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Anti-Epstein-Barr virus antibodies as serological markers of multiple sclerosis: a prospective study among United States military personnel

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Abstract

Background—Elevated Epstein-Barr virus (EBV) antibody titers are risk factors for MS, but the strength and consistency this association are not well characterized.

Objectives—To determine whether this association is confounded by vitamin D or modified by gender or race, and the usefulness of EBV nuclear antigen (EBNA) antibodies as a marker for MS.

Methods—We conducted a prospective study among US military personnel. Antibody titers against EBV antigens were measured in serum samples from 222 individuals who developed MS and 444 age, sex, and race/ethnicity matched controls. Conditional logistic regression was used to estimate relative risks.

Results—MS risk increased with increasing titers of anti-EBNA complex ($p < 10^{-9}$) and anti-EBNA-1 ($p = 5.8 \times 10^{-9}$) titers. MS risk was 36-fold higher among individuals with anti-EBNA complex IgG titers ≥ 320 than among those with titers < 20 (95% CI: 9.6–136), and 8-fold higher among those with anti-EBNA-1 ≥ 320 than among those with anti-EBNA-1 < 20 (95% CI: 2.6–23). These associations were consistent across gender and race/ethnicity groups and independent from 25-hydroxyvitamin D levels. Areas under the ROC curves were 0.67 for EBNA complex and 0.65 for EBNA-1.

Conclusions—Serum titers of pre-onset anti-EBNA antibodies are strong, robust markers of MS risk and could be useful in an MS risk score.

Keywords

Epstein-Barr virus; epidemiology; nested case-control study; risk factor

Introduction

Multiple sclerosis (MS) is a complex disease in which both genetic and environmental factors contribute to the etiology.[1-3] Infection with Epstein-Barr virus (EBV) [4] and levels of IgG antibody titers against the EBV nuclear antigen complex (EBNAc) and EBNA-1 are well-established risk factors for MS. [1] Previous studies, however, were too small to determine whether these associations were sufficiently strong and robust to be clinically useful. [5-8] Further, none included a sufficient number of men or minorities to examine risk of MS in these groups or accounted for vitamin D status, which, by modulating the immune response to EBV and MS risk, could confound the results. Here we report the results of an independent validation of these biomarkers in a large and ethnically diverse sample of U.S. young adults with serially collected blood samples and previously measured vitamin D status.

Materials and Methods

This study was approved by the Institutional Review Boards of the Harvard School of Public Health and the Walter Reed Army Institute of Research, both of which waived the requirement of informed consent to use archived medical records and previously collected serum samples.

Case ascertainment and control selection

MS is a medically disqualifying condition in the US military and active-duty personnel receiving an MS diagnosis will undergo a review for medical separation by the Physical Disability Agency (PDA) of their branch of service. We searched the records of the US Army and US Navy (which includes the Marines) PDAs for members with an MS diagnosis between 1993 and 2004 (Army) and 1992 and 2004 (Navy), and we identified 515 potential MS cases for inclusion in our study. Upon medical record review, 315 were confirmed as having definite or probable MS according to the study diagnostic criteria, as previously described.[4, 9]

The Department of Defense Serum Repository (DoDSR) houses over 40 million serum samples that have been collected on average every two years from approximately eight million active-duty US military personnel since 1985. [10] At least one and up to three serum samples collected prior to their date of first MS symptoms (onset date determined from the medical record) were retrieved from the DoDSR for each case. These specimens included the earliest sample available, the last sample collected prior to the date of onset, and one sample collected in the interval between these two. Two controls were randomly selected from the DoDSR and matched to each case on age (± 1 year), sex, race/ethnicity (non-Hispanic white, non-Hispanic black, Hispanic, other), dates of blood collection (± 30 days), and branch of military service (Army, Navy, Marines); controls had to be on active duty on the date of first symptoms of the matched case.

Laboratory analysis

Serum EBV IgG antibody titers against the viral capsid antigen (VCA) and the EBV nuclear antigens (EBNA) EBNAc, EBNA-1, and EBNA-2 were determined by immunofluorescence, as previously described,[4] at the Swedish Institute for Infectious

Disease Control (SMI) (Solna, Sweden). EBNA-1 and EBNA-2 IgG titers were not measured in 56 cases and their 112 matched controls. Antibody titers were measured in 2-fold dilutions. Serum levels of 25-hydroxyvitamin D (25(OH)D) were measured using a radioimmunoassay (RIA), as previously described.[9, 11] For both EBV and 25(OH)D assays, samples were sent to the respective laboratories organized in triplets (1 case/2 controls) and laboratories were blinded to the case-control status of the samples. Further, we included quality control samples in triplicate from the same parent sample that were interspersed throughout the study samples to monitor assay variation. The intra-assay coefficients of variation ranged from 9-17% for the EBV IgG antibody titers and were <8% for the 25(OH)D RIA.

