

Serum concentration of immunoglobulin G-type antibodies against the whole Epstein–Barr nuclear antigen 1 and its aa35–58 or aa398–404 fragments in the sera of patients with systemic lupus erythematosus and multiple sclerosis

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Summary

Several studies suggest that infection by Epstein–Barr virus (EBV) might be one of the environmental factors which facilitates the development of autoimmune disorders in genetically susceptible individuals. Recent data indicate that high anti-Epstein–Barr nuclear antigen 1 (EBNA)-1 immunoglobulin (Ig)G titre is a strong risk factor for multiple sclerosis (MS) in patients both with and without the main genetic predisposing trait, human leucocyte antigen (HLA)-DRB1*15:01. Because no similar studies have been published in systemic lupus erythematosus (SLE) patients, we determined the HLA-DRB1*15:01 carrier state and the serum titres against the whole EBNA-1 and its small fragments aa35–58 and aa398–404 in 301 SLE patients, 135 MS patients and in 345 healthy controls. The carrier state of the HLA-DRB1*15:01 allele was deduced from genotyping of a tagSNP (rs3135388) by applying a *Taqman*-based assay. The serum concentrations of antibodies to EBNA-1 and its aa35–58 or aa398–404 fragments were determined using a commercial assay (ETI-EBNA-G) and home-made enzyme-linked immunosorbent assays, respectively. The serum concentration of anti-EBNA-1 antibodies was significantly ($P < 0.001$) higher both in MS and SLE patients than in controls. Similar significant differences were found both in HLA-DRB1*15:01 carriers and non-carriers. Furthermore, titres of antibodies against the aa35–58 EBNA-1 fragment were elevated both in MS and SLE patients. By contrast, the levels of aa398–404 EBNA-1 antibodies were elevated significantly only in the SLE patients. These findings indicate that high anti-EBNA-1 IgG titres are HLA-DRB1*15:01-independent risk factors not only for MS, but also for SLE, while high antibody titres against the aa398–404 fragment are characteristic for SLE.

Keywords: Epstein–Barr virus, HLA, multiple sclerosis, systemic lupus erythematosus

Introduction

Several studies indicate that infection by the Epstein–Barr virus (EBV) is associated strongly with certain autoimmune diseases, such as multiple sclerosis (MS), systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) or myasthenia gravis (MG) [1]. Most studies addressed the EBV association of the former two [2–4]. Perhaps the strongest evidence for the association of the EBV infection and MS is the observation that patients with a high

Epstein–Barr nuclear antigen 1 (EBNA-1) immunoglobulin (Ig)G titre face a several-fold higher chance of developing MS subsequently, compared to those with a low titre. Some papers, published several years ago [5,6], have already reported that the titres of certain antibodies against EBV (EBNA-1, EBNA-2 and EA-D) are higher in blood samples obtained from future patients before the onset of MS than in those from age- and sex-matched controls. Recently, four longitudinal studies [7–10] provided strong support for these findings. In 2005, Levin *et al.* [7]

measured the titres of various antibodies against EBV in serial blood samples from 83 patients with MS and controls, using banked blood samples and archived data maintained by the US Army. Beyond the age of 20 years, anti-EBNA-1 titres were significantly higher in the samples from future patients, but remained unchanged in those from controls. Studies by Levin *et al.* showed that people who have not yet been infected by EBV face an extremely low chance of contracting MS; however, the risk increases dramatically after infection [9]. Theoretically, regarding the relationship between high anti-EBNA-1 antibody level and MS, it could be hypothesized that the susceptibility to MS and predisposition to the elevation of antibody titre might be attributed to the same genetic factors. However, this assumption has been largely debated by the latest studies [9,11–13] that compared the presence of human leucocyte antigen (HLA)-DRB1*15:01 (the strongest hereditary risk factor for MS) and high titres of anti-EBNA-1 IgG antibodies in patients with MS and in healthy controls. As demonstrated by these studies, both indicate a high risk for MS but are independent of each other. The evidence for the association between high titres of the anti-EBV antibodies and SLE is much more limited than that obtained in MS patients [14,15]. In a recent new study, Esen *et al.* [16] did not find any difference between the anti-EBNA-1 antibody levels of SLE patients and controls; however, in agreement with earlier findings [17], they detected significantly higher levels of antibodies reacting with the EA/D antigen in the patients. The HLA-DRB1*15:01 allele is less important in the genetic background of SLE than that of MS; however, several studies also indicate an elevated risk of SLE in the carriers of this allele or haplotype [18–20].

