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PAPER

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**Objectives:** To study in relapsing–remitting (RR) multiple sclerosis (MS) whether exacerbations and brain activity as measured by magnetic resonance imaging (MRI) are associated with plasma levels of anti-Epstein Barr (EBV) antibodies and EBV DNA.
**Methods:** This was a prospective study with 73 RR MS patients followed for an average of 1.7 years with frequent neurological examination and blood sampling. Antibodies to various EBV proteins were measured by ELISA and plasma EBV DNA was measured by PCR.
**Results:** All MS patients had IgG antibodies to EBV (viral capsid antigen (VCA) and/or EBV nuclear antigen (EBNA)), irrespective whether samples were taken at stable disease or exacerbation. A significantly elevated percentage of the patients (48%) had antibodies against EBV antigens (early antigen, EA) that indicate active viral replication, compared with the age matched healthy controls (25%). Antibodies against a control herpesvirus, cytomegalovirus, were similar between the two groups. The percentage of EA positive individuals and EA titres did not differ between stable disease or exacerbation. Anti-VCA IgM was positive in three cases, unrelated to disease activity. Using a highly sensitive PCR on 51 samples taken at exacerbation visits, only three patients were found to have one timepoint with viraemia, and this viraemia was unrelated to disease activity. Of special note was the fact that anti-EA seropositive patients remained seropositive during follow up, with stable titres over time. We hypothesised that these patients may constitute a subgroup with higher disease activity, due to the triggering effect of a chronic attempt of the virus to reactivate. The EA positive group did not differ from the EA negative with respect to clinical disease activity or other characteristics. However, in the EA positive group, analysis with gadolinium enhanced MRI indicated more MRI disease activity.
**Conclusions:** There was no evidence for increased clinical disease activity in the subgroup of MS patients with serological signs of EBV reactivation. However, the observation that chronic EBV reactivation may be associated with increased inflammatory activity as assessed by gadolinium enhanced MRI lesions should be reproduced in a larger and independent dataset.

There is accumulating indirect evidence for a role of Epstein-Barr virus (EBV) infections in the pathogenesis of multiple sclerosis (MS).[1–4] The risk for MS is significantly increased after infectious mononucleosis, and MS is rare among individuals without serum anti-EBV antibodies. Moreover, a recent large prospective epidemiological study showed that serum antibody titres against EBV were increased before onset of MS.[5 6] In addition, T cell reactivity to EBV epitopes is different in MS,[7] and in the cerebrospinal fluid, T cells of MS patients recognise EBV transformed B cells.[8] It may be the age at primary EBV infection that influences the risk for future development of MS, analogous to the relation between EBV infection and development of infectious mononucleosis.[9 10]

Apart from these studies aiming at the aetiology of MS, studies that investigate the relation between EBV and MS disease activity are scarce. The striking property of herpesviruses such as EBV to show periodic reactivation makes EBV an attractive candidate as a co-factor in the relapsing remitting course of MS. It has been suggested that EBV reactivation is associated with the occurrence of exacerbations.[11] Recently, we conducted a prospective study to investigate the role between infections and exacerbations in MS. Using the material obtained during that study, we questioned whether EBV antibody and EBV DNA levels in plasma are increased during exacerbations in MS and whether serological signs of EBV activity correlate with activity measured by magnetic resonance imaging (MRI).

## METHODS

### Patients and samples

Data and material for this study were collected in the Rotterdam Study on Exacerbations in MS (ROSE), a prospective cohort study specifically designed to investigate the relation between MS exacerbations and infections.[12] Patients with relapsing–remitting (RR) MS were followed for a mean of 1.7 years. EDSS scores were performed at regular 8 week visits and at two additional visits (3 weeks apart) in case of either exacerbation or clinical infection, when also blood samples were collected.

In a random subgroup, a series of three magnetic resonance imaging (MRI) examinations were planned following every infection over a period of 6–7 weeks after the onset of infection. In addition, control plasma samples were collected from healthy individuals (n = 52). All plasma samples were immediately frozen in −80°C until use.

