An altered immune response to Epstein-Barr virus in multiple sclerosis

A prospective study

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Abstract—*Objective:* To investigate the association between human herpesviruses and multiple sclerosis (MS), as well as between measles virus and MS. *Methods:* The authors identified prospectively collected serum samples from 73 MS cases and retrospective sera from 161 MS cases in two population-based serum bank registers. Analyses of IgG antibody responses in cases and matched referents were performed for Epstein-Barr virus (EBV [EBNA-1 and VCA]), human herpesvirus 6 (HHV-6), herpes simplex virus (HSV), varicella zoster virus (VZV), and measles. *Results:* All cases showed signs of past EBV infection. High activity to EBNA-1 and HHV-6 significantly (borderline significance for HHV-6) increased the risk for MS in prospective sera. A discrepancy between activities to EBNA-1 and VCA was striking in MS samples collected less than 5 years before relapsing-remitting MS onset, where high activity to EBNA-1 significantly increased, and high VCA activity significantly decreased the risk for MS. There was no support for major causal roles for HSV, VZV, or measles. *Conclusion:* Individuals who will develop MS exhibit an altered immune response against the EBV virus characterized by a high IgG activity to EBNA-1 in the absence of high activity to VCA, this being most pronounced in the 5-year period preceding MS onset.

NEUROLOGY 2004;62:2277–2282

Evidence supports that agents associated with multiple sclerosis (MS) are common and act on a population level, and that the age of acquisition might be from 13 to 20 years.[1,2] Of all environmental agents studied, viruses remain the most suspected etiologic agents. Among these, Epstein-Barr virus (EBV) and human herpesvirus 6 (HHV-6) have particularly been implicated as associated with MS.[3] The pathogenetic mechanism discussed for infectious agents includes the activation of autoreactive lymphocyte clones through molecular mimicry.[4] Thus far only two studies have been published that have prospectively analyzed the prevalence of virus titers among MS patients. Both studies only investigated EBV, which was implicated in the pathogenesis of the disease.[5,6]

From the family of eight DNA viruses constituting the human herpesviruses, four were selected for this study. EBV, the cause of infectious mononucleosis, is often regarded as the virus with the strongest association with MS.[7] The EBV is tropic for B cells and persists normally throughout life as a latent infection in some of these. Unlike in the developing countries, as many as 50% of individuals in developed countries may have their primary infection after childhood.[8] The cause of roseola, HHV-6, is tropic for T cells and has received increased attention as a putative candidate in MS pathogenesis.[9] The usually benign childhood infection is a recognized cause of febrile seizures, and has been implicated as a cause of meningoencephalitis.[10] The neurotropic herpes simplex virus (HSV) and varicella zoster virus (VZV) have been identified as the main viral agents causing complications such as meningoencephalitis and myelitis.[11] The measles virus, morbilli, is unlike the herpesviruses RNA-encoded, and has been associated with MS previously.[12]

The design of the present study was suggested close to 40 years ago by Kurland: we studied the IgG antibody responsiveness to EBV, HHV-6, HSV, VZV, and measles virus in both prospectively and retrospectively collected MS serum samples together with matched referents derived from two regional serum banks.[13]

Methods. *Subjects and study design.* MS cases database. In order to study the epidemiology of MS in Västerbotten County, northern Sweden, a case register was created. A high risk for MS was identified by estimates of the prevalence in 1990 and 1997, and the incidence in 1988 through 1997.[14,15] On the latter prevalence day, December 31, 1997, there were 259,163 inhabitants in the study area. Case ascertainment and reliability of clinical and epidemiologic data have been assured from medical records, the results of MRI, CSF electrophoresis analysis, and other laboratory and paraclinical tests, together with a follow-up interview and neurologic examination.

Serum sample database. The Northern Sweden Health and

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Supported by grants from the Swedish Association of Neurologically Disabled.

Received October 1, 2003. Accepted in final form February 11, 2004.

