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Virology

Low intrathecal immune response of anti–EBNA-1 antibodies and EBV DNA from multiple sclerosis patients

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Abstract

Numerous studies have been carried out to determine whether an Epstein–Barr virus (EBV) infection can be considered a risk factor for multiple sclerosis (MS), following the evidence of an increase in IgG response to nuclear antigen-1 (EBNA-1) in both serum and cerebrospinal fluid (CSF) from MS patients. However, the possible interaction between EBV and MS has still not been well characterized, and the possible pathogenic role is yet to be determined. A case-control study (76 cases and 75 controls) was conducted to investigate anti-EBV antibodies synthesis in serum and CSF through intrathecal specific IgG synthesis to EBNA-1, as well as the presence of EBV DNA in plasma, peripheral blood mononuclear cells, and CSF from MS patients. Intrathecal EBNA-1 specific IgG synthesis was detected in 6.6% MS patients and in 17.3% controls. No EBV DNA was found in plasma or CSF, and our findings showed no evidence of high intrathecal EBNA-1 specific IgG synthesis or of significant EBV DNA in CSF in MS patients.

Keywords: Epstein–Barr virus; Multiple sclerosis; Intrathecal synthesis; DNA; Cerebrospinal fluid

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) that leads to demyelination and neurodegeneration (Noseworthy et al., 2000). It is thought that Epstein–Barr virus (EBV) may contribute to the pathogenesis of MS, but the precise mechanisms are unknown.

There are numerous reports of cases in which MS patients have a higher seroprevalence and a higher concentration of anti-EBV antibodies as compared to controls (Ascherio et al., 2001; Buljevac et al., 2005; Cepok et al., 2005; Myhr et al., 1998). This difference in seroprevalence is more obvious in studies carried out in MS pediatric cases (Alotaibi et al., 2004; Pohl et al., 2006).

Differently to seroprevalence studies, the study of intrathecal synthesis is a sensitive and quantitative method to determinate local synthesis of specific antibodies at CNS level. IgG intrathecal response is the most frequent pathologic sign in CNS in patients with MS. Specific immune response can be determined by the cerebrospinal fluid (CSF)-to-serum antibody index (AI), which is highly sensitive (Jacobi et al., 2007; Reiber and Lange, 1991; Reiber and Peter, 2001). The quantification of intrathecal antibody response supports discrimination between antibody synthesis against persistent infection causing antigen and concomitant polyspecific synthesis of antibodies (Robinson-Agramonte et al., 2007).

The aim of this study was to determine intrathecal EBNA-1 specific IgG synthesis in the CNS of patients with MS and of control patients, and to research the presence of EBV DNA.

2. Materials and methods

2.1. Clinical samples and controls

A total of 151 paired serum and CSF samples were extracted from patients recruited at the University Hospital

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San Cecilio, Granada, between January 2002 and April 2005. All subjects signed informed consent forms.

2.1.1. Group I

Paired serum and CSF samples were collected from 76 patients (62 females, 14 males) with MS, 55 with relapsing–remitting MS (RRMS) and 21 with secondary progressive MS (SPMS). Mean patient age was 34.9 ± 9.7 years (34.3 ± 9.8 for women and 37.5 ± 9.6 for men), and the age range was 18–59 years. The mean age of patients with RRMS was 32 ± 8.0 while that of patients with SPMS was 42.6 ± 9.9. Mean disease progress was 8 ± 8.1 years, with a range between 15 days and 29.5 years (5.2 ± 5.9 for patients with RRMS and 15.3 ± 8.6 for patients with SPMS). The neurologic dysfunction of these patients was assessed using the Poser criteria (Hobart et al., 2000).

2.1.2. Group II

Paired serum and CSF samples were collected from 75 patients (49 females, 26 males) scheduled for minor surgery through lumbar puncture, without any history of neurologic or autoimmune diseases, following the established diagnostic criteria. Mean control age was 45.6 ± 12.9 years (43.9 ± 12.6 for women and 48.6 ± 13.1 for men), and the age range was 20–73. This group was used as control.

Two milliliters of blood were taken from each subject under sterile conditions; blood samples were then centrifuged at 1100 × *g*, and sera were stored at −80 °C until the analysis. Two milliliters of CSF were collected from each subject through lumbar puncture and immediately stored at −80 °C.