### Definitions

Exacerbation was defined as worsening of existing or appearance of new symptoms lasting more than 24 hours and following a period of at least 30 days of improvement or stability.[13] Neurological deterioration only temporarily associated with a period of fever was not considered as exacerbation. Infection was defined as the appearance of

**Abbreviations:** EA, early antigen; EBNA, Epstein-Barr nuclear antigen; EBV, Epstein-Barr virus; MS, multiple sclerosis; RR, relapsing–remitting; VCA, viral capsid antigen

coryza, sore throat, flu-like feeling, myalgia, fever, diarrhoea, or a urinary infection lasting for more than 24 hours.

Exacerbations were categorised according to severity and duration. Exacerbations with an EDSS increase ⩾1.0 and lasting more than 3 months were defined as sustained progression.[12]

### Detection of viral antibodies

The following antibodies to Epstein-Barr antigens were determined: IgM and IgG against EBV viral capsid antigen (VCA), IgG against EBV early antigen (EA) and IgG against Epstein-Barr nuclear antigen (EBNA).[14] Antibodies to these antigens were determined by ELISA (Biotest, Dreieich, Germany) following the manufacturer's instructions. Anti-cytomegalovirus (CMV) IgG was tested with ELISA (ETI-Cytok G; Diasorin, Saluggia, Italy).

For each measurement of an antibody concentration, a ratio was calculated as sample optical density (OD) value divided by cutoff OD value. The cutoff values were defined as the mean OD+3SD of the negative control. For all assays, a ratio of >2.0 was considered as seropositive.

### PCR

*Taq*man PCR primers were selected from the EBV DNA genome encoding for the non-glycosylated membrane protein BNRF1 P143, and generated a DNBA product of 74 bp.[15] A known EBV copy number based on a reference standard quantified by electron microscopy (ABI Advanced Biotechnologies, Columbia, MS, USA) was used for standardisation. Serial dilutions ranging from 10 to $10^7$ genome equivalents per ml (gEq/ml) were made to characterise linearity, precision, specificity, and sensitivity. The *Taq*man assay appeared to detect viral DNA in plasma over a linear span between 50 and $10^7$ gEq/ml with an average coefficient of variation of 1.56% (range 0.7 to 7.0%). Test results below 50 gEq/ml were considered negative. No viral DNA was detected in plasma of healthy EBV seropositive individuals.

### Magnetic resonance imaging protocol

In case of symptomatic infection, a series of three MRI scans was planned in a random subset of patients: the first soon after the onset of infection (MRI1); the second after a period of 3 weeks (MRI2), and the third after 6 weeks (MRI3),[12] using a 1.5 T imaging unit (Philips Gyroscan ACS-NT). Initially, 5 mm slices from T1 weighted sequences were obtained using 0.1 mmol/kg gadolinium diethylene triamine penta-acetate (Gd-DTPA; single dose). Halfway through the study, 0.3 mmol/kg Gd-DTPA (triple dose) with 3 mm slice protocol was applied to enhance sensitivity.[12] We quantified the number of Gd enhanced lesions, the number of new lesions in the second and third MRIs of the same series and the number and percentage of active MRI series per patient. An active MRI series was defined as a series with at least one enhanced lesion in any of the three MRIs. Only scans of patients not treated with interferon (IFN)-β were analysed.

### Statistics

Between group differences in different demographic, MRI, and serological parameters (ratios) were analysed by independent *t* tests or Mann-Whitney test. Difference in mean EDSS value was tested using the Mann-Whitney test. Frequency distribution between groups for number of patients with sustained progression and for sex, number, and type of exacerbations were tested by $\chi^2$ test. Comparison of different MRI parameters between groups was performed using Mann-Whitney or Fisher's exact test.

## RESULTS

### Clinical characteristics

Of 73 patients, 58 had at least one exacerbation during study follow up and were selected for further analysis. Plasma samples from 54 of the 58 patients were available for analysis. Average age was 39.3 years, with average disease duration of 10.4 years, and an average baseline EDSS of 2.5 (median 2.0, range 0 to 6.0). The total follow up time was 5130 weeks (98.7 years), during which 141 exacerbations occurred.

