean antibody titer from all the available serum samples collected before MS onset, and for each control the geometric mean of the corresponding matched samples. To explore dose-response relationships, in these conditional logistic regression models the antibody titers were initially treated as categorical variables, with each doubling of titers (eg, 20, 40, 80) as a separate category. However, the lowest and highest categories had to be collapsed in some analyses because of small numbers.

Table 1. Characteristics of Multiple Sclerosis Cases and Controls

Characteristic	Cases (n = 83)	Controls (n = 166)
Sex, No. (%)		
Male	54 (65)	108 (65)
Female	29 (35)	58 (35)
Age at collection of baseline blood, y		
Mean (SD)	24 (5.1)	24 (5.1)
Range	17-39	17-39
Race/ethnicity, No. (%)		
White	50 (60)	100 (60)
Black	29 (35)	58 (35)
Hispanic	1 (1)	2 (1)
Other	3 (4)	6 (4)
Education, No. (%)		
High school	65 (78)	127 (77)
Some college	7 (8)	11 (7)
Completed college	7 (8)	22 (13)
Graduate school	4 (5)	6 (4)

To take advantage of the longitudinal design of the study, we further examined whether an increase in antibody titers within person during the follow-up was associated with an increased risk of MS. For each antibody, we conducted a conditional logistic regression analysis restricted to the 69 case-control sets with more than 1 serum sample available. In these models, we used an indicator (with value 0 or 1) for a 4-fold or greater increase in titers during the follow-up as the independent variable and case status as the dependent variable. Because age is strongly related to risk of MS and to exposure to EBV, we further examined whether the relationship between anti-EBV antibody and MS was modified by age at blood collection, by conducting stratified analyses and by adding an interaction term (equal to the product between antibody titers and an indicator variable for age 20 years or younger vs 21 years or older) to the conditional logistic regression models. All *P* values are 2-tailed and significant at $P<.05$. We used SAS version 8.2 (SAS Institute Inc, Cary, NC) for all analyses.

RESULTS

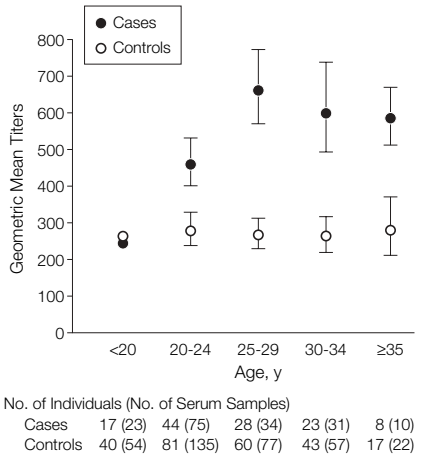
Baseline characteristics of cases and controls are shown in TABLE 1. For cases, the mean (SD) age at MS onset was 27

Table 2. Geometric Mean Titers of Antibodies in Baseline Serum Samples*

	All Subjects			Cases With Blood Collected ≥5 Years Before MS Onset		
Antibodies	Cases (n = 80)	Matched Controls (n = 153)†	*P* Value	Cases (n = 26)	Matched Controls (n = 50)	*P* Value
IgG to EBV VCA	859	700	.04	792	605	.08
IgA to EBV VCA	3.0	2.7	.25	3.3	2.7	.34
EBNA complex	469	282	<.001	465	229	<.001
EBNA-1	326	230	.05	376	192	<.001
EBNA-2	22	16	.09	21	17	.25
Diffuse early antigen	5.3	3.6	.02	6.0	4.4	.18
Restricted early antigen	3.3	3.1	.46	3.4	3.0	.15
Cytomegalovirus	13	14	.98	11	14	.95

Abbreviations: EBNA, Epstein-Barr nuclear antigen; EBV, Epstein-Barr virus; MS, multiple sclerosis,VCA, viral capsid antigen.
*EBV-negative cases and controls (VCA IgG<1:20) were excluded.
†Baseline antibody titers were missing for 1 control.