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Table 1 *Characteristics of the prospective and retrospective multiple sclerosis cases*

Characteristics	Prospective	Retrospective
No. cases	73	161
Female sex	67	125
Source of samples		
Northern Sweden Health and Disease Study	12	98
Northern Sweden Maternity Cohort	61	63
Serum collection		
Median age, y (range)	28 (17–59)	40 (19–68)
Median year (range)	1984 (1976–1995)	1992 (1975–2000)
Duration between symptom onset and serum collection, y		
≤1	12	26
2–4	16	17
5–9	26	40
10–14	12	18
≥15	7	60

Disease Study (NSHDS) Cohort (Medical Biobank of Umeå University) contains three subcohorts: The Västerbotten Intervention Program (VIP) cohort, the MONICA cohort, and the Mammography Screening cohort. Originally, VIP is a long-term project intended for health promotion of the population in the Västerbotten County. Every year, all individuals aged 40, 50, and 60 years are invited. The project started in 1985 and the cohort included 74,000 individuals by December 2002, of whom 67,000 had donated blood samples. The Mammography Screening Cohort started in 1995 and supplies the bank with 44,000 blood samples from 25,700 individuals. From 1997, the Västerbotten Mammography Screening Program has invited all women aged 50 to 69 years biannually. The Northern Sweden MONICA (Monitoring Trends and Determinants in Cardiovascular Disease) project (screening 1986, 1990, 1994, and 1999) consists of 11,000 random blood samples from 7,500 individuals aged 25 to 64 years. These three subcohorts provide at present 130,000 samples from 85,000 individuals. The blood samples have been subdivided into plasma (elsewhere referred to as serum), buffy-coat, and erythrocytes and are stored in freezers at −70 °C. The NSHDS cohort is population-based.

The Northern Sweden Maternity Cohort (NSMC) was collected from 1975 to 1999. The serum samples were acquired at the first visit to the antenatal care clinic after pregnancy was diagnosed, with the original purpose of estimating immunity for the rubella virus. The material has been kept in freezers at −20 °C. The NSMC is population-based and comprises 102,788 sera from 78,700 women.

Study population. The MS database was linked with the databases of the NSHDS and NSMC cohorts. From the 850 MS cases in our database we identified 251 cases in either of the two serum bank registers. For 234 MS cases, serum samples from cases and matching referents were available. Cases without any possible MS symptoms preceding the serum collection were defined as prospective cases (n = 73), and the remaining cases were defined as retrospective cases (n = 161). All analyses on antibody activities to viruses were performed separately for these two groups (table 1). The same list of MS-onset symptoms being used in the epidemiologic surveys was adopted and used for calculations of age at onset and the duration between serum collection and symptom onset.[14] Owing to the content of the serum banks there was an excess of females (192 females and 42 males), giving a female to male ratio of 4.6. Otherwise the diagnostic categories and characteristics in the MS populations did not differ substantially from a population-based MS sample (table 2).[16,17]

Of the 234 serum samples, 124 were from the NSMC, and 110 from the NSHDS cohort. Detailed data on clinical characteristics were available from the epidemiologic surveys for 80% (n = 187) of these, and 178 cases belonged to the December 31, 1997 Västerbotten County prevalence population. The remaining 20% were resident in other areas in northern Sweden (n = 40) or in Västerbotten County but not belonging to the epidemiologic survey populations (n = 7). For each case, three referents matched for serum bank, sex, age, and year of serum collection were selected. Except for five cases from the MONICA subcohort where referent samples were received from the VIP subcohort, the referents were also matched for subcohort. The mean year of serum collection was 1989 for both cases and referents, the absolute mean difference was 7.8 days, and the maximum difference was 758 days. The absolute mean difference in age at serum collection was 114 days between cases and referents, and the maximum difference was 621 days.

The local ethics committee and the Swedish Data Inspection Board approved the study.

