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Antibodies to specific EBNA-1 domains and HLA DRB1*1501 interact as risk factors for multiple sclerosis ☆

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ABSTRACT

Epitope reactivity of multiple sclerosis (MS) plasma antibodies against the Epstein–Barr virus protein EBNA-1 and its association with HLA DRB1*1501 status was investigated in a case-referent study. Based on EBNA-1 fragment reactivity and the effect of peptide blocking, four 29–36 amino acid long EBNA-1 fragments were selected for detailed studies. MS cases had increased antibody reactivity against several EBNA-1 domains, of which antibodies against EBNA-1 (amino acid 385–420) in HLA DRB1*1501 positive individuals were associated with a 24-fold risk increase for MS. The data need confirmation in a larger sample but suggest a role for this epitope in the autoimmune pathogenesis of MS.

1. Introduction

To date, increased reactivity of plasma antibodies with specificity against Epstein–Barr virus (EBV) is the strongest known single risk factor for the autoimmune disease multiple sclerosis (MS) (Ascherio and Munger, 2007). An association between MS and EBV was suggested already in 1980, and it has since been strengthened repeatedly (Alotaibi et al., 2004; Ascherio et al., 2001; Levin et al., 2003; Sumaya et al., 1980; Sundstrom et al., 2004). It is an open question whether anti-EBV antibodies reflect a non-specific autoimmune dysfunction or are a marker for a specific immune mechanism.

There are serological data from several prospective and retrospective studies suggesting that past EBV infection is a prerequisite for MS development (Ascherio et al., 2001; Levin et al., 2003; Sundstrom et al., 2004). In addition, EBV seronegativity is associated with an extremely low risk for MS (Ascherio and Munger, 2007). MS cases also have a changed antibody repertoire against EBV as compared with referents. This is characterized by a marked increase of antibodies against the latent Epstein–Barr Nuclear Antigen-1 (EBNA-1) that is accompanied by a less pronounced increase of antibodies towards other EBV antigens (DeLorenze et al., 2006; Levin et al., 2003; Lunemann et al., 2006; Sundstrom et al., 2004). The most recognized T cells controlling EBV infected B-cells are CD8 positive (Rickinson and Kieff, 1996). However, EBNA-1 specific CD4 positive T cells may play a central role in maintaining the EBV-specific immune control (Munz, 2004). The fact that immunoreactivity appears towards EBNA-1 is paradoxical, since the peptide has a unique glycine-alanine repeat that interferes with proteasomal degradation (Sharipo et al., 1998), which decreases antigen presentation in persistent EBV infection. In MS, enhanced Th1 responses exclusive for EBNA-1, but not for other latent or lytic EBV proteins, have been demonstrated (Lunemann et al., 2006).

The evidence is growing stronger that EBV plays a central role in the aetiology of several autoimmune diseases. Serological results tie EBV to autoimmune diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). Molecular mimicry between EBNA-1 and disease-specific antigens has been suggested for both RA and SLE (Fox et al., 1986; McClain et al., 2005). A similar immune response as that shown for MS (high antibody reactivity to EBNA-1 in the absence of the same response to VCA) has been shown in RA (Blaschke et al., 2000). Also in paediatric SLE an antibody reactivity preferentially directed against EBNA-1 has been found (McClain et al., 2006). Increased antibody reactivity against EBNA-1 has been shown in synovial fluid in RA as well as in the cerebrospinal fluid (CSF) in MS (Bray et al., 1992; Fox et al., 1986).

The genetic association between MS and the HLA class II haplotype was shown 35 years ago (Dyment et al., 2004). While new genes have been identified since then, their linkage to MS is not as strong as the linkage between MS and the HLA region (Hafler et al., 2007). The DR 15 allele HLA DRB1*1501 is the best studied MS risk allele and carries

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a 2 to 4-fold relative risk increase for MS. It has been shown that the HLA DRB1*1501 allele and antibody reactivity against EBNA-1 interact as risk factors for MS (De Jager et al., 2008; Sundstrom et al., 2008). The antibody reactivity to EBNA-1 is higher in HLA DRB1*1501-positive individuals than in those without the allele (Sundstrom et al., 2008). Our previous EBNA-1 analyses were performed using a commercial ELISA assay from Biotest, Germany, which covers the C-terminal 47.2% of the EBNA-1 amino acids without the glycine-alanine repeat (Hinderer et al., 1990).

The aim of this study was to extend our previous study (Sundstrom et al., 2008) by investigating the serological response to specific epitopes of EBNA-1 and to study their association with HLA DRB1*1501 status as risk factors for MS.

2. Materials and methods

2.1. MS patients and referents

A database containing 850 cases fulfilling MS diagnostic criteria (Poser et al., 1983) was created from an epidemiological survey in Västerbotten County 1997–2001 (Sundstrom et al., 2003). The database was linked with the Northern Sweden Health and Disease Study (NSHDS) Cohort (Medical Biobank of Umeå University) (Sundstrom et al., 2004). We got access to plasma and DNA from 109 cases. For each case two matched referents were selected (best match for sex, age, and year of blood collection), which yielded 212 referents (for six cases DNA was only available from one referent per case).

The samples were well matched: for both cases and referents the median year of plasma collection was 1994 (range 1987–2000), and the difference was not more than one day for ≥90% of the subjects. The absolute mean difference of age at plasma collection between cases and referents was 103 days, and the maximum difference was 435 days.