Figure 1. Geometric Mean Titers of Epstein-Barr Nuclear Antigen (EBNA) IgG by Age at Blood Collections



No. of Individuals (No. of Serum Samples)

Cases	17 (23)	44 (75)	28 (34)	23 (31)	8 (10)
Controls	40 (54)	81 (135)	60 (77)	43 (57)	17 (22)

Figure includes all samples (baseline plus samples during follow-up). Error bars indicate standard error.

(5.5) years (range, 18-41 years). The diagnosis of MS was confirmed in 53 (64%) and probable in 30 (36%). Mean (SD) time between baseline blood collection and MS onset was 4.0 (2.4) years (range, <1-11 years). Three of 83 cases and 7 of 166 controls were EBV negative (VCA IgG <1:20) at baseline; the 3 seronegative cases converted before MS onset. The baseline geometric mean serum antibody titers to VCA (IgG), EBNA complex, EBNA-1, and EA-D were significantly higher among EBV-positive individuals who later developed MS than among their matched controls, whereas there were no significant differences in antibodies to other EBV antigens or CMV (TABLE 2). Similar results were observed in analyses restricted to cases with blood samples collected at least 5 years before the onset of MS (Table 2).

Because the incidence of MS increases sharply between the ages of 20 and 30 years, we examined whether the serum titers of antibodies to EBV changed with age. Among individuals who developed MS, but not among controls, we observed a sharp and significant increase in mean serum titers of antibodies to EBNA complex in early adulthood followed by a plateau (FIGURE 1). Titers to EBNA complex of cases were similar to those of controls younger than 20 years, but 2- to 3-fold higher at age 25 years or older (Figure 1). The difference in geometric mean titers between cases and controls at age 25 years or older was highly significant ($P<.001$, using a generalized linear model). Results were similar for antibodies to EBNA-1. Modest increases with age were also seen for mean antibody titers to EBNA-2 and EA-R, but not VCA IgG, EA-D, or CMV (data not shown).

To examine whether this increase in antibody titers with age was explained by a shorter interval between blood collection and MS onset, we conducted a regression analysis among MS cases using antibodies to EBNA complex or EBNA-1 as the dependent variable, and, simultaneously, age at blood collection and the time interval between blood collection and MS onset as the independent variables. In this regression model, age at blood collection was significantly and positively associated with mean titers of antibodies to EBNA complex ($P=.04$) and EBNA-1 ($P=.007$), whereas there was no relationship between these antibody titers and the time interval between blood collection and MS onset.

The risk of MS increased with increasing serum levels of antibodies to EBNA complex and less strongly to VCA IgG. Compared with individuals with the lowest antibody titers for EBNA complex (<40) and VCA (<160), the RR was 35.9 (95% confidence interval [CI], 4.0-322; *P* for trend <.001) for individuals in the highest category of EBNA complex and 8.7 (95% CI, 0.93-82; *P* for trend = .009) for individuals in the highest category of VCA. To obtain more stable RR estimates, we repeated the analyses using as the reference category titers less than 320 for VCA, and titers less than 80 for EBNA complex (FIGURE 2). Positive associations were also found with EBNA-1 (*P* for trend = .003) and EA-D (*P* for trend = .05), whereas no significant associations were found for VCA IgA, EBNA-2, EA-R, and CMV (data not shown).

In within-person analyses, a 4-fold increase in EBNA complex titers between the sample collected at baseline (typically at time of entry into the Army) and a subsequent serum sample was associated with a 3-fold increase in risk of developing MS (RR, 3.0; 95% CI, 1.3-6.5; $P=.007$); this association was stronger among individuals with the first blood sample collected at or before age 20 years (RR, 18; 95% CI, 2.2-138; $P=.006$). Similar results were obtained for EBNA-1, whereas no significant overall associations were found for other EBV antibodies or antibodies to CMV (TABLE 3).