No studies have yet been performed to clarify whether the genetic factors and the high titres of anti-EBV antibodies are independent and additive risk factors not only for MS, but also for SLE. Therefore, the primary aim of the present work was to compare the levels of anti-EBNA-1 antibodies in patients with MS or SLE and in healthy controls, as well as to determine simultaneously the proportion of the carriers of the HLA-DRB1*15:01 allele in these three groups. The secondary objective of the study was to compare the fine specificity of the anti-EBNA-1 antibodies in the two diseases investigated. Several studies in SLE patients and experimental animals demonstrated a strong cross-reaction between anti-Sm B'/B antibodies and those to an epitope (aa398–404, PPPGRRP) of the EBNA-1 molecule [21–24]. Furthermore, the N-terminal 35–58 sequence of EBNA-1 is highly homologous to the C-terminal 95–119 region of the ribonucleoprotein SmD [25]. The levels of the antibodies for the former epitope, however, have not been tested in the sera of MS patients. Therefore we introduced new enzyme-linked immunosorbent assay (ELISA) methods for measuring the serum concentrations of antibodies against the EBNA-1 fragments aa35–58 and aa398–404, and compared the median levels in the sera

of high number of SLE and MS patients and healthy controls.

Patients and methods

Subjects tested

A total of 301 SLE patients [275 females, aged 44.0 (37.0–57.0) years] were included into the study. The median [interquartile (IQ) range] the age of the patients at the onset of disease was 28.0 (21.0–41.0) years; 6.0 (3.0–12.0) years elapsed between diagnosis and blood sampling, whereas the patients were followed-up for 7.0 (2.0–12.0) years from the diagnosis of SLE. The SLE disease activity index (SLEDAI) score at the time of blood sampling was 4.0 (2.0–8.0).

The most frequent organ manifestations included skin manifestations (86%), polyarthritis (71%), cytopenia (58%), central nervous system (CNS) manifestations (33%), nephritis (32%), pulmonary symptoms (17%), lymphadenopathy (15%) and cardiac (12%) manifestations, as well as myopathy (9%) (percentages of the whole SLE patient group). In detail, SLE patients experienced the following symptoms: anaemia (49%), leucopenia (44%), alopecia (16%), thrombopenia (15%), pericarditis (11%), mental dysfunction (7%), serositis (6%), vasculitis (3%), erythema of palm/sole (3%) and myocarditis (2%).

Cyclophosphamide was administered to 8% of patients, and azathioprine was used in 28% of patients. Thirteen per cent of SLE patients received both these immunosuppressants, whereas 51% of the SLE patients received none of these drugs. Steroid preparations were used in 80% of the patients, either transiently or continuously.

One hundred and thirty-five MS patients [76 females, aged 41.0 (34.8–48.0) years] were included into the study. The median (IQ range) the age of the patients at the onset of the disease was 30.0 (25.0–34.5) years, and 5.1 (1.0–9.5) years elapsed between the onset of disease and blood sampling. The patients were followed for a mean 11.0 (7.0–17.0) years from the diagnosis of MS. The mean Expanded Disability Status Scale (EDSS) scores for the cohort were 1.7 (range: 1.0–3.5) at the time of diagnosis of MS and 2.5 (range: 1.0–4.0) at the time of blood sampling. Seventy-five patients had oligoclonal bands (OCB) in the cerebrospinal fluid. The distribution of the subtypes of MS were as follows (percentage of the whole MS patient group): 73% with relapsing–remitting MS, 14% with secondary progressive MS, 8% with primary progressive MS and 5% with clinically isolated syndrome (including relapsing inflammatory optic neuropathy). MS patients were treated as follows at the time of blood sampling: 11% with intramuscular (i.m.) interferon (IFN)- β 1a, 10% with subcutaneous (s.c.) glatiramer acetate, 8% with s.c. IFN- β 1a, 4% with s.c. IFN- β 2b, 2% with intravenous (i.v.) mitoxantrone and 1% with

oral fingolimod. Sixty-four per cent of the patients received no treatment at the time of blood sampling.

Healthy controls, comprising 107 subjects who had volunteered at the time of a regular medical screening, as well as 238 parents of patients scheduled for bone marrow transplantation – that is, 345 individuals [189 females, aged 46.0 (37.0–57.0) years] in total – were included.

Genetic determination

The carrier state of the HLA-DRB1*15:01 allele was deduced from genotyping of a tagSNP (rs3135388), by applying a *Taqman*-based assay [26].