EBNA-1 short fragment analyses, see below, were not possible to interpret for one case and five referents. That left 108 cases and 207 referents for the analysis of the interaction between HLA status and the specificity of antibody reactivity to EBNA-1.

The characteristics of these 108 cases are shown in Table 1. Twenty-five out of these (matched 1:1) were used for the screening analyses (characteristics in Table 1, analyses corresponding to Tables 2 and 3). The local ethics committee approved the study.

2.2. Generation and purification of thioredoxinA-EBNA-1 fusion proteins

The fragments were amplified using the plasmid pCMVEBNA-1 (Invitrogen) by PCR with Pwo polymerase (Roche Applied Science, Mannheim, Germany) and specific primers (listed in Table E-1) carrying sites for restriction endonucleases at the 5′ ends. Then the fragments were ligated into the plasmid pET-M20 (Dr G. Stier, EMBL, Heidelberg) (EMBL, 2001) after sequence coding for TEV-site and 6 histidines at the 3′ end of the gene encoding for Thioredoxin A (TrxA).

PCR amplification: 0.1 μg pCMVEBNA-1, 300 nM of each primer, 200 nM dNTPs, 1× PCR-buffer with 2 mM MgSO4 and 2.5 U Pwo DNA polymerase in 100 μl. *Cycle conditions*: 94 °C 2 min followed by 2 cycles of 94 °C 15 s, 50 °C 25 s, 72 °C 20 s then 23 cycles of 94 °C 15 s, 60 °C 25 s, 72 °C 20 s and finished by 5 min at 72 °C. The fusion proteins TrxA-ΔEBNA-1 were expressed in *E. coli* strain Rosetta (Novagen). The bacteria were collected, washed and disrupted by sonication in lysis buffer (NaH_2PO_4 50 mM, NaCl 300 mM, glycerol 5%, imidazole 10 mM and PMSF 10 μg/ml, pH 7.2). The soluble material was passed over a column with Ni^{2+} saturated chelating agarose (Qiagen). After washing with lysis buffer, bound material was eluted with 300 mM imidazole. TrxA-fragment EBNA-1 (amino acids 478–578) was found to be insoluble in lysis buffer after sonication. In this case the insoluble fraction was dissolved in 6 M guanidine hydrochloride (GuHCl), which also was included in all buffers used for purification. GuHCl was later removed by dialysis against TBS with stepwise lowered concentration of GuHCl. Purity of the fusion proteins was checked by Coomassie brilliant blue stain of proteins separated by 12% SDS-PAGE. Concentration of fusion proteins was calculated from $OD_{280\ nm}$.

2.3. Enzyme-linked immunoabsorbent assays (ELISA)

In-house ELISA was used to analyze and quantify the binding of IgG to the fusion proteins. Nunc immunoplates were coated with 5 μg/ml of fusion proteins in TBS + 0.05% NaN_3 for 24 h in 6 °C, the wells were emptied before blocking with BSA 50 μg/ml for 1 h and washed four times with PBS Tween 0.1%. Plasma samples were diluted 1:150 in TBS with 5% Western blot blocking reagent and incubated for 2 h at +37 °C. After washing four times with PBS Tween 0.05%, 100 μl of alkaline phosphatase labeled anti-human IgG (Sigma, St Louis, OH, USA), diluted 1:36000, was added and incubated for 1 h in 37 °C. After washing four times, 100 μl 1 mg/ml p-Nitrophenyl phosphate (Sigma) in 1 M di-ethanolamin pH 9.8 was added and incubated 1 h at room temperature. The reaction was stopped with 50 μl NaOH 3 M, and the $OD_{s405\ nm}$ was read in a Multiscan spectrophotometer (Labsystems, Helsinki, Finland).

All ELISA analyses were performed blind and in duplicate simultaneously for all fragments. In each set of analyses, positive and negative controls were included on every third plate. The mean absorbance from the TrxA-6xHis control was subtracted from the mean absorbance of every sample. The antibody reactivity of the samples was expressed in arbitrary units (AU) as a percentage of the absorbance of the positive control. In each analysis 2 wells coated with each antigen were incubated with sheep anti-TrxA (a kind gift from A. Holmgren, Karolinska Institute, Sweden) and with alkaline phosphatase labeled secondary antibody to control the coating procedure.

Table 1
Characteristics of the screening and total multiple sclerosis populations.

	Screening		Total	
Number of				
Cases	25		108	
Females:males	17:8		68:40	
Clinical subtype				
Relapsing–remitting (RRMS)	9		49	
Secondary progressive (SPMS)	8		29	
Progressive from onset	6		23	
Unclear whether RRMS or SPMS	0		4	
Unclear clinical subtype	2		3	
Clinical characteristics				
Median disease duration (range)	25	(0–49)	14	−9 to +49
Median age at disease onset (range)	36	(14–63)	34	(14–65)
Serum collection				
Median age (range)	60	(50–68)	50	(29–68)
Median year (range)	1995	(1988–1998)	1994	(1987–2000)

Table 2
Median antibody reactivities against EBNA-1 fragments in 25 multiple sclerosis cases and 25 matched referents.

EBNA-1 fragment	Antibody reactivity				*p*-value
	Cases		Referents		
	Median	IQR	Median	IQR	
1–90	0.67	0.16–0.92	0.22	0.036–0.59	0.011
402–502	0.98	0.82–1.1	0.51	0.21–0.91	<0.001
478–578	0.043	0.028–0.083	0.026	0.0090–0.044	0.014
553–641	0.19	0.098–0.41	0.15	0.060–0.23	NS

IQR = interquartile range.