Covariates

In addition to the matching factors, information on place of residence at entry into the military was also available. Cases and controls were categorized into tiers based on the latitude of their state of residence at entry (North, Middle, South, outside the continental US), as previously described. [9]

Statistical analyses

Of the 315 confirmed MS cases, 83 were included in our previous study of EBV antibody titers and MS,[7] and are thus not included in this study. For ten of the remaining 232 cases, matched controls could not be identified, leaving 222 MS cases and 444 matched controls available for this analysis. EBV seronegativity was defined as having no detectable antibodies to any of the EBV antigens measured (VCA <1:20, EBNAc, EBNA-1, or EBNA-2 <1:5).[4] Of the cases and controls included in this study, seven cases and 25 controls were EBV negative in the earliest sample available; all seven cases became positive prior to MS onset and 17 controls remained EBV negative. Because the main exposure of interest in this study was the humoral immune response to the virus in EBV infected subjects, EBV negative samples were excluded from analyses of case-control comparisons.

Baseline geometric mean antibody titers in cases were compared with controls using generalized linear models that accounted for the matched design of the study. Likewise, among controls the geometric mean antibody titers were compared across age, sex, and race/ethnicity. Because there was no change in the EBV IgG titers over time within cases or controls, the following analyses were conducted using the average of all available EBV-positive pre-symptom onset samples for each case, and the EBV-positive matched samples for controls. Conditional logistic regression was used to examine the dose-response relationship between MS and each of the EBV IgG antibody titers, modeling the EBV titers categorically as the reciprocal of the 2-fold titer dilution. The categories have been modified with respect to our previous study[7] to account for differences in mean antibody titers between Virolab, Inc. (the laboratory used in the previous study, now closed) and the SMI. Conditional logistic regression was also used to estimate the relative risk (RR) of MS associated with a 4-fold increase in a given EBV antibody titer using the base two logarithm of the reciprocal of the dilution as a continuous variable.

In our previous investigation of serum 25(OH)D levels and risk of MS in this military population,[9] an inverse association was seen only among whites as the sample size for blacks was small and results inclusive; therefore, in this study we assessed whether the association between EBV and MS was independent from that between vitamin D and MS among whites. We ran conditional logistic regression models including both average EBV titer levels and average season-, age-, sex-, and laboratory assay batch-adjusted 25(OH)D levels (created as previously described [9]). The EBV antibody titer levels were modeled as continuous for a 4-fold increase in the specific antibody. We also tested for interaction

between EBV and the following: 25(OH)D levels, age at first sample collection, sex, and race/ethnicity, by entering cross-product terms in conditional logistic regression models and using likelihood ratio tests to determine statistical significance of the interaction, where lack of a statistical interaction implies that the effects of EBV titers and 25(OH)D combine in a multiplicative manner.

Receiver operating characteristic (ROC) curves were plotted (sensitivity of continuous log₂ anti-EBNA IgG antibody titer against 1–specificity using the sample collected most recently before MS symptom onset to more closely simulate the situation of a patient presenting in a clinical setting and being evaluated for MS risk) to ascertain how well anti-EBNA IgG titer levels discriminate between individuals who developed MS and their matched controls. The area under the ROC curves was determined using the *C* statistic. All *P* values are 2-tailed. The statistical software SAS v9.1 (SAS Institute Inc, Cary, NC) was used for all analyses.

Results

Characteristics of the MS cases and matched controls are shown in Table 1. Consistent with the demographics of the US military, nearly 70% of cases and controls were male and 56% were non-Hispanic white. MS cases were on average 28 years old when they developed symptoms consistent with MS, 75% onset with a relapsing-remitting course, and there was a mean of 5.1 years between collection of their first blood sample and first MS symptoms.