Synthesis of the aa398–404 EBNA-1 peptide conjugate

Because of the small size of the EBNA-1 epitope aa398–404, it was not possible to use the peptide as a target antigen in ELISA; therefore, this peptide was prepared as a multiple antigenic peptide (MAP) conjugate to facilitate its adsorption to the ELISA plates. (³⁹⁸Pro-Pro-Pro-Gly-Arg-Arg-Pro⁴⁰⁴-Ttds)-Lys(³⁹⁸Pro-Pro-Pro-Gly-Arg-Arg-Pro⁴⁰⁴-Ttds)-Lys[(³⁹⁸Pro-Pro-Pro-Gly-Arg-Arg-Pro⁴⁰⁴-Ttds)]-Lys(³⁹⁸Pro-Pro-Pro-Gly-Arg-Arg-Pro⁴⁰⁴-Ttds)]-NH₂ (aa398–404 EBNA-1 peptide conjugate) was prepared by the solid-phase peptide synthesis method on 4-methylbenzhydrylamine resin, with a mixed Boc/Bzl-Fmoc/'Bu strategy. First, the Boc-Lys(Boc) amino acid derivative was coupled to the resin with diisopropylcarbodiimide/1-hydroxybenzotriazol coupling agents. Then, after cleaving the Boc protecting group with 33% (V/V) trifluoroacetic acid (TFA), dichloromethane, Boc-Lys(Boc) was coupled again and the protecting groups were removed, thus producing a tetramer dendrimer or multiple antigenic peptide (MAP) core structure [27]. To enhance the solubility of the conjugate, Fmoc-4,7,10-trioxa-1,13-tridecanediaminosuccinic acid (Fmoc-Ttds) [28] was coupled to all four amino groups, and then the aa398–404 EBNA-1 peptide chains were built up with with Fmoc/'Bu strategy. Once the synthesis was complete, the side chain protecting groups were cleaved with TFA, a mixture of 1,2-ethanedithiol, thioanisole, phenol and water (10:0.125:0.25:0.75:0.25 V/V/V/m/V) for 2.5 h. Then, the peptide conjugate was cleaved from the resin with liquid hydrogen fluoride. The peptide conjugate was purified by reverse-phase high-performance liquid chromatography (RP-HPLC); its homogeneity was determined by analytical RP-HPLC and was found to be >90% pure. It was identified by a Bruker Esquire 3000 + iontrap mass spectrometer (M_{calc} : 4642.6, M_{meas} : 4644.4), and its amino acid composition was verified after acidic hydrolysis and post-column ninhydrin derivatization on a SYKAM automated amino acid analyser [Pro: 15.5 (16), Gly: 4.08 (4), Lys: 3.3 (3), Arg: 7.04 (8)].

Synthesis of aa35–58 EBNA-1 peptide

Peptide ³⁵GDDNHGRGRGRGRGGGRPGAPG⁵⁸-NH₂ (EBNA-1 aa35–58) was prepared by the solid-phase peptide synthesis method on 4-methylbenzhydrylamine resin, with Fmoc/'Bu strategy. The Fmoc-amino acid residues were coupled with diisopropylcarbodiimide/1-hydroxybenzotriazol or O-benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate/N,N-diisopropylethylamine/1-hydroxybenzotriazol coupling agents and the Fmoc group was cleaved with piperidine – 1,8-diazabicyclo[5.4.0]undec-7-ene – DMF (2:2:98 V/V/V). After the completion of the synthesis the side chain protecting groups and the peptide were cleaved with TFA: a mixture of 1,2-ethanedithiol, thioanisole, phenol and water (10:0.125:0.25:0.75:0.25 V/V/V/m/V) for 2.5 h. The peptide conjugate was purified by RP-HPLC; its homogeneity was determined by analytical RP-HPLC and was found to be >90% pure. It was identified by a Bruker Esquire 3000 + iontrap mass spectrometer (M_{calc} : 2270.4, M_{meas} : 2271.9), and its amino acid composition was verified after acidic hydrolysis and post-column ninhydrin derivatization on a SYKAM automated amino acid analyser Asp/Asn: 1.59 (2) (hydrolytic loss), Pro: 2.61 (2), Gly+Ala: 12.2 (12 + 1) (determined as co-eluted hydrolysis products), His: 1.08 (1), Arg: 6.53 (6).