### EBV seropositivity

As markers of previous EBV infection, anti-VCA IgG and anti-EBNA IgG were tested in 54 patients (total 188 samples). Anti-VCA IgG was positive in each sample of all 54 patients (100%) and anti-EBNA IgG in 51 of 54 patients (94%). We tested anti-VCA IgM and anti-EA IgGA as markers for reactivation of EBV.[16 17] All 188 samples tested for anti-VCA IgM were negative except three, collected on a single occasion in three different patients. IgG seropositivity to EA was tested in 214 samples from 54 patients (average 3.9 samples per patient, median 3, range 2 to 18). Anti-EBV EA IgG was positive in 26 of 54 (48%) of the patients (table 1).

Because others previously reported a difference in anti-EA positivity between MS patients and healthy individuals,[18 19] we also tested anti-EA IgG in a group of 52 age matched healthy individuals. Significantly fewer of these controls were positive (13/52, 25%, $p = 0.013$). In addition, the concentrations of anti-EA IgG were significantly higher in the MS group (median 1.8, mean (SD) 2.2 (0.2)) than in the healthy controls (0.6, 1.3 (0.2), $p<0.001$; Mann-Whitney *U* test, fig 1). Differences remained after exclusion of patients that received IFN-β. IgG titres against the control herpesvirus, CMV did not differ significantly between MS patients and healthy individuals. In the MS group, 44% were anti-CMV IgG positive versus 56% in the healthy controls ($p = 0.2$).

### Exacerbations and EBV seropositivity

We analysed if the occurrence of exacerbations was associated with a higher percentage in EBV seropositivity. We compared samples collected at the baseline visits (when there was no exacerbation and/or infection) with samples taken during the first exacerbation. Exacerbation samples were collected at the second exacerbation related visit, 3 weeks after the onset of exacerbation, to allow sufficient time for development of a titre increase. In total, 45 paired baseline exacerbation samples were available. Anti-VCA IgG was positive in 100% of cases, at both timepoints. None of the samples was positive for anti-VCA IgM, either at the baseline or at exacerbations. Anti-EBNA IgG was positive in 43/45 patients, both at baseline and exacerbation visits. Anti-EA IgG were positive in 24 of 45 baseline (53%) and in 22 of 45

**Table 1** Frequency of seropositivity for different anti-EBV antibodies in the study population*

| Anti-VCA IgG | Anti-EBNA IgG | Anti-EA IgG | No. of patients |
|---|---|---|---|
| + | + | + | 24 |
| + | + | – | 27 |
| + | – | + | 2 |
| + | – | – | 1 |
| | | | Total 54 |

*Based on the measurement of at least two different plasma samples taken per patient (total 188 samples for anti-VCA and anti-EBNA and 214 samples for anti-EA).

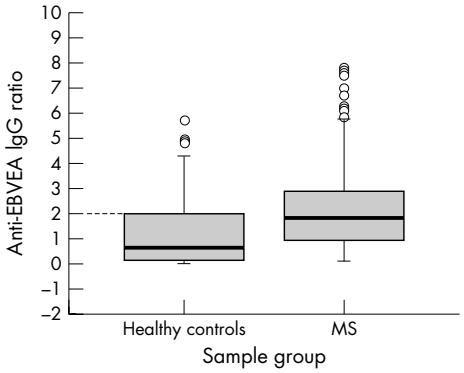


**Figure 1** Anti-EBV EA IgG ratio in MS patients (n = 54) and age matched healthy controls (n = 52). Boxplot represents interquartile range including 50% of the values. Line across the boxplot represents median. Whiskers extend to the highest and lowest value, excluding outliers, which are given separately. Mean (SE) anti-EBV EA was 2.2 (0.2) in the MS group and 1.3 (0.2) in the healthy controls, p<0.001. Dotted line represents the cutoff value.

exacerbation samples (49%) (p = 0.7). Thus, no significant difference in EBV seropositivity was found between baseline and exacerbation samples.