The geometric mean IgG antibody titers against VCA, EBNAc, EBNA-1, and EBNA-2 in the earliest pre-onset MS sample collected were higher among cases than among controls, both overall and in samples collected 5 or more years before onset of symptoms (Table 2). The relative risk of MS increased linearly with increasing average anti-EBNAc and EBNA-1 titers: comparing individuals with average titers ≥ 320 to those with titers < 20 , the relative risk of MS was 36.1 (95% CI: 9.6 to 136) for EBNAc and was 7.7 (95% CI: 2.6 to 23.0) for EBNA-1 (Figure 1). Weaker, but still significant, positive associations were also found for average IgG antibody titers to EBNA-2 and VCA (Fig). Further analyses were conducted using the log₂-transformed average anti-EBV titers as continuous variables. Overall, a 4-fold increase in anti-EBNAc IgG titers was associated with a 2.9-fold increase in MS risk and a 4-fold increase in anti-EBNA-1 IgG titers was associated with a 2.4-fold increase in MS risk (Table 3). Similarly strong associations were seen in subgroup analyses. Both men and women had statistically significant 2-3-fold increases in MS risk with every 4-fold increase in anti-EBNA-1 IgG titers and a significant 2-fold increase in MS risk was seen among both non-Hispanic whites and non-Hispanic blacks (Table 3). None of the interactions between these factors and anti-EBNAc or anti-EBNA-1 titers were statistically significant (*p* for interaction: EBNAc/EBNA-1: sex, *P*=0.34/0.49; race/ethnicity, *P*=0.30/0.43; age at first sample collection, *P*=0.78/0.77). Among white controls, average season-, age-, and sex-adjusted 25(OH)D levels were weakly inversely correlated with EBNA complex IgG and EBNA-2 IgG (EBNA: *r*= −0.13, *P*=0.05; EBNA-2: *r*= −0.16, *P*=0.03). Further, controls in the bottom quintile of 25(OH)D (< 63.3 nmol/L) had higher EBNA complex IgG titers as compared with controls in the top quintile (> 99 nmol/L), but the difference was not statistically significant (EBNA IgG titers in 25(OH)D q1: 65.5 vs q5: 50, *p*=0.26). To assess whether the associations between EBNAc and EBNA-1 and MS risk were confounded by 25(OH)D levels, we also adjusted the analyses for average season-, age-, and sex-corrected 25(OH)D levels, but the results did not change (RR for a 4-fold increase in anti-EBNAc IgG comparing all cases and controls=2.9, 95% CI: 2.2 to 3.9, *P*<0.0001; EBNA-1: RR=2.4, 95% CI: 1.8 to 3.2, *P*<0.0001; data not shown for other categories in Table 3). Because seasonal variation in 25(OH)D may influence the immune response to EBV, we also adjusted the analysis for 25(OH)D levels that were not corrected for season, but the results were similar (data not shown).

To determine the usefulness of anti-EBNAc and anti-EBNA-1 titers to predict MS risk, we also estimated ROC curves (Figure 2). These curves were derived using only the anti-EBNAc and anti-EBNA-1 titers measured in the sample collected most recently before MS symptom onset (average eight months before), to simulate the situation of a patient presenting in a clinical setting and being evaluated for MS. The area under the curve (C-statistic) was 0.67 for serum anti-EBNAc titers and 0.65 for anti-EBNA-1.

Discussion

In this prospective study of US military personnel, we confirmed our previous observation in this [7] and other populations [5, 6, 8] that IgG antibodies to EBNAc and EBNA-1 are monotonically and strongly associated with an increased risk of MS. This is the largest prospective study conducted to address this question and the first to show that a strong association is present in both men and women, is similar in the race/ethnic groups represented in this study, and is not confounded or modified by serum 25(OH)D levels.

Although previous prospective studies of EBV and MS risk have included both men and women [6-8] and were heterogeneous with respect to race, [7, 8] sample sizes were small and precluded stratified analyses. In this study, we were able to examine potential effect modification of the EBNA-MS association by sex and race/ethnicity. While among the controls, women and blacks had significantly higher EBNAc and EBNA-1 IgG titers as compared with men and whites, respectively, their risk of MS associated with a 4-fold increase in titers was similar and we did not observe any significant statistical interactions with sex or race/ethnicity and anti-EBNAc or EBNA-1 IgG on MS risk, suggesting that differences in MS risk between men and women and whites and blacks are not due to variations in the immune response to EBV within these groups.