Laboratory methods. In-house enzyme-linked immunosorbent assays (ELISA) were used to detect IgG antibodies to HSV (group-reacting antigen to HSV-1 and HSV-2), VZV, and measles.[18] All analyses were performed blind in duplicate in a pair of wells coated with a viral antigen and a control antigen. In each run, high and low positive controls and a negative control were included. The antibody activity of the sample was expressed in arbitrary units (AU) as a percentage of the net-absorbance (absorbance of virus coated well − absorbance of control antigen well) of the high positive control. Samples with <5 AU were negative. The IgG anti-EBNA-1 response was measured by a commercial ELISA from Biotest, Germany, according to the manufacturer's instruction. The antibody activity of the sample was expressed in AU as a percentage of the absorbance of the positive control. The manufacturer's cut-off value for presence of antibodies was used. The IgG response to HHV-6 (HHV-6B) was measured by a commercial ELISA from Biotrin, Ireland. The antibody reactivity was expressed in AU as for the anti-EBNA assay. The cut-off value for presence of antibodies was determined by using 30 serum samples obtained from children aged 12 to 18 months. An absorbance value <0.20 was considered as negative and was recorded in 11 children. Their mean value (SD) was 0.078 (0.057). The cut-off was determined as the mean + 3 SD; that is, 0.25. The 19 antibody positive children had an absorbance median of 1.5 (range 0.33 to 2.3).

The IgG response to EBV viral capsid antigen (VCA) was determined by immunofluorescence assay in a high screening dilu-

Table 2 *Clinical characteristics of the prospective (n = 73) and retrospective (n = 161) multiple sclerosis (MS) cases*

Characteristics	Prospective	Retrospective
Median symptom onset age, y (range)	34 (22–65)	27 (12–63)
Poser diagnostic classification		
Definite MS	73	154
Probable MS		3
Not classified		4
Clinical subtype, n (%)		
RR/SPMS	65 (89)	132 (82)
PPMS	7 (10)	17 (11)
PRMS	1 (1)	7 (4)
Not classified	0 (0)	5 (3)
MRI positive for MS	56/61 (92)	110/121 (91)
CSF positive for MS	63/68 (93)	125/137 (91)

RR/SPMS = relapsing-remitting MS and secondary progressive MS; PPMS = primary progressive MS; PRMS = progressive-relapsing MS.

Table 3 *Prevalence of seronegativity in multiple sclerosis cases and referents*

	Prospective*				Retrospective*			
Antibody	Cases, n = 73	Referents, n = 219	OR	95% CI	Cases, n = 161	Referents, n = 483	OR	95% CI
EBV-EBNA-1	0	4.1	0.24	0–1.5	0.6	5.0	0.12	0.0029–0.75
EBV-VCA	0	0.91	1.2	0–16	0	1.4	0.31	0–2.1
HSV	24.6	18.3	1.5	0.73–2.8	16.8	19.7	0.81	0.48–1.3
VZV	9.6	4.6	2.2	0.68–6.6	1.2	2.1	0.60	0.064–2.8
Measles	0	0	—	—	0	0.8	0.57	0–4.5
HHV-6	0	0	—	—	0	0.4	1.2	0–16

* Values are percentages.

EBV = Epstein-Barr virus; HSV = herpes simplex virus; VZV = varicella zoster virus; HHV = human herpesvirus.

tion (1/320) using butyric acid stimulated P3HR1 lymphoma cells.[19] All assays were read blind by the same experienced microscopist. The fluorescence activity grading was aggregated to three categories: "low" (negative in 1/320 dilution), "intermediate" (weak positive), and "high" (intermediate and strong positive). Negative samples in 1/320 dilution were identified by re-analysis in 1/20 dilution.

All methods, except HHV-6 ELISA, are accredited according to EN 45001 at The Swedish Board for Accreditation and Conformity Assessment.