### Titres of anti-EBV IgG and exacerbations

To examine whether exacerbations in MS are associated with a change in anti-EA IgG ratio, we performed a quantitative analysis in the same set of 45 baseline–exacerbation paired samples. The mean (SD) anti-EA IgG ratio was 2.4 (0.3) at baseline, compared with 2.3 (0.3) at exacerbation (p = 0.2, paired *t* test, fig 2). Therefore, exacerbations were not found to be associated with a change in anti-EA Ig titre.

### Anti-EA IgG ratios: a stable phenomenon

Anti-EA IgG antibodies have been described to fluctuate over time, in association with EBV reactivation.[11] We were surprised to observe that nearly all patients who tested EA positive at one timepoint remained positive at all other timepoints tested. A total of 214 samples from 54 patients was tested for EA-IgG positivity (average 3.9 samples per patient, median 3, range 2 to 18) covering a period of 1537 weeks or 30% of follow up time (on average 28.5 weeks per patient, median 19, range 2 to 104). Levels of anti-EBV EA IgG remained remarkably stable within individual patients (intraclass correlation (ICC) = 0.96) (fig 3).

### Anti-EA IgG positive and negative individuals: different clinical characteristics

In total, 26 patients were EA seropositive (mean (SD) ratio 3.7 (0.1)) and 28 seronegative (1.1 (0.2)). Testing the samples of these groups for IgG titres against control viruses (HHV6, CMV, and parainfluenza 1) revealed no significant titre differences between the EA seropositive group (EA+) and EA negative group (EA−) groups (p>0.2, data not shown). Furthermore, the levels of the IgG EA titres did not show any correlation with the IgG titres against these control viruses, which makes it unlikely that EA seropositivity was merely part of a more generalised aspecific immune activation.

Because of the stable anti-EA IgG positivity over time, we were interested whether the EA+ group had intrinsic clinical characteristics that were different from the EA−. Baseline demographic data for these two groups are given in table 2, showing no significant differences between the two groups in sex, age, disease duration, or progression up to the start of the study.

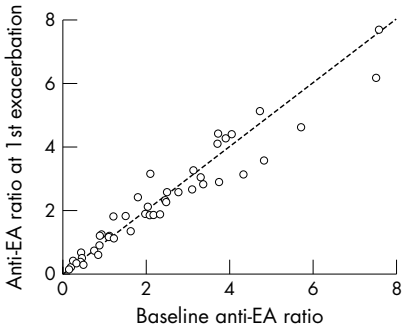


**Figure 2** The scatterplot representing anti-EBV EA IgG ratio at the baseline and at the first exacerbation, n = 45. Dotted line represents the line of identity.

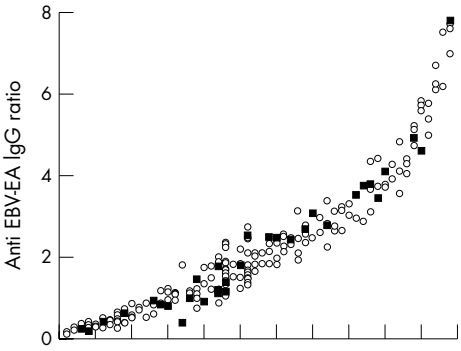


**Figure 3** Stability of anti-EBV EA IgG titre during follow up. The y axis depicts anti-EBV EA ratio for each sample. Individual patients, sorted on mean EA ratio from lowest to highest (n = 54), are represented along the X-axis. Filled squares represent samples taken during exacerbation.

Clinical features observed during follow up were also similar between EA+ and EA− patients. EA+ patients were followed for 47.9 years with 60 exacerbations, 13 of which were sustained, while EA− patients were followed up for 49.2 years with 80 exacerbations, 15 of which were sustained. In both groups, 13 patients had at least one exacerbation with sustained progression. Mean annual relapse rate was 1.4 in the EA+ and 1.8 in the EA− group (p = 0.1, Mann-Whitney). Mean annual relapse rate for exacerbations with sustained progression was 0.28 in the EA+ and 0.27 in the EA− group (p = 0.96, Mann-Whitney). Disease progression rate (in EDSS points; mean (SE)) during the follow up also did not differ between the groups (EA+ patients 0.5 (0.2), EA− patients 0.6 (0.3), p = 0.96 Mann-Whitney).

