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81	Abstract	Epstein-Barr virus (EBV) has been implicated in multiple sclerosis (MS) pathogenesis. We aimed to assess the frequency of EBV-specific IgG and IgM oligoclonal bands (OCB) in cerebrospinal fluid (CSF) of 50 patients with clinically isolated syndrome (CIS) and in 27 controls with Guillain-Barré syndrome (GBS). Furthermore, we assessed correlations between the presence of OCB and CIS patients' CSF, MRI, and clinical variables. There was no difference in the proportion of CIS and GB patients with positivity for anti-EBV-specific IgG/IgM OCB. There were no correlations between OCB and analyzed variables, nor were they predictive of a higher disability at 3 years.

82	Keywords separated by ' - '	Epstein-Barr virus - Clinically isolated syndrome - Multiple sclerosis - Oligoclonal IgG bands - Oligoclonal IgM bands

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SHORT COMMUNICATION

Cerebrospinal fluid anti-Epstein-Barr virus specific oligoclonal IgM and IgG bands in patients with clinically isolated and Guillain-Barré syndrome

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Abstract Epstein-Barr virus (EBV) has been implicated in multiple sclerosis (MS) pathogenesis. We aimed to assess the frequency of EBV-specific IgG and IgM oligoclonal bands (OCB) in cerebrospinal fluid (CSF) of 50 patients with clinically isolated syndrome (CIS) and in 27 controls with Guillain-Barré syndrome (GBS). Furthermore, we assessed correlations between the presence of OCB and CIS patients' CSF, MRI, and clinical variables. There was no difference in the proportion of CIS and GB patients with positivity for anti-EBV-specific IgG/IgM OCB. There were no correlations between OCB and analyzed variables, nor were they predictive of a higher disability at 3 years.

Keywords Epstein-Barr virus · Clinically isolated syndrome · Multiple sclerosis · Oligoclonal IgG bands · Oligoclonal IgM bands

Introduction

Epidemiological data, including a seroprevalence >99% in multiple sclerosis (MS) patients (compared to 94% in controls) (Ascherio and Munger 2007) and a higher risk of MS in subjects with a history of infectious mononucleosis (Handel et al. 2010), indicates that EBV may play a role in MS pathogenesis. The high infection prevalence in MS patients has suggested that it may be a prerequisite for the development of MS, although pathogenetic mechanisms are still unclear and hypotheses include promotion of autoimmunity by EBV-infected autoreactive B cells, bystander damage related to anti-EBV responses, and cross-reactivity between EBV and central nervous system (CNS) antigens (Holmøy, Kvale, and Vartdal 2004; Lang et al. 2002; Lünemann et al. 2008).

A debated issue is whether EBV represents an antigenic target for antibody production within the CNS.

Castellazzi et al. (Castellazzi et al. 2014) recently reported, among other findings, that local synthesis of cerebrospinal fluid (CSF) specific IgG oligoclonal bands (OCB) directed against Epstein-Barr virus (EBV) occurred in 21% of 100 patients with relapsing-remitting multiple sclerosis (RRMS), and that they had a low affinity in all patients. Authors concluded that the EBV-specific intrathecal oligoclonal IgG production is probably unrelated to the cause of the disease, but may occur in a subset of MS patients as part of a humoral polyreactivity directed against many different pathogens.

We hereby report on similar findings in a group of 50 patients with clinically isolated syndrome (CIS). We furthermore report results on the frequency of CSF-restricted EBV-specific IgM OCB in the CIS cohort, on the correlations between the presence of anti-EBV-specific IgG and IgM bands and CSF, MRI and clinical variables (including disability and the risk of conversion to clinically definite MS within the subsequent 3 years), and on the frequency of EBV-specific IgG and IgM OCB in a control group of 27 patients with Guillain-Barré syndrome (GBS).

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Methods and materials

Patients

Of 201 consecutive patients with a first demyelinating event (CIS) seen at our center, whose CSF and serum had been stored at −80 °C after collection, we selected those patients (nr = 50) who proved positive for both CSF-restricted IgG OCB and CSF IgM OCB (the latter were CSF-restricted in 40 patients and present as a "mirror pattern", i.e., identical bands in CSF and serum, in 10 patients). Furthermore, of 38 patients diagnosed with GBS, we selected 27, who showed a CSF IgG mirror pattern, as controls.