COMMENT

These results confirm those obtained in a smaller study of women with MS.[6] Although the date of onset of MS is difficult to establish accurately, and many

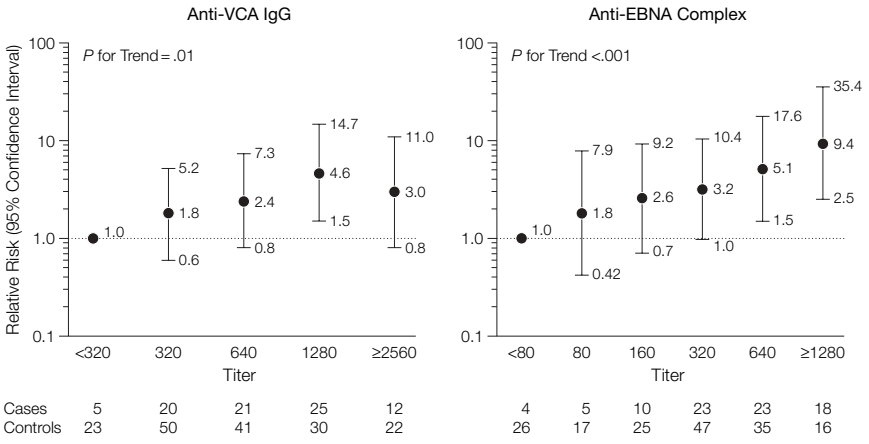
patients at the time of clinical onset have multiple silent MRI lesions,[15] the observation that anti-EBV antibody titers among cases compared with controls were already significantly elevated 5 or more years before the onset of MS suggests that the increased antibody response to EBV is not a consequence of MS, but rather may be an early event in the pathological process that leads to demyelination and clinical disease. In particular, elevated risks were found for EBNA complex and EBNA-1. A significant increase in anti–EBNA-1 titers 5 or more years before the onset of MS was also found in a study in Sweden,[7] although in that study an opposite association was reported for anti-VCA titers; the reason for this difference is unclear.

The pattern of antibody response that we observed among individuals who developed MS is different from the pattern observed in immunocompromised hosts or in chronic infectious mononucleosis, in which there are elevated anti–EBNA-2 and reduced anti–EBNA-1 titers,[12] and from that observed in Burkitt lymphoma, where there are prediagnostic elevations of anti-VCA but not anti-EBNA antibodies.[1] Rather, the elevation of titers to EBNA complex and EBNA-1 suggests a more severe or more recent primary infection or reactivation of infection accompanied by a vigorous cellular immune response.[16-18] Anti-VCA and anti-EBNA IgG elevation in prediagnostic serum samples has been associated with risk of Hodgkin disease[3] and nasopharyngeal carcinoma,[19] but in the latter, the strongest predictors of risk are IgA antibodies to VCA.[20]

The age-related increase in serum titers of anti-EBNA and anti–EBNA-1 antibodies among individuals with MS was a striking and unexpected finding. The fact that this increase occurred between the late teens and the mid to late 20s, independently from the age of MS onset, supports the hypothesis of an age of vulnerability for the acquisition of MS.[21] The incidence of infectious mononucleosis peaks at this age, but since most participants in our study were already EBNA-1 seropositive at the time of first blood collection, a newly acquired EBV infection is an unlikely cause of this antibody response. More likely, the antibody response is due to either infection with a separate microorganism or other factors that alter the immune response to EBV[22] or, more speculatively, to infection with a strain of EBV different from that originally carried by the host. There is increasing evidence that coinfection with multiple EBV strains, either acquired sequentially or simultaneously, is common even in healthy individuals,[23] but little is known about serological response or other consequences of coinfection.

It has been suggested that multiple infections in early childhood may reduce

Figure 2. Relative Risk of Multiple Sclerosis According to Anti-VCA IgG and Anti-EBNA IgG Antibody Titers



VCA indicates viral capsid antigen; EBNA, Epstein-Barr nuclear antigen. Epstein-Barr virus titer levels were missing for 1 control.