Measurement of the serum concentration of the IgG-type antibodies for the whole EBNA-1 molecule and the aa35–58 or aa398–404 EBNA-1 peptide regions

The serum concentration of antibodies to EBNA-1 was determined using a commercial enzyme-linked immunosorbent assay (ETI-EBNA-G; DiaSorin, Saluggia, Italy). The plates of the kit were coated with a 59-amino-acid synthetic peptide containing several domains of the EBNA-1, which are known as immunodominant epitopes of EBNA-1. The Gly-Ala repeat was excluded, owing to its known cross-reactivity with autoantigens. Interassay coefficient of variation was found to be 5%, while intra-assay coefficient of variation was 4%.

The levels of antibodies to the aa35–58 or aa398–404 EBNA-1 epitope regions were measured by home-made ELISA methods, as follows. Maxisorb ELISA plates (NuncTM, Roskilde, Denmark) were coated with 10 µg/ml of the aa35–58 peptide or aa398–404 conjugate of EBNA-1 in phosphate-buffered saline (PBS), 100 µl/well (ON, 4°C). After blocking the plates with blocking buffer [PBS, 3% bovine serum albumin (BSA), 0.05% Tween 20] for 1 h at room temperature (RT), the serum samples, as well as the positive and negative controls, were diluted 1:40 for the anti-aa35–58 EBNA-1 detection or 1:100 for the anti-aa398–404 EBNA-1 detection in dilution buffer (PBS containing 0.05% Tween 20 and 2% BSA). A standard, twofold dilution series (1:10–1:640) of pooled serum was applied to

each plate along with diluted analytes in duplicate, and incubated for 1 h at RT. The standard serum pool was prepared from 20 individual samples which contained high levels of antibodies against EBNA-1. Plates were then washed with PBS-T (PBS, 0.05% Tween 20), and incubated with peroxidase-conjugated rabbit anti-human IgG (Jackson ImmunoResearch, West Grove, PA, USA), 1:5000 diluted in dilution buffer (1 h, RT). After washing steps, 100 µl/well *o*-phenylenediamine dihydrochloride (OPD) substrate (Dako, Glostrup, Denmark) was applied and the plates were developed for 15 min at RT. The enzymatic reaction was terminated with 50 µl/well 1 M sulphuric acid and optical density was measured at 492/620 nm. The concentrations are expressed in arbitrary units; 100 units correspond to the levels of anti-aa35–58 EBNA-1 antibody or anti-aa398–404 EBNA-1 antibody in 1 ml undiluted standard serum pool. Interassay coefficient of variation was found to be 8%, while intra-assay coefficient of variation was 6% for the anti-aa35–58 EBNA-1 assay; the corresponding values were 7% and 6% for the anti-aa398–404 EBNA-1 assay, respectively.

Determination of anti-SmB/B' and anti-SmD titres

Commercial kits were used for the determination of anti-SmB/B' titres (Varelisa® Sm antibody assay; ThermoFisher Scientific, Phadia GmbH, Freiburg, Germany) and anti-SmD titres (Hycor Biomedical, Indianapolis, IN, USA), according to the provider's instructions.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism version 5.0 (GraphPad Software Inc, San Diego, CA, USA; <http://www.graphpad.com>) and SPSS version 13.0 (SPSS Inc., Chicago, IL, USA) software. The differences between the groups were evaluated with the Mann–Whitney *U*-test. Correlations between the variables were expressed using the non-parametric Spearman's correlation coefficients. The categorical variables were compared with the Fisher's exact test. All the tests were two-tailed.

Results

Differences in the serum concentration of anti-EBNA-1 IgG-type antibodies comparing patients with MS, SLE and healthy subjects

Compared to the healthy controls, the levels of IgG-type anti-EBNA-1 antibody were significantly higher in the sera of both the MS and the SLE patients (Fig. 1). Comparing the two patient groups revealed a higher anti-EBNA-1 antibody concentration in the MS than in the SLE group (Fig. 1). Seronegative (< 20 U/ml) samples occurred significantly less frequently among both the MS patients (five of