More conservative calculation, using only clinical data from the follow up period between samples (that is, from the first to the last samples per patient) gave similar results (table 2). The total follow up in this calculation was 29.6 years, with 66 exacerbations, 15 of which were exacerbations with sustained progression. Mean annual exacerbation rate and exacerbation rate for exacerbations with sustained progression again did not differ between the two groups. In the EA+ group, median annual RR was 3.0 (3.3 (0.4), and in the EA− group 2.6 (mean (SE) 3.2 (0.5), p = 0.4, Mann-Whitney). For exacerbations with sustained progression the median annual exacerbation rate in the EA+ group was 0.0 (1.2 (0.5), and in the EA− group 0.0 (mean (SE) 0.4 (0.2)), p = 0.3, Mann-Whitney). We were thus unable to detect any difference in the clinical course of MS between these two groups.

**Table 2** Clinical characteristics of patients in EBV EA seropositive and negative groups

| | Mean anti-EBV EA IgG ratio | |
|---|---|---|
| | >2.0 (EA+) | <2.0 (EA−) |
| Demographic data | | |
| No. of patients (F/M) | 26 (22/4) | 28 (20/8) |
| Mean (SE) age in years | 41.3 (1.5) | 37.3 (2.0) |
| Mean (SE) disease duration in years | 10.7 (1.2) | 10.0 (1.4) |
| Mean baseline EDSS (median, range) | 2.3 (2.0, 0 to 5.5) | 2.7 (2.0, 0 to 6.0) |
| Annual progression rate of disease (SE)† | 0.3 (0.1) | 0.4 (0.2) |
| Study data* | | |
| Years of follow up | 11.4 | 18.2 |
| No. of exacerbations | 28 | 38 |
| Median (mean) (SE) annual exacerbation rate | 3.0 (3.3) (0.4) | 2.6 (3.2) (0.5) |
| No. of exacerbations with sustained progression‡ | 8 | 7 |
| Median annual exacerbation rate for exacerbations with sustained progression (mean (SE)) | 0.0 (1.2) (0.5) | 0.0 (0.4) (0.2) |

*Follow up data presented here relate only to the follow up period from the first to the last collected sample per patient, which is shorter than clinical follow up per patient; †calculated as baseline EDSS per number of years since disease onset; ‡for definition of sustained progression, see text.

**Table 3** MRI data for EA+ and EA− patients

| | Mean anti-EBV EA IgG ratio | | |
|---|---|---|---|
| | <2.0 (EA−) | >2.0 (EA+) | p value |
| 1-Gd | | | |
| Number of patients | 15 | 12 | |
| Mean no. of Gd enhanced lesions | 0.14 (0.07 | 0.7 (0.5 | 0.3 |
| Mean no. of new lesions | 0.05 (0.05 | 0.3 (0.2 | 0.09 |
| No. of patients (%) with ⩾1 active MRI series | 4 (27%) | 5 (42%) | 0.4* |
| 3-Gd | | | |
| Number of patients | 11 | 9 | |
| Mean no. of Gd enhanced lesions | 0.39 (0.2 | 1.87 (0.8 | 0.03 |
| Mean no. of new lesions | 0.05 (0.05 | 0.48 (0.3 | 0.02 |
| No. of patients (%) with ⩾1 active MRI series | 5 (45%) | 8 (89%) | 0.07* |

One MRI series per patient was analysed;, lesion numbers are depicted as mean (SEM). All calculations Mann-Whitney except for *Fisher's exact test.

### EBV antigenaemia and exacerbations

Because of the serological indication of a high percentage of EBV reactivation, we used an ultrasensitive PCR to test for the presence of EBV antigen in 51 samples drawn during the exacerbation visits of 48 patients. Only 3 of 51 samples (6%) were positive. From these three patients we performed the PCR analysis in all samples that were obtained during study follow up; 17 samples per patient (a total of 51). None tested positive.