Table 3. Relative Risk of Multiple Sclerosis Corresponding to a 4-Fold Increase in Serum Antibody Titers During Follow-up

	All Cases (n = 69)		Age First Sample Collected: ≤20 y (n = 25)		Age First Sample Collected: >20 y (n = 44)	
Antibodies	RR (95% CI)	*P* Value	RR (95% CI)	*P* Value	RR (95% CI)	*P* Value
IgG to EBV VCA	1.3 (0.6-2.9)	.49	3.3 (0.6-19)	.17	1.0 (0.4-2.5)	>.99
IgA to EBV VCA	2.0 (0.13-32)	.62	NA		NA	
EBNA complex*	3.0 (1.3-6.5)	.007	17.6 (2.2-138)	.006	1.5 (0.5-4.1)	.43
EBNA-1*	3.0 (1.2-7.3)	.01	15.6 (2.0-124)	.009	1.5 (0.4-5.0)	.52
EBNA-2	2.1 (0.9-4.8)	.07	1.2 (0.3-5.7)	.79	2.8 (1.0-7.3)	.04
Diffuse early antigen	0.81 (0.27-2.5)	.72	2.0 (0.3-14)	.49	0.42 (0.09-2.0)	.28
Restricted early antigen	1.5 (0.4-5.0)	.52	2.0 (0.3-14)	.49	1.1 (0.2-5.2)	.89
Cytomegalovirus	1.2 (0.6-2.5)	.61	1.3 (0.4-4.5)	.67	1.2 (0.46-3.0)	.75

Abbreviations: CI, confidence interval; EBNA, Epstein-Barr nuclear antigen; EBV, Epstein-Barr virus; NA, not available; RR, relative risk; VCA, viral capsid antigen.
*In conditional logistic regression model, interaction between age at blood collection ≥20 years and EBNA complex, or EBNA-1 were statistically significant (*P* = .02 and *P* = .03, respectively).

the risk of MS by downregulating autoimmune responses that could be triggered by infection with the same or related microbes later in life.[24,25] This hypothesis, often called the "hygiene hypothesis," has also been invoked to explain more generally a positive relationship between incidence of autoimmune and allergic diseases and level of sanitation.[26] A confirmed prediction of this hypothesis is an increased risk of MS among individuals with a history of infectious mononucleosis, which is a strong marker of late age for EBV infection.[27] A key question, however, is whether there is a specific role for late infection with EBV in triggering MS. If so, the hygiene hypothesis would predict a low MS risk among EBV-uninfected individuals. In contrast, in the absence of a specific role of EBV, the lack of anti-EBV antibodies would only be relevant as a marker of low exposure to infection in childhood,[28] and EBV-uninfected individuals would be predicted to have a high MS risk. Consistent with the first formulation, the risk of MS among EBV-seronegative individuals is several fold lower than among EBV-positive individuals.[29] Innate resistance to both EBV and MS could be invoked to explain this association, but this explanation is virtually ruled out by the recent finding of an 8-fold higher risk of MS among children infected with EBV than among those not infected.[30] This strong association also provides evidence to counter the explanation that the increase in anti-EBNA titers in our study is a consequence of a change in the immune system occurring years before the clinical onset of MS.

Overall, the results of our investigation therefore support a specific role of EBV as a risk factor for MS. Because of the central role of EBV infection in the hygiene hypothesis, we suggest that it should be called the "EBV hypothesis" or the "EBV variant of the hygiene hypothesis" of MS, to differentiate it from the more general version of the hygiene hypothesis that refers to asthma and other immune conditions, but does not seem to include MS. Similar epidemiological evidence relates EBV infection to systemic lupus erythematosus,[31] suggesting that EBV may be a risk factor for autoimmune diseases.

Author Contributions: As principal investigator, Dr Ascherio had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
Study concept and design: Levin, Rubertone, Spiegelman, Ascherio.
Acquisition of data: Levin, Munger, Rubertone, Peck, Lennette, Ascherio.
Analysis and interpretation of data: Levin, Munger, Peck, Spiegelman, Ascherio.
Drafting of the manuscript: Levin, Ascherio.
Critical revision of the manuscript for important intellectual content: Levin, Munger, Rubertone, Peck, Lennette, Spiegelman, Ascherio.
Statistical analysis: Munger, Peck, Spiegelman, Ascherio.
Obtained funding: Levin, Lennette, Ascherio.
Administrative, technical, or material support: Levin, Peck, Lennette, Ascherio.
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