The association that we found between anti-EBNA antibodies and MS risk is unlikely to reflect confounding by genetic factors because we have previously shown that the HLA-DRB1*1501 allele, which is the strongest genetic predictor of MS [12], and anti-EBNA titers contribute independently and multiplicatively to MS risk. [13] Although numerous other genetic variations associated with MS risk have been reported [14, 15], their overall contribution is modest and could not explain the over 30-fold risk gradient associated with anti-EBNAc titers. Confounding by non-genetic factors is also unlikely. The main environmental risk factors for MS, besides EBV infection, are cigarette smoking and low vitamin D levels [1, 2]. Smoking history was not available in this study, but smoking is only a moderate risk factor for MS, and thus cannot explain the much stronger anti-EBNA-MS association. Further, in a pooled analysis of data from three studies conducted in the US, Sweden, and Australia, a significant association between anti-EBNA titers and MS risk was found both in smokers and never smokers. [16] Vitamin D could modulate the immune response to EBV, [17, 18] and a biological interaction between 25(OH)D levels and anti-EBNA antibodies is thus plausible. In our white controls, we did observe weak inverse correlations between the average EBV antibody IgG titer and average 25(OH)D levels, which did reach significance for anti-EBNAc and anti-EBNA-2 IgG antibody titers. However, levels of anti-EBNAc IgG titers, while lower in the top than in the bottom quintile of serum 25(OH)D, were not significantly different, and, most importantly, the association between anti-EBNA titers and MS risk was independent from 25(OH)D levels.

These results add to the already strong evidence that EBV infection is a risk factor for MS. The risk of MS is extremely low in individuals who are not infected with EBV [19-21] and higher in individuals with a history of infectious mononucleosis than in those whose primary EBV infection was asymptomatic. [22, 23] More recently, we have shown in a longitudinal follow-up study that EBV negative young adults are protected from MS until the time of

EBV infection, and that their risk rises sharply within a few months from seroconversion. [4]

Insufficient progress, however, has been made in understanding the biological mechanisms underlying this association. The presence of large numbers of EBV infected cells in the brain of MS patients reported a few years ago [24] has not been corroborated in other studies [25-27] and remains controversial. [28] The existence of cross-reacting T-cells recognizing both EBV peptides and myelin antigens has been documented [29-31], but their role in MS pathogenesis is still unproven. Other proposed mechanisms are still speculative. [32] The strong and monotonic increase in MS risk with increasing anti-EBNAc IgG antibody titers reported here suggest that either these antibodies have a direct role in MS pathogenesis or are strongly correlated with the relevant pathogenic mechanisms. Although a few studies have examined antibody responses to specific EBNA-1 epitopes [33, 34], the stronger association with anti-EBNAc titers than anti-EBNA-1 titers in our study, as well as the presence of a weaker but still significant association between anti-EBNA-2 titers and MS risk, seems to suggest a broader role of antibody titers against latent EBV antigens rather than a single specific cross-reacting epitope.

Independently from the underlying molecular mechanisms, the existence of a strong and consistent association between serum titers of anti-EBNAc antibodies and MS risk could contribute to identifying individuals at a higher risk of MS. Our estimated C statistic for a model that included only anti-EBNAc titers was 0.67 and is clearly too low to advocate the clinical use of anti-EBNAc titers by itself. It is noteworthy, however, that this value is slightly higher than the C statistic of 0.635 reported by De Jager et al. [35] for a genetic risk score comprising the HLA risk allele DRB1*1501 and 16 other polymorphisms related to MS; this C statistic increased to 0.683 when EBV antibody titers, determined predominately after the diagnosis of MS and only in a subset of white women, and smoking were added to the genetic risk score. The current study comprises white, black, and Hispanic men and women and is novel in using pre-MS onset EBV antibody titers. When combined with family history and genetic markers, anti-EBNAc titers may thus contribute more substantially to determine MS risk. The lack of genetic material and family history of MS is an important limitation of this study and prevents the derivation of a combined risk score incorporating these factors. However, this does not affect the validity of our findings on anti-EBNAc titers, because of the previously demonstrated independence of anti-EBNAc titers as a risk factor for MS. [13]

In conclusion, in this prospective study, we found that MS risk increases by more than 30-fold with increasing serum titers of anti-EBNAc IgG antibodies and by more than 7-fold with increasing titers of anti-EBNA-1 IgG antibodies. Further, the association was not confounded by vitamin D, and was consistent in both men and women and the race groups included in this study. These titers are thus the first reliable serological biomarkers of MS risk, and future studies with the aim of developing a combined risk score to predict an MS diagnosis should consider including anti-EBNA antibody titer levels in their criteria.

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All authors, external and internal, had full access to all of the data (including statistical reports and tables) in the study and can take responsibility for the integrity of the data and the accuracy of the data analysis.

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Figure 1.

RR of MS by levels of EBV IgG antibody titers

A. EBNAc: 217 cases/422 controls; excludes 5 cases and 4 controls missing average EBNAc, 17 controls negative for EBV infection in all samples, and 1 control deleted due to suspect lab values; B. EBNA-1; C. EBNA-2 EBNA-1 and EBNA-2 IgG antibodies were not measured in 56 cases and 112 controls. Both include 161 cases/313 controls: excluded from analysis were 5 cases and 4 controls missing EBNA-1 and EBNA-2, and 15 controls negative for EBV infection in all samples. D. VCA: 218 cases/ 421 controls; excludes 4 cases and 5 controls missing average VCA, 17 controls negative for EBV infection in all samples, and 1 control deleted due to suspect lab values.