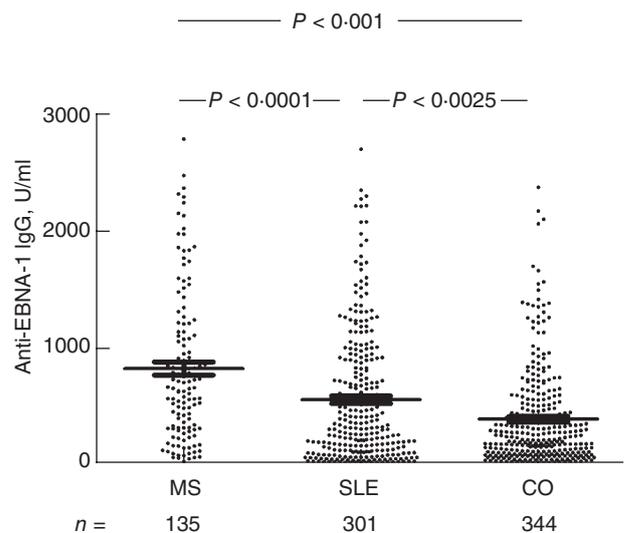


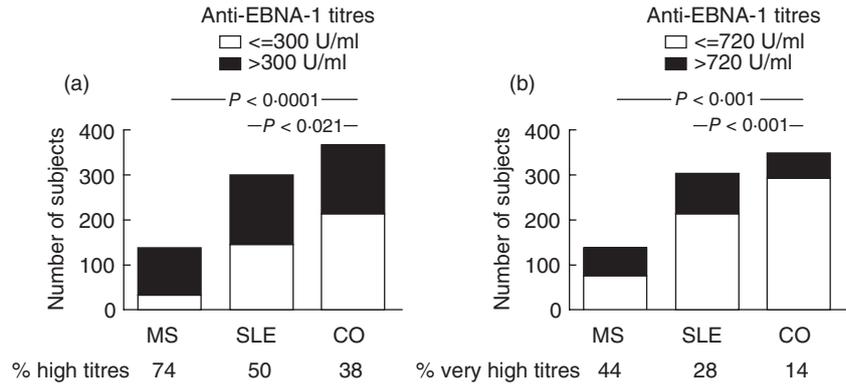
Fig. 1. The levels of immunoglobulin (Ig)G-type antibodies to whole Epstein–Barr nuclear antigen 1 (EBNA-1), in the sera of EBNA-1-positive patients with multiple sclerosis (MS) or systemic lupus erythematosus (SLE) and in healthy subjects. EBNA-1 positivity (> 20 U/ml) was found in 96% (130 of 135) of MS patients, in 95% (286 of 301) of SLE patients and in 90% (310 of 345) of healthy control subjects. *P*-values of non-parametric analysis of variance (Kruskal–Wallis) test followed by Dunn's *post-hoc* test are shown.

135) ($P = 0.0261$) and SLE patients (15 of 301) ($P < 0.0258$, Fisher's exact test) than among the control samples (35 of 345). When the EBV-negative subjects were excluded from the comparative analysis, significant differences were still found between the IgG EBNA-1 response levels of EBV-positive MS patients [654.8 (313.8–1138.0) AU/ml], SLE patients [344.4 (120.2–890.7) AU/ml] and healthy controls [202.0 (87.2–453.9) AU/ml]. Both high (> median, 300 U/ml) and very high (in the highest quartile (> 720 U/ml) values were found significantly more frequently in both the MS and SLE patients' sera compared to healthy controls (Fig. 2). When comparing the subgroups of MS patients, no difference was found between MS patients without treatment [578.0 (246.0–1060.0) AU/ml] or with treatment [641.9 (290.0–1591.0) AU/ml] ($P = 0.2581$). Similarly, no difference was found between SLE patients without treatment [301.7 (71.1–949.4) AU/ml] or with treatment [343.9 (103.5–712.3) AU/ml] ($P = 0.9099$).

The frequency of HLA-DRB1*15:01 carriers among MS or SLE patients and healthy subjects

The frequency of this allele was determined in representative groups of 268 SLE patients and 90 MS patients, as well as 282 healthy controls (no DNA samples were available for the remaining patients) (Fig. 3). Carriers of the HLA-DRB1*15:01 allele were significantly more common among the MS patients than in the group of healthy controls. When the subjects were divided according to sex, the

Fig. 2. Frequency of the (a) 'high' (> 300 U/ml, median of all values) and (b) 'very high' (> 720 U/ml, in the highest quartile of all values) anti-Epstein-Barr nuclear antigen 1 (EBNA-1) titres in the sera of multiple sclerosis (MS) and systemic lupus erythematosus (SLE) patients, as well as healthy controls (CO). Both 'high' and 'very high' titres were found significantly more frequently in both the MS and SLE patients' sera compared to the healthy controls, as shown by the percentages.



frequency of the HLA-DRB1*15:01 carrier state was significantly higher among MS patients than among healthy subjects, both in females and in males ($P = 0.0084$ and $P = 0.0058$). This frequency was also significantly higher in SLE patients than in healthy controls, although the difference was smaller (Fig. 3).