2.2. Determination of intrathecal IgG synthesis

A commercial ELISA kit from Vircell (Granada, Spain) was used to detect specific IgG antibodies to EBNA-1. Serum and CSF samples were examined using the technique and following the manufacturer's instructions. The reproducibility of the results was previously tested by repeating the procedure in the first 25% of samples, accepting variations of <5% in absorbance results.

To determine intrathecal EBNA-1 specific IgG synthesis, serum and CSF were diluted to the same IgG concentration. CSF and serum were tested for albumin and IgG by means of standard immunochemical nephelometry assay on a Dade Behring Nephelometer (Siemens, Marburg, Germany), while EBV-specific antibodies in CSF and serum samples were assessed using the ELISA technique. IgG intrathecal production was detected quantitatively by analyzing the CSF-to-serum IgG versus albumin quotient diagram (Reiber and Peter, 2001).

Intrathecally synthesized EBV-specific CSF antibodies were determined through the AI, calculated as AI = $Q_{spec.}/Q_{total}$ (Reiber and Lange, 1991). Intrathecal synthesis of specific antibodies is present if the CSF/serum quotient of specific IgG antibodies ($Q_{spec.}$) is significantly higher than the CSF/serum quotient of the total IgG (Q_{total}) or with Reiber correction; in the case where $Q_{total} > Q_{lim}$ with AI = Q_{spec}/Q_{lim}. Q_{lim} was calculated according to Reiber and Lange (1991). The normal reference range for AI is between 0.7 and 1.3 (1.0 ± 2 SD). A corrected AI ≥ 1.5 indicated local specific antibody synthesis in the CNS and was taken as evidence for intrathecal IgG production to EBNA-1.

2.3. Detection of EBV DNA through nested PCR

To obtain further evidence of EBV infection, we also assessed the presence of EBV DNA both in the case group and in the control group, using nested PCR (n-PCR) in peripheral blood mononuclear cells (PBMC), plasma, and CSF. PBMC and plasma were separated by means of Ficoll–Hypaque gradient centrifugation (20 min, 1000 × *g*) of additional blood samples collected from 151 subjects.

DNA was extracted from plasma, PBMC, and CSF by means of High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannhein, Germany) following the manufacturer's instructions.

PCR was carried out to detect EBV sequences, using primers of the virus capsid antigen p23 region of the *blrf2* EBV gene (Meerbach et al., 2001). To detect p23 EBV DNA, p23-1 primer 5′-TCAGCTCCACGCAAAGTC-3′ and p23-2 primer 5′-CACTTTCTTTGCTTC-3′ were used to amplify a 471-bp fragment. To detect EBV-specific PCR products, a set of internal primers (p23-3 and p23-4) were used to amplify a 363-bp segment, with the following sequences: p23-3: 5′-TTGACATGAGCATGGAAGAC-3′; p23-4: 5′-CTCGTGGTCGTGTTCCCTCAC-3′.

In brief, aliquots (1 μL, 1–2 μg) of total DNA isolated from PBMC, plasma, and CSF were used for n-PCR in a total volume of 25 μL. The reaction mixtures contained 1× reaction buffer (pH 8.5), 160 μmol/L each of dNTP, 1.5 mmol/L $MgCl_2$, 0.8 μmol/L of each oligonucleotide primer, and 1 U of GoTaq® DNA Polymerase (Promega, San Luis Obispo, CA). The cycle, which consisted of 95 °C for 60 s of denaturation, 60 °C for 60 s of annealing, and 72 °C for 60 s of elongation, was repeated 30 times; in addition, 5 min at 95 °C was precycle and 2 min at 72 °C was postcycle. For the n-PCR, 1 μL of the PCR product from the first amplifications was added to 24 μL of the PCR reaction mixture and amplified 35 cycles with the inner primers.

DNA extracted from the recombinant plasmid containing the p23 region of EBV described below was used as positive control; negative controls were included at all steps of sample preparation and PCR. Ten microliters of each amplified product were loaded onto a 1.5% agarose gel. The presence of a band with the predicted length of 363 bp for the p23 primers indicated a PCR-positive sample for EBV DNA. Researchers were blinded to the origin of the sample (from patient or control) and to previous test results.