Statistical analysis. SPSS was used to perform matched bivariate and multiple logistic regression analysis for estimating the risk of activity against virus on MS in terms of OR and 95% CI. The categories were created from the activities to viruses in prospective and retrospective referents. Owing to the sample size, the activity levels were split into tertiles: high, medium, and low. To estimate the risk of seronegativity of different viruses on MS, the exact method in logXact was applied to estimate the OR and 95% CI owing to the presence of zero cells.

Results. *Prospective analyses.* All prospectively collected samples from MS cases were positive for EBNA-1 and VCA. Among referents, all but nine individuals were positive for EBNA-1 and all but two for VCA. These high seropositivity rates among both patients and controls precluded the possibility to find significant risk associations as to seroconversion status as such. Likewise, all cases and referents were positive for measles and HHV-6 (table 3). Analyzing antibody activities, however, revealed that high

Table 4 *Activities to viruses in multiple sclerosis prospective samples*

			Prevalence, %		Bivariate analysis		Multivariate analysis	
Antibody	Categories	Arbitrary units	Cases	Referents	OR	95% CI	OR	95% CI
EBV-EBNA-1	Low	<126	21.9	33.3	1		1	
	Medium	126–142	15.1	34.3	0.70	0.30–1.6	0.71	0.29–1.8
	High	>142	63.0	32.4	4.2	1.9–9.2	4.5	1.9–11
EBV-VCA	Low		38.4	38.4	1		1	
	Medium		35.6	38.8	0.91	0.48–1.7	0.59	0.28–1.2
	High		26.0	22.8	1.1	0.57–2.3	0.86	0.38–2.0
HSV	Low	<72	35.6	32.9	1		1	
	Medium	72–126	46.6	33.8	1.4	0.72–2.5	1.8	0.89–3.7
	High	>126	17.8	33.3	0.63	0.32–1.2	0.59	0.25–1.4
VZV	Low	<48	28.8	33.8	1		1	
	Medium	48–68	37.0	32.0	1.3	0.71–2.5	0.98	0.46–2.1
	High	>68	34.2	34.2	1.1	0.60–2.2	0.94	0.44–2.0
Measles	Low	<122	27.4	33.8	1		1	
	Medium	122–148	26.0	33.8	1.1	0.49–2.4	0.88	0.35–2.2
	High	>148	46.6	32.4	2.4	1.1–5.6	1.4	0.52–3.6
HHV-6	Low	<50	21.9	33.3	1		1	
	Medium	50–68	27.4	34.3	1.2	0.59–2.6	1.4	0.60–3.1
	High	>68	50.7	32.4	2.4	1.2–4.8	2.3	1.0–5.1

ORs and 95% CIs estimated in bivariate and multivariate logistic regression analysis.

EBV = Epstein-Barr virus; HSV = herpes simplex virus; VZV = varicella zoster virus; HHV = human herpesvirus.

Table 5 *Activities to viruses in relapsing-remitting multiple sclerosis prospective samples by duration between serum collection and symptom onset*

		Arbitrary units		≥5 y		<5 y	
Antibody	Categories	≥5 y	<5 y	OR	95% CI	OR	95% CI
EBV-EBNA-1	Low	<126	<127	1		1	
	Medium	126–140	127–143	0.68	0.20–2.3	0.98	0.16–5.8
	High	>140	>143	4.2	1.2–14	11	1.5–75
EBV-VCA	Low			1		1	
	Medium			0.54	0.19–1.5	0.37	0.078–1.7
	High			1.4	0.40–5.1	0.16	0.032–0.86
HSV	Low	<72	<73	1		1	
	Medium	72–127	73–120	1.0	0.38–2.7	2.6	0.52–13
	High	>127	>121	0.36	0.11–1.2	0.95	0.22–4.1
VZV	Low	<50	<46	1		1	
	Medium	50–72	46–67	1.5	0.53–4.3	0.39	0.083–1.8
	High	>72	>67	0.40	0.12–1.4	1.3	0.34–5.3
Measles	Low	<125	<122	1		1	
	Medium	125–153	122–147	1.2	0.36–4.0	0.52	0.10–2.5
	High	>153	>147	1.7	0.44–6.6	0.75	0.12–4.6
HHV-6	Low	<51	<47	1		1	
	Medium	51–75	47–66	1.9	0.60–6.0	5.7	0.80–41
	High	>75	>66	2.5	0.78–8.2	12	1.8–76
No of cases							
Total				39		26	
Females				38		23	
Males				1		3	
Median age, y (range)				26 (17–59)		27 (21–49)	