The relationship between the carrier state of the HLA-DRB1*15:01 allele and anti-EBNA-1 antibody levels in patients with MS or SLE and in healthy controls

Next, we compared the levels of anti-EBNA-1 antibodies separately in DRB1*15:01 carriers and non-carriers in patients and controls. Both in carriers and non-carriers, compared to the healthy controls, the serum concentration of IgG antibodies to EBNA-1 was significantly higher both in MS and in SLE patients, although the difference was greater in the case of the former (Table 1).

Next we investigated if the effect of the HLA-DRB1*15:01 carrier state and the high titre of the anti-EBNA-1 antibodies are additive. When the distribution of subjects with neither, and both of these factors, was calculated, we found highly significant ($P = 0.0008$) differences between controls subjects and SLE patients: 152 of 282 (54%) and 15 of 282 (5%) of the controls had none of the risk factors and both

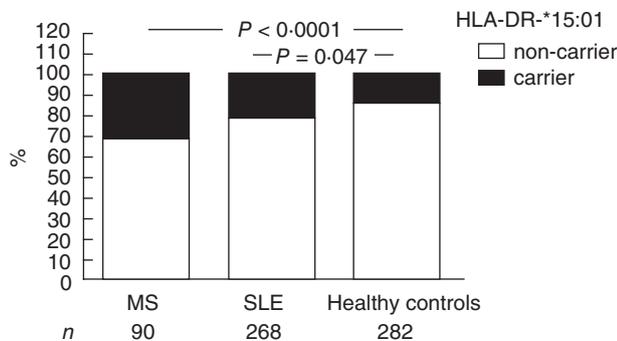


Fig. 3. The distribution of human leucocyte antigen (HLA)-DRB1*15:01 carriers within the study groups. *P*-values for Fisher's exact test are indicated.

risk factors, respectively, while for the SLE patients the same proportions were 107 of 268 (40%) and 32 of 268 (12%), respectively. In the case of MS, even higher differences ($P < 0.0001$) were observed: no risk factor, 21 of 90 (23%); both risk factors, 27 of 90 (30%).

Differences in the serum concentration of the antibodies to the aa35–58 and aa398–404 fragments of the EBNA-1 in patients with MS or SLE, and in healthy subjects

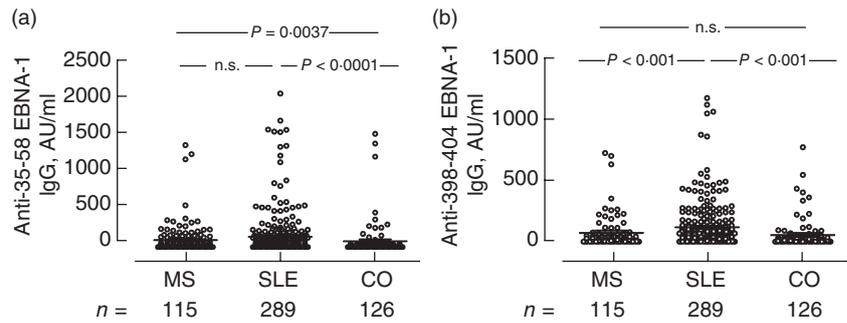
Antibodies against the aa35–58 epitope region of EBNA-1 presented significantly elevated levels both in MS patients [26.6 (2.9–103.0) AU/ml; $P = 0.0037$] and in SLE patients [33.2 (3.3–129.5) AU/ml; $P < 0.0001$], compared to healthy controls [7.5 (0–32.6) AU/ml] (Fig. 4a). We could not find a difference in the study group regarding the frequency of patients with undetectable antibodies against the aa35–58 fragment of the EBNA-1. As the N-terminal 35–58 sequence of EBNA-1 is highly homologous to the C-terminal 95–119 region of the ribonucleoprotein SmD, we also determined

Table 1. The levels of immunoglobulin (Ig)G-type antibodies to Epstein-Barr nuclear antigen 1 (EBNA-1) in the sera of patients with multiple sclerosis (MS) or with systemic lupus erythematosus (SLE) and in healthy subjects, who carry or do not carry the human leucocyte antigen (HLA)-DRB1*15:01.