2.4. Sensitivity of n-PCR

Serial dilution of a quantified DNA was used to determine the sensitivity of the developed n-PCR. For the quantification of the DNA, a standard curve was determined by real-time PCR, while quantified EBV DNA was used to check n-PCR sensitivity.

Table 1
Frequency and intensities of EBNA-1 IgG in serum and CSF, and intrathecal EBNA-1 IgG synthesis in MS patients (n = 76) and controls (n = 75)

	MS patients		Controls	
	Positive	Median (range)	Positive	Median (range)
EBNA-1 IgG serum	66/76 (86.8%)	0.65 (1.88–0.03)	62/75 (82.6%)	0.46 (2.13–0.03)
EBNA-1 IgG CSF	62/76 (81.6%)	0.50 (1.67–0.03)	52/75 (69.3%)	0.38 (1.32–0.02)
Intrathecal EBNA-1 IgG synthesis AI ≥ 1.5[a]	5/76 (6.6%)	2.3 (1.57–4.64)	13/75 (17.3%)	2.8 (1.5–12.24)

Intrathecal EBNA-1 IgG displays frequencies for increased antibody index ≥ 1.5.
[a] Wilcoxon test: $P > 0.123$; Welch test: $P > 0.155$.

The same 471-bp fragment of the p23 gene was amplified using the outer primers described above. The PCR program used for amplification was: 95 °C for 60 s, 42 cycles at 95 °C for 20 s, 58 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 3 min. The specific fragment was extracted from the gel using the QIAEX II Gel Extraction Kit (Qiagen, Valencia, CA). The amplified fragment was bound into a plasmid pGEM-T easy vector system (Promega) and subsequently cloned into *Escherichia coli* (Strain JM-109). Transformed cells were spread in an LB agar plate with ampicillin (100 μg/mL), X-gal (0.08 mg/μL) (Fermentas, Canada), and IPTG (0.5 mmol/L) (Fermentas, Ontario, Canada). White colonies were selected as carriers of the specific EBV fragment.

Transformed *E. coli* were grown and pelleted; plasmids were extracted and purified with the QIAGEN Plasmid Mini Kit (Qiagen).

The EBV was cultured in P3HR1-infected cells. Then, the culture was centrifuged at 2900 × *g* for 15 min, and 0.1 g of EBV culture was treated with 500 μL of lysis solution (guanidine isothiocyanate 4 mol/L, *N*-lauril sarcosine 0.5%, β-mercapto-ethanol 100 mmol/L) and 50 μL sodium acetate 2 mol/L pH 4.

Purified plasmid solution was used to build the standard curve. Plasmid dilutions from 1.04×10^7 to 1.33×10^2 copies/μL were used. The 5-fold dilutions of extracted EBV genomic DNA were analyzed to adjust to a 12 800 copies/μL EBV genomic DNA dilution. The same PCR conditions as indicated above were used for real-time PCR quantification, using Kapa SYBR FAST qPCR Kit (KapaBiosystems, Woburn, MA) and p23 specific oligo pairs (0.25 μmol/L).

Serial dilutions (1/10-fold) of the quantified EBV genomic DNA were used to check the sensitivity of n-PCR.

2.5. Identification of PCR products

After electrophoresis, the identity of positively amplified fragments was confirmed by means of enzymatic cleavage. This assay was done from PCR product using the restriction enzyme *Hin*cII (Fermentas).

2.6. Statistics

Fisher exact χ^2 test was used to associate percentages of positivity with qualitative variables. Wilcoxon test and Welch t test were employed to compare the differences between the values of intrathecal antibodies synthesis and of antibodies in serum and CSF.

3. Results

3.1. Synthesis of intrathecal anti–EBNA-1 antibodies

Intrathecal anti–EBNA-1 IgG synthesis in the CSF from MS patients and controls did not differ significantly ($P > 0.123$, Wilcoxon test; $P > 0.155$, Welch test). Table 1 shows the frequency of EBV-specific IgG with increased AI (≥ 1.5). Of MS patients, 6.6% (5/76) showed specific IgG