ORs and 95% CIs estimated in multivariate logistic regression analysis.

activity against EBNA-1, measles, and HHV-6 significantly increased the risk for MS in the bivariate logistic regression analysis whereas VCA was not associated with risk for developing MS. In the multivariate analysis only EBNA-1 and HHV-6 remained significant (OR = 4.5; 95% CI: 1.9 to 11, and OR = 2.3; 95% CI: 1.0 to 5.1) (table 4). The risk for MS related to antibody levels at different time intervals between sample collection and MS onset was analyzed in the 65 cases with relapsing-remitting onset (table 5). The discrepancy between EBNA-1 and VCA activities was striking in cases where samples were collected less than 5 years before MS onset. High activity to EBNA-1 was associated with marked increased risk for MS in this group (OR = 11; 95% CI: 1.5 to 75), while high activity to VCA significantly decreased the risk for MS (OR = 0.16; 95% CI: 0.032 to 0.86). Similarly, HHV-6 was exclusively associated with significantly increased risk for MS in samples collected less than 5 years before MS onset (OR = 12; 95% CI: 1.8 to 76).

Retrospective analyses. The pattern of seronegativity was similar as compared to the prospective population. In this group, however, EBNA-1 seronegativity was associated with a significant decreased risk for MS (OR = 0.12; 95% CI: 0.0029 to 0.75) (see table 3). In accordance with findings in the prospectively collected samples, significantly increased risks for MS were found for medium and high activities to EBNA-1. In addition, high activities against VZV and measles were associated with an increased MS risk in the retrospective group (table 6).

Discussion. The biobanks provide unique opportunities to study events prior to disease onset; that is, factors of potential etiologic importance. Studies on biobank samples are clearly superior to the ordinary case-referent study regarding, for example, the availability of matching referents and standardization of sample collection.

Our findings support the view that past EBV infection is a prerequisite for the acquisition of MS. Together with the results from two recent prospective serologic studies, the association between EBV and MS may seem indisputable.[5,6]

The fact that all prospective MS cases in these three studies show signs of past EBV infection also suggests that EBV requires a latency period to increase the risk for MS. This is consistent with the time interval between the common ages for EBV infection and MS onset in the western world. Moreover, it is consistent with the scarcity of child-onset

Table 6 *Activities to viruses in multiple sclerosis retrospective samples*

Antibody	Categories	Arbitrary units	Prevalence, %		Bivariate analysis		Multivariate analysis	
			Cases	Referents	OR	95% CI	OR	95% CI
EBV-EBNA-1	Low	<110	6.2	33.3	1		1	
	Medium	110–144	44.1	33.6	6.5	3.2–13	6.0	2.9–12
	High	>144	49.7	33.1	8.4	4.1–17	7.5	3.6–16
EBV-VCA	Low		26.7	40.5	1		1	
	Medium		42.2	34.0	2.0	1.3–3.2	1.5	0.93–2.5
	High		31.1	25.5	2.0	1.2–3.2	1.7	0.96–2.8
HSV	Low	<77	30.4	33.4	1		1	
	Medium	77–121	38.5	33.3	1.3	0.82–2.0	1.3	0.78–2.0
	High	>121	31.1	33.3	1.0	0.65–1.6	1.0	0.60–1.6
VZV	Low	<42	19.9	33.3	1		1	
	Medium	42–59	27.3	33.6	1.5	0.88–2.5	1.2	0.71–2.2
	High	>59	52.8	33.1	3.2	1.9–5.3	2.6	1.5–4.6
Measles	Low	<109	21.1	33.0	1		1	
	Medium	109–133	36.0	33.5	1.7	1.0–2.7	1.4	0.86–2.5
	High	>133	42.9	33.5	2.2	1.3–3.7	1.9	1.1–3.3
HHV-6	Low	<47	34.1	34.2	1		1	
	Medium	47–64	25.5	32.5	0.79	0.50–1.2	0.70	0.42–1.2
	High	>64	40.4	33.3	1.2	0.80–1.9	1.0	0.63–1.7