Group	Number of subjects	Anti-EBNA-1 IgG, U/ml median (interquartile range)
HLA-DRB1*15:01 carriers		
MS patients	31	596.0 (338.8–1100.8)***+++
SLE patients	53	386.9 (102.7–963.1)*
Healthy subjects	42	198.7 (71.7–427.0)
HLA-DRB1*15:01 non-carriers		
MS patients	59	438.0 (146.0–825.0)**+++
SLE patients	215	304.7 (103.1–726.1)***
Healthy subjects	240	183.7 (76.3–455.8)

+++ $P < 0.001$ between the two patient groups. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$ compared to the healthy control group. Non-parametric analysis of variance (Kruskal-Wallis) test followed by Dunn's *post-hoc* test.

Fig. 4. The levels of immune globulin (Ig)G-type antibodies to the (a) aa35–58 or to the (b) aa398–404 fragments of Epstein–Barr nuclear antigen 1 (EBNA-1) molecule, in the sera of patients with multiple sclerosis (MS) or systemic lupus erythematosus (SLE) and in healthy subjects. *P*-values of non-parametric analysis of variance (Kruskal–Wallis) test followed by Dunn's *post-hoc* test are shown. n.s.: not significant.



the anti-SmD titres in SLE patients. A significant correlation was found between the anti-35–58 EBNA-1 levels and the anti-SmD titres ($r = 0.2474$, $P < 0.0001$), as well as a moderate but significant correlation with the anti-SmB/B' titres ($r = 0.1402$, $P = 0.0258$).

Compared to healthy controls [7.3 (0.0–31.1) AU/ml], the serum concentration of antibodies against the aa398–404 epitope of the EBNA-1 was, significantly, sixfold higher in the sera of the SLE patients [44.8 (12.7–127.6) AU/ml]. By contrast, no significant difference could be found between the MS patients [10.3 (0.5–41.6) AU/ml] and healthy controls (Fig. 4b). The same reactivity patterns were ascertained when the results were stratified according to the HLA-DRB1*15:01 carrier state (data not shown). Antibodies against the aa398–404 epitope of the EBNA-1 were undetectable in 28 of 115 MS patients ($P = 0.0091$ compared to the controls, $P < 0.0001$ compared to the SLE patients, Fisher's exact test), in 22 of 289 SLE patients ($P < 0.0001$) and in 51 of 126 control samples.

We found a significant correlation between the titres of antibodies against the aa398–404 epitope of the EBNA-1 and the anti-SmB/B' titres ($r = 0.3286$, $P < 0.0001$), and a slight but still significant correlation with the anti-SmD titres ($r = 0.1517$, $P = 0.0095$).

Discussion

Our paper reports two novel findings. First, we extended to SLE patients the previous observations of several authors, indicating that the HLA-DRB1*15:01 carrier state and the high serum concentration of IgG-type anti-EBNA-1 antibodies are independent and additive risk factors of MS. We have demonstrated that, compared to healthy controls, the level of anti-EBNA-1 IgG is higher in SLE patients, whether or not they carry the HLA-DRB1*15:01 allele, and the effect of the two risk factors are additive. Secondly, we found that the serum concentration of IgG-type antibodies against a particular EBNA-1 epitope (aa398–404, PPPGRRP), which has been found previously to cross-react with an autoantibody (anti-SmB/B') characteristic for SLE, is elevated in SLE but not in MS. Interestingly, titres of antibodies against the aa35–58 EBNA-1 epitope region, which is

highly homologous to the C-terminal 95–119 region of SmD, were elevated both in MS and SLE patients.

Sundstrom *et al.* [29] were the first to demonstrate by multivariate logistic regression analysis that EBNA-1 reactivity and HLA-DRB1*15:01 are statistically independent risk factors for MS. Later, the same group [12] found that the levels of antibodies against several epitopes of EBNA-1 are also significantly higher in MS patients than in healthy controls, regardless of whether or not they carry the HLA-DRB1*15:01 antigen. In accordance with these findings, Waubant *et al.* [30] found that EBNA-1 seropositivity was associated with an almost fourfold risk of MS in patients with early paediatric MS. Interestingly, in a further study children with MS did not differ from age-matched healthy controls in seroprevalence of further childhood viruses [31].