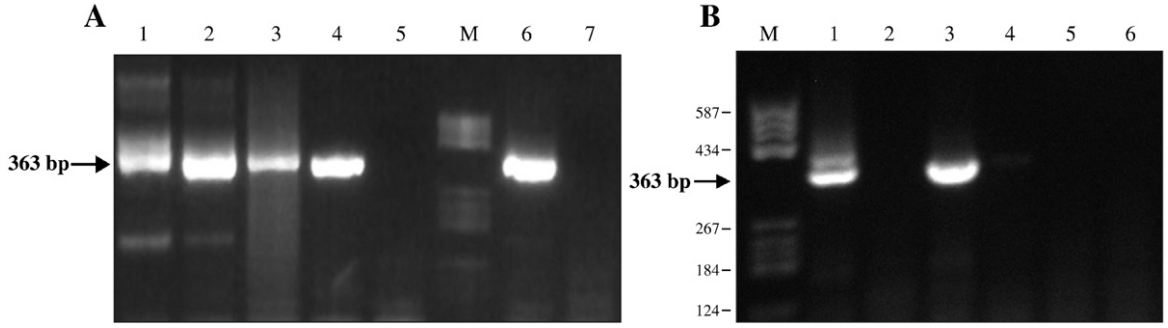

Fig. 1. Sensitivity of n-PCR for EBV p23 DNA. (A) 1–5: serial dilutions of the quantified EBV genomic DNA (1: 1.28×10^4; 2: 1.28×10^3; 3: 1.28×10^2; 4: 1.28×10^1; 5: 1.28×10^0 copies); 6: positive control; 7: negative control. (B) 1: positive control; 2: negative control; 3–6: adjusted dilutions of the quantified EBV genomic DNA (12, 6, 3, and 1.2 copies, respectively). DNA from EBV was amplified by n-PCR and analyzed by 1.4% agarose gel electrophoresis and ethidium bromide staining. M: DNA molecular weight marker V (Roche Diagnostics). Deionized distilled water was used as a negative control. The amplified nested EBV PCR product is indicated (363 bp).

Table 2
Results obtained from detection of EBV DNA in PBMC, plasma, and CSF among MS patients and controls

	MS patients			Controls		
	PBMC[a]	Plasma[b]	CSF[c]	PBMC[a]	Plasma[b]	CSF[c]
EBV-positive	19	0	1	14	0	0
EBV-negative	57	76	75	61	75	75
% positive	25	0	1.3	18.7	0	0

[a] No significant difference between MS patients and controls, $P = 0.432$.
[b] No significant difference between MS patients and controls, $P = 1.000$.
[c] No significant difference between MS patients and controls, $P = 1.000$.

intrathecal production against EBNA-I versus 17.3% (13/75) of the control patients.

EBNA-1–specific AIs that showed intrathecal immune response against EBV ranged from 1.57 to 4.64 with a mean of 2.3 in the group of MS patients, while it ranged from 1.5 to 12.24, with a mean of 2.8, in the control group (Table 1).

CSF and serum levels of anti–EBNA-1 IgG from controls and from patients with MS were also subject to statistical analysis. Anti–EBNA-1 IgG responses were higher both in the serum and the CSF of MS patients in comparison to those of control patients, but these differences were not statistically significant.

3.2. Sensitivity of n-PCR

Serial dilutions (1/10-fold) of the quantified EBV-genomic DNA were used to assess the sensitivity of n-PCR for EBV p23. EBV p23 DNA was consistently detected in 6 copies of the generated EBV p23 DNA fragment, the lowest n-PCR detection limit using the primers described (Fig. 1). n-PCR specifically yielded a predicted 363-bp DNA fragment. In each PCR experiment, a positive control of plasmid DNA of EBV p23 was included, and it was always found positive.

3.3. Use of n-PCR to detect EBV DNA in CSF, plasma, and PBMC

EBV DNA was detected in PBMC for 19/76 MS patients and for 14/75 (Table 2) control patients. The percentages of positive results were 25% and 18.7%, respectively. The percentage of EBV DNA in MS patients was higher than in control patients, but not statistically significant ($P > 0.432$, Fisher χ^2 test). All plasma samples, both of MS and control patients, were EBV DNA negative. No EBV DNA was detected in any CSF sample studies in the control group. In MS patients, EBV DNA was detected in one CSF sample (1.3%) that also showed EBV DNA in PBMC. Fig. 2 illustrates representative results obtained with samples from MS patients.

When the primers generated amplicons from the correct molecular weight, the positive PCR products were confirmed by enzymatic cleavage with *Hinc*II and all of them were digested.