Figure 2. Receiver Operating Characteristic curves for EBV IgG antibodies in the sample collected most recently before MS symptom onset. A) anti-EBNAc IgG antibodies (C=0.67); B) anti-EBNA-1 IgG antibodies (C=0.65)

Table 1
Selected Characteristics of Cases and Controls, US Active-Duty Army and Navy Personnel (1992-2004)

	Cases (n=222)	Controls (n=444)
Sex, N (%)		
Male	148 (67)	296 (67)
Female	74 (33)	148 (33)
Age at first blood draw, yr		
Mean (SD)	23.4 (5.5)	23.4 (5.5)
Range	16 - 40	17 - 41
Race/Ethnicity, N (%)		
White	125 (56)	250 (56)
Black	64 (29)	128 (29)
Hispanic	26 (12)	52 (12)
Other	7 (3)	14 (3)
Tier residence at entry, N (%) [*]		
North	32 (14)	87 (20)
Middle	87 (39)	143 (32)
South	85 (38)	176 (40)
Outside US	4 (1.8)	5 (1.1)
Education, N (%) [*]		
High school	174 (78)	370 (83)
Some college	11 (5)	22 (5)
Completed college	25 (11)	40 (9)
Graduate school	10 (5)	9 (2)
Age at symptom onset, yr		
Mean (SD)	28.4 (6.2)	NA
Range	18 - 48	
MS Type, N (%) [*]		
RRMS	167 (75)	NA
PMS	3 (1)	
Other	23 (10)	
Time between first blood draw and MS onset, yrs		
Mean (SD)	5.1 (3.1)	
Range	<1 - 13	NA

NA: not applicable

^{*} Does not add to total due to missing values

Table 2
Geometric Mean Titers of EBV Antibodies in Baseline Serum Sample among EBV Seropositive Cases and Controls

IgG Antibodies	All Subjects		Blood collected ≥ 5 years Before onset			
	Cases ^a (215)	Controls (418)	p-value	Cases (95)	Controls (192)	p-value
EBV VCA ^b	1658	1109	<0.0001	1528	1068	0.0026
EBNAc ^b	114	60	<0.0001	114	60	<0.0001
EBNA-1 ^b	64	29	<0.0001	74	32	<0.0001
EBNA-2 ^b	14	9	0.0013	14	9	0.05

EBNAc titers were not available for 7 cases and 13 controls (208/405)
EBNA-1 titers were not available for 61 cases and 118 controls (122/300).
EBNA-2 titers were not available for 61 cases and 117 controls (122/301).

^aExcludes 7 cases and 25 controls that were EBV negative at baseline, and 1 control with equivocal laboratory results

^bVCA missing for 6 cases and 13 controls (n in analysis: 209 ca/405 co)

Table 3
Relative Risk of MS Corresponding to a 4-Fold Increase in Average anti-EBNA IgG Serum Antibody Titers*

Characteristic	EBNAc		EBNA-1	
	N Cases/controls	RR (95% CI)	N Cases/controls	RR (95% CI)
All	217/422	2.9 (2.2, 4.0)	161/313	2.4 (1.8, 3.3)
Sex				
Males	147/278	3.3 (2.3, 4.7)	114/215	2.6 (1.8, 3.8)
Females	70/144	2.4 (1.5, 3.9)	47/98	2.1 (1.3, 3.5)
Race/Ethnicity				
White	124/234	3.5 (2.3, 5.3)	101/190	2.3 (1.6, 3.3)
Black	61/126	2.5 (1.5, 4.3)	33/71	2.3 (1.2, 4.3)
Hispanic/other	32/62	2.4 (1.2, 4.4)	27/52	3.3 (1.5, 7.6)
Age at first sample collection				
≤20	91/175	3.2 (2.0, 5.0)	66/127	2.9 (1.7, 4.7)
>20	126/247	2.8 (1.9, 4.2)	95/186	2.2 (1.5, 3.2)
Definite MS	173/337	3.0 (2.1, 4.1)	129/252	2.3 (1.7, 3.2)
Relapsing-remitting MS	164/315	3.3 (2.3, 4.7)	122/234	2.3 (1.7, 3.2)

* excludes 5 cases and 4 controls missing average EBNA, 17 controls negative for EBV infection in all samples, and 1 control deleted due to suspect lab values