ORs and 95% CIs estimated in bivariate and multivariate logistic regression analysis.

EBV = Epstein-Barr virus; HSV = herpes simplex virus; VZV = varicella zoster virus; HHV = human herpesvirus.

MS and MS as a complication from primary EBV infection.[20]

One proposed mechanism for how EBV infection may lead to MS is through molecular mimicry. This phenomenon has been implicated in several studies in which homologies between MBP and EBNA-1 have been demonstrated.[21,22] Furthermore, naturally processed EBV peptides may potently activate MBP-specific T cells restricted by HLA-DR2, the most common HLA type of MS patients.[23,24]

An alternative, or complementary, hypothesis is that EBV may serve as a risk factor for autoimmune diseases in a non organ-specific way. Some interesting observations may support this view. One biologic property of EBV is to prevent its own antigens from being presented on infected B cells and thereby escaping immune surveillance by the host. This is primarily mediated by blockage of proteasomal processing via the unique glycine-alanine repeat in EBNA-1.[25] Interestingly, autoantibodies against proteosomal subunits have been demonstrated to occur in several autoimmune conditions, including MS.[26] If such proteosomal autoantibodies may block some of the biologic action of EBNA-1, this could lead to an increased immunogenicity of EBV proteins, especially EBNA-1. The selectively elevated EBNA-1 response in MS cases in the present study is in agreement with such mechanisms. An increased antigen presentation capacity of EBV-infected B cells may then lead to T cell activation by infected B cells, and induction of an autoimmune response.[27]

We found a striking discrepancy between the response against EBNA-1 as compared to VCA. This discrepancy was not obvious from the two previous prospective EBV studies, which however reported a statistically significant association between high EBNA-1 (but not VCA) titers and the risk of developing MS. Since acknowledged laboratory methods have been used both in the present and in the two previous prospective studies, different laboratory methodology is probably not a plausible explanation for the observed difference, which could instead just reflect MS heterogeneity in different study populations. Interestingly, the same discrepancy (an increased response against EBNA-1 but not VCA) has been reported in a German study on another autoimmune disease, rheumatoid arthritis.[28] One possibility is that this immune response against EBV is autoimmune-specific rather than MS-specific, and that the susceptibility of the individual determines which autoantigen will be targeted. Also, in systemic lupus erythematosus patients, EBV infection has been shown to confer a risk for developing the disease.[29] Furthermore, molecular mimicry between EBV and autoimmune target antigens has been found in other autoimmune diseases than MS.[29-31]

The importance of the significant association between MS and HHV-6 in prospective samples is un-

clear. HHV-6 is latent in CD4 positive T cells, and an inflammatory response involving the activation of these cells may boost a host immune response against HHV-6. However, there is experimental support for a potential role for HHV-6 in MS pathogenesis. Cross-reactivity between MBP and HHV-6, as well as significantly higher levels of soluble CD46 (the cellular receptor used by HHV-6) in MS cases compared to referents, has been demonstrated.[32,33]

The prospective part of this study did not support an MS association for HSV, VZV, or measles. For the significant, exclusively retrospective associations for VZV and measles, these may not be attributed any causal value with certainty.

Acknowledgment

The authors thank Maria Nyström for laboratory support.

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