4. Discussion

Given the conflicting reports over how EBV may contribute to MS, we studied the involvement of EBV infection in MS using 2 methods: the sensitive method of intrathecal anti-EBV IgG synthesis determination and a highly sensitive n-PCR.

Intrathecal IgG production and oligoclonal IgG bands formation in the CSF were found in more than 90% of MS patients (Salvetti et al., 2009). MS is characterized by a polyclonal intrathecal immune response with nonspecific synthesis of antibodies, but recognizing several neurotropic viruses. The detection of intrathecal synthesis of specific antibodies, known as the AI, is an established method to prove brain viral infection. Oligoclonal IgG in the CSF is typically found in CNS infections but disappears with time; however, it is a persistent phenomenon in the case of MS

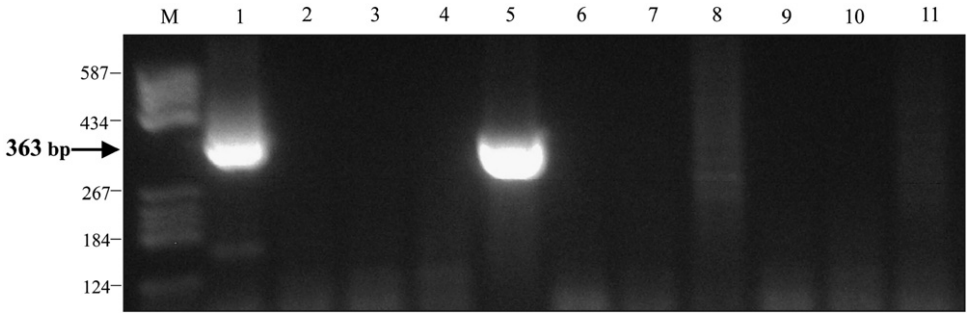

Fig. 2. Representative results obtained by n-PCR with samples from MS patients. M: DNA molecular weight marker V (Roche Diagnostics); lane 1: positive control; lane 2: negative control; lanes 3, 6, and 9: CSF samples; lanes 4, 7, and 10: plasma samples; lanes 5, 8, and 11: PBMC samples. n-PCR analyzed by 1.4% agarose gel electrophoresis and ethidium bromide staining. One hundred femtograms of plasmid DNA of EBV p23 was used as a positive control, and deionized distilled water was used as a negative control. The amplified nested EBV PCR product is indicated (363 bp).

(Salvetti et al., 2009). Anti–EBNA-1 antibody response is a latent marker; it correlates well with what has been isolated from the CSF of MS patients and it is associated with evidence of MS disease activity (Castellazzi et al., 2010).

Our study revealed that only 6.6% of MS patients showed evidence of intrathecal synthesis of IgG against EBNA-1 compared to 17% of control patients. Our findings are in line with those obtained by Pohl et al. (2010) who observed 8% of intrathecal synthesis against EBNA-1 in MS adult patients. Another study found no significant difference in the frequency of intrathecal anti-EBV IgG synthesis (anti–EBNA-1 IgG and anti-VCA IgG) in adult patients with MS as was reported by Castellazzi et al. (2010). They observed intrathecal synthesis of anti–EBNA-1 and anti-VCA IgG in a range of 6.3–2.5%, respectively, in MS patients and in a range of 1.3–3.8% in control patients. Other recent studies that have found no evidence of intrathecal IgG synthesis using other techniques like ELISA or immunoblot analysis, respectively, are those reported by Sargsyan et al. (2010) and Jafari et al. (2010). As in these studies, and contrary to the general belief, our results indicate that the frequency of detection of intrathecal IgG synthesis in MS patients was lower than in control patients.

However, several studies have described an increased IgG antibody response against EBV in the CSF of MS patients in comparison with control groups (Cepok et al., 2005; Bray et al., 1992; Jaquiéry et al., 2010; Sumaya et al., 1985). Bray et al. (1992) determined that 85% of MS patients had CSF that reacted to EBNA-1, whereas only 11% of control patients reacted. Jaquiéry et al. (2010) have reported higher VCA and EBNA-1 IgG antibody indexes in early MS as compared to patients with other inflammatory neurologic diseases and non-inflammatory neurologic diseases. Cepok et al. (2005) and Sumaya et al. (1985) should be cited with regard to studies that assessed the specificity of oligoclonal IgG antibodies against EBV in the CSF. They suggested an important role in the pathogenesis of disease, but none of these studies determined and stated whether the antibodies in the CSF were intrathecal or through blood flow.