Furthermore, another group [11] showed that the association between anti-EBNA-1 titres and MS risk was not affected by adjustment for DR15, and was similar in DR15-positive and DR15-negative women. More recently [13], the same group reported on the combined effects of smoking, anti-EBNA antibodies and HLA-DRB1*15:01 on MS risk. Sundqvist *et al.* [32] studied the interactions of three MS risk factors in association with high anti-EBNA-1 titres (and those of the anti-385–420 fragment of the EBNA-1, HLA-DRB1*15:01) as well as in the absence of HLA-A*02. They found a strong interaction among these risk factors and concluded that the mechanism through which HLA genes influence the risk of MS may be related to the immune control of EBV infection. Our present findings are in complete accord with these results. Compared to healthy control subjects, the serum concentration of anti-EBNA-1 antibodies was significantly ($P < 0.001$) higher in the sera of both HLA-DRB1*15:01 carrier and non-carrier MS patients. By contrast, our present findings are at variance with the recent data from Irizar *et al.* [33], as in the Hungarian population we did not find the association between HLA-DRB1*15:01 and MS to be female-specific.

Only speculative explanations have been published to date for the presumed causes of the elevated anti-EBNA-1 IgG antibody levels in MS. According to Levin *et al.* [7], the elevation of antibody titres to EBNA-1, seen in future MS

patients during their early 20s, suggests a more severe or more recent primary infection or reactivation of a pre-existing infection, accompanied by a vigorous cellular immune response. Very recently, Santon *et al.* [34] detected dual infection by both types (1 and 2) of EBV in 63 patients (90%) and 46 controls (37.4%). Logistic regression models showed a significant ($P < 0.001$) association between MS and dual-type infection. It can be assumed that this dual infection may result in the elevation of anti-EBNA-1 IgG titres.

Considering that SLE also seems to be associated with EBV infection [14,15,35,36], and HLA-DRB1*15:01 is one of the genetic risk factors for SLE [19,20], we performed a similar study of SLE patients. To the best of our knowledge, until now the interaction between high-titre anti-EBNA-1 antibodies and the carrier state of HLA-DRB1*15:01 has not been studied. In accordance with earlier work [14,15], but in contrast to a recent study [16], we found that the titre of the anti-EBNA-1 antibody is significantly higher in SLE patients than in healthy controls. Performing this comparison in carriers and non-carriers of HLA-DRB1*15:01, we found the same difference for both groups, indicating that high EBNA-1 reactivity and the carrier state of HLA DRB1*15:01 are statistically independent and additive risk factors for both MS and SLE.

In the second part of the study, we found substantial differences between MS and SLE patients regarding the level of IgG-type antibodies to an EBNA-1 epitope (aa398–404, PPPGRRP). The serum concentration of this type of antibody was significantly higher in SLE patients than in healthy controls, while no difference could be found between controls and MS patients. The high serum concentration of anti-aa398–404 EBNA-1 antibodies is not surprising in SLE, as many observations in patients [14] and data from animal experiments [37] demonstrated a strong cross-reaction between this EBNA-1 epitope and an epitope (B'/B) of the Sm autoantigen, and autoantibodies against the latter are characteristic in SLE. However, it was demonstrated recently [12] that, in MS, the elevated level of anti-EBNA-1 includes antibodies directed against several epitopes of the protein, but anti-PPPGRRP antibodies have not been studied previously in MS patients. Nevertheless, Sundstrom *et al.* [12] measured antibodies to a larger EBNA-1 fragment (391–410, SGSPRRRPPGRRPFFH-PVG), which includes the 398–404 fragment, and found only a small difference ($P = 0.047$) in the antibody levels of MS patients and controls. Although antibodies against the aa35–58 epitope region of EBNA-1 showed a significantly higher level in SLE or MS patients, no difference was found among the study groups regarding the frequency of patients with undetectable antibodies against the aa35–58 epitope region of the EBNA-1. Our results are in accordance with the findings of Marchini *et al.* [25], who described that antibodies to the aa35–58 EBNA-1 fragment are produced in controls and in EBV-related diseases, but only SLE sera

contain anti-viral antibodies cross-reactive with the SmD autoantigen. Thus, our present findings suggest that the elevated level of the anti-PPPGRRP antibody is characteristic of and specific for SLE.

Earlier, we summarized [4] findings indicating differences in the relationship of EBV with the two autoimmune diseases, MS and SLE. The incidence of MS [38], but not of SLE [39], is markedly higher in those who had had infectious mononucleosis previously. While the viral load is extremely high in SLE [40,41], in MS it is similar to that seen in the healthy population [42]. Our present finding on anti-PPPGRRP antibody levels also supports this conclusion. Thus, our present observations are in line with the literature data, and indicate that there are both similarities and differences in the relationship of EBV with two autoimmune diseases, MS and SLE. Studies into these connections may provide new data on the nature of the associations between EBV infection and these autoimmune disorders.

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