### MRI lesions and EA serology

A series of three sequential MRI scans from 37 patients without immunomodulatory treatment was available for analysis. The time lapse between the onset of infection and the MRI recordings was 7, 24, and 47 days for the first, second, and third MRI respectively.[12] We performed the analysis separately for single and triple Gd MRIs.

In the Gd single dose group, a series from 12 EA+ and 15 EA− and patients was available, and in the triple dose group, a series from 9 EA+ and 11 EA− patients was available. The results are shown in table 3. In both single and triple Gd series we observed more MRI activity in the EA+ group. These patients had on average more Gd enhanced lesions, more new lesions, and a more active MRI series. This difference showed a trend in the single Gd MRI series, and reached statistically significant levels in the triple Gd MRI series.

## DISCUSSION

This study confirms earlier reports that 100% of the MS patients have serological signs of a previous EBV infection,[1 20] and that a disproportionally high percentage of MS patients have elevated titres of anti-EA antibodies.[18 19] The presence of anti-EA antibodies indicates acute or chronic active EBV infection and onset of viral replication.[16 17] There are indications that the primary EBV infection in MS patients has occurred years before the onset of neurological symptoms,[9–11 20] with a possible risk enhanced role for primary infections occurring relatively later in life (adolescence).[10 21] A remarkable finding of this study was the fact that during follow up, EA seropositivity remained stable over time. Normally, EA titres decline within weeks or months after a primary infection or a reactivation of EBV.[16 17] Therefore, we investigated whether these patients had active viral replication, resulting in continuous viraemia. However, with a highly sensitive PCR, we could only detect viraemia at three timepoints, and this was found to be transient. How can the findings of elevated immune responses against an early lytic EBV protein, but a lack of viraemia be reconciled? Chronic T cell responses against EBV proteins of the early replication phase, such as EA and polymerases, can prevent viral replication and shedding.[22 24] In this respect it is of extra interest that the EA associated DNA polymerase bears a mimicry motif for myelin basic protein reactive T cell clones.[25–28]

We observed no relation between increased EA titres or viraemia on the one hand and exacerbations or clinical phenotype on the other. Recently, Wandinger *et al* reported some indications for relatively more active EBV replication in a subgroup of 11 MS patients with clinical exacerbations than in eight clinically stable patients.[11] What could account for the discrepancy between the two studies? There were technical differences, such as the use of a different PCR and the lack of IgA serology in our study. In the Wandinger study, significance was only reached after making a composite score of several serological tests together with PCR. Furthermore, we specifically selected samples taken during exacerbation for PCR analysis, whereas Wandinger considered time windows up to 12 months around exacerbations.

A particular aspect of our study was the possibility of analysing the association between EA seropositivity and MRI activity. We observed higher numbers of gadolinium enhanced lesions in the EA+ than in the EA− group, both on single and triple dose scans. In the single dose Gd group, with relatively lower sensitivity to detect lesions, a trend was observed. In triple dose Gd series, 89% of EA+ patients had at least one active MRI series and a significantly higher mean number of gadolinium enhanced lesions. These findings could perhaps be seen as a reflection of a higher rate of lesional activity in the EA+ group.

As there are many recordings of elevated antibody production against a variety of antigens in MS,[29] it could be argued that the observed enhanced anti-EA production is merely part of a non-specific polyclonal B cell response. In this light, elevated production of EA antibodies would simply be related to a higher active state of the immune system, thus associated with more inflammation in the brain. However,

antibody production against other control viruses was not heightened in the EA seropositive group. Although we did not observe a higher clinical exacerbation rate in EA seropositive MS patients, it cannot be ruled out that EBV reactivation is related to lesion activity as detected by MRI. This could be of interest, because MRI is more sensitive in recording the occurrence of new lesions. However, this study was not powered to investigate a relationship between anti-EA titre status and MRI activity. This preliminary observation should thus be reproduced in a larger dataset before drawing firm conclusions.

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The Rotterdam Study on Exacerbations in MS (ROSE) has been approved by the medical ethics committee of Erasmus MC and all patients gave their informed consent before entering the study.

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