The mean AI for EBV was 2.3 and 2.8 in MS and control patients, respectively, but a higher AI value (12.24) was observed in control patients. A recent study (Pohl et al., 2010) obtained a mean AI for EBNA-1 of 2 in both adults and children. In this study, the mean AI for EBV was lower than for other neurotropic viruses, data which seem to indicate that those values were generally independent from the clinical diagnosis and that the severity of tissue inflammation does not correlate with the value of AI.

Although the AI and PCR methods may identify similar infectious agents in similar clinical conditions, both methods are not interchangeable (Denne et al., 2007). Only one patient with intrathecal IgG synthesis against EBV also turned out to be EBV DNA positive in their PBMC.

Regarding the detection of EBV DNA, all the CSF and plasma samples were negative in both groups, except one CSF sample from an MS patient, which was positive for EBV DNA. These findings suggest that the presence of EBV DNA is not a common event in sera and/or CSF from MS patients and do not support a direct role for systemic EBV or for EBV in the intrathecal compartment in the pathogenesis of MS. Similar findings have been reported before by several different groups (Alvarez-Lafuente et al., 2008; Franciotta et al., 2009; Mancuso et al., 2007; Pohl et al., 2010; Santiago et al., 2010).

We obtained the sensitivity limit for n-PCR with the p23 set of primers of 6 EBV genome copies, showing that our methodology was capable of detecting very few EBV genome copies.

The qualitative detection of the virus does not discriminate between colonization (chronic infection) and reactivation. This poor behavior improves, however, with samples of spinal fluid, serum, and plasma. Studies of serum and plasma would only detect viral genome in currently ill patients or in those with a high systemic EBV load, but because only one sample was positive, quantification was not necessary.

Yamamoto et al. (1995) reported that the presence of cell-free EBV DNA in plasma is a common phenomenon in patients with EBV-associated diseases. This may be in patients with the more severe clinical categories of EBV diseases, but our findings indicated that it is not a common phenomenon in MS.

As regard the presence of EBV DNA in PBMC, we obtained a greater frequency of EBV DNA detection in MS patients (25%) than in control patients (18.7%), but this difference was not statistically significant. We must consider that EBV DNA detection in PBMC has no clinical relevance because the virus can be latent in PBMC.

Our EBV DNA results are consistent with previous studies. Lindsey et al. (2009) and Lünemann et al. (2006) found that EBV DNA was frequently detectable in PBLs from both MS and control patients and that the levels in MS were not significantly higher than in control patients. Several studies (Alvarez-Lafuente et al., 2006; Sotelo et al., 2007) have reported the presence of a similar percentage of EBV DNA in PBMC from MS patients during a relapse and from control patients.

However, an association has been suggested between clinical disease activity and EBV reactivation in MS. Wandinger et al. (2000) detected positive EBV DNA in sera in 72.7% of patients with MS exacerbations but in none of the patients with clinically stable disease, whereas others were only able to amplify EBV DNA in 3 of 51 serum samples in patients with an active phase of MS (Buljevac et al., 2005) or to inform that the proportion of positive samples was similar for cases with blood collected before (28%) or after (31%) the onset of neurologic symptoms (Wagner et al., 2004).

5. Conclusions

Elevated anti–EBNA-I serum concentrations are a known phenomenon in MS, and numerous studies have reported increased humoral immune response against EBV in the

CNS of MS patients. Little is however known about EBV-specific antibodies synthesis in the CNS of people with MS. Our findings show a lack of intrathecal anti-EBV antibody synthesis in over 93% of MS patients, and this is a strong argument against the direct role of this pathogen in the humoral immune response of the CNS in MS, while the absence of a high viral load of EBV in MS patients indicates lack of evidence of an active EBV infection. These findings agree with most published studies examining EBV in MS.

Detection of intrathecal synthesis of antibodies against infectious agents has proven to be a stable and sensitive diagnostic method in later stages of the disease, although the existence of an association between EBV reactivation and MS activity cannot be ruled out.

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