Data collection

Of all CIS patients, we recorded demographic (sex, age at onset), clinical (symptoms at onset, EDSS at onset, and clinical recovery), MRI (presence and number of brain and spinal cord MRI lesions, presence and number of gadolinium-enhancing lesions on MRI, and presence of spinal/infratentorial lesions), and routine CSF data collected at onset (CSF proteins, cell count, Link's IgG Index, presence of IgG OCB and CSF/serum albumin quotient, indicating a blood-brain barrier dysfunction if value >6.5 × 10^3), as well as follow-up data including the occurrence of relapses and disability status (EDSS) (Kurtzke 1983) in the subsequent 3 years.

Laboratory procedures

Cerebrospinal fluid and serum sampling

Laboratory procedures were carried out on CSF, and serum samples from each patient, which, at the time of the diagnostic spinal tap, had been centrifuged at 3000 rpm for 10 min and stored in cryovial tubes at −80 °C within 2 h from collection.

CSF and serum samples were analyzed for the presence of IgM OCB by means of agarose gel isoelectric focusing (IEF) followed by immunoblotting with polyclonal specific anti-human IgM antibodies (Dako), according to the method proposed by Villar et al. (L. M. Villar et al. 2001), with some modifications (Ferraro et al. 2015). We obtained the approval of the Modena Ethics Committee (protocol nr. 116/09).

EBV antigen-specific immunoblotting

EBV-specific OCB were investigated by antigen-specific immunoblotting as reported by Castellazzi et al. (Castellazzi et al. 2014), using the same mixture of antigens (Genway Biotech, Inc. San Diego, CA, USA, a P3H3 extract crude viral lysate containing a high concentration of EBV antigens, including VCA, EBNA, early antigen diffuse, and early antigen restricted). However, we did not use a commercial kit to carry out IEF. For IEF, we used agarose gel, polyvinylidene fluoride membrane, rabbit anti-human IgG (primary antibody), polyclonal swine anti-rabbit Ig/AP (secondary antibody conjugated to alcaline phosphatase) (Dako Cytomation), and nitro blue tetrazolium and bromo-cloroindoleyl phosphate as dyes.

The presence of CSF IgM OCB and of EBV-specific IgG and IgM OCB (at least two) was blindly assessed by two independent neurologists (DF and PS) and by a biologist (RB) in case of discrepancies.

Statistical methods

We calculated absolute frequencies and percentages for categorical variables and mean ± standard deviation and median for continuous variables. Mann-Whitney's test and Fisher's exact test were used to explore differences between groups. We used logistic regression to assess the relationship between anti-EBV-specific OCB status and the risk of disability or of a relapse (with subsequent diagnosis of clinically definite MS-CDMS) at 1 and 3 years in CIS patients.

Results

CIS and GBS patient characteristics

CIS patients' demographic, clinical, MRI, and CSF characteristics are shown in Table 1.

CIS patients with positivity for IgM OCB, and thus selected for this study, do not differ from IgM OCB-negative patients of the initial CIS cohort (nr = 201) with respect to baseline variables, except for a higher number of gadolinium-enhancing lesions on baseline MRI in IgM OCB-positive patients (1.1 vs 0.5, $p = 0.01$).

GBS patients' demographic and CSF characteristics are shown in Table 2.

Frequency of EBV-specific IgG and IgM in CIS and GBS patients

In CIS, CSF-restricted EBV-specific IgG OCB (see Fig. 1) were present in 14 (28%) of patients and a EBV-specific IgG OCB in a mirror pattern in three (11%), while CSF-restricted EBV-specific IgM OCB (Fig. 2) were present in three (6%).

In GBS, EBV-specific IgG OCB (mirror pattern) were present in six (22 %) of patients, while EBV-specific IgM OCB were present in three (16 %).

There were no statistically significant differences in the proportion of CIS and GB patients with positivity for EBV-specific IgG OCB or IgM OCB ($p = 0.78$ and $p = 0.34$, respectively).

Q2 **Table 1** Baseline demographic, clinical, MRI, and CSF characteristics of CIS patients (nr = 50)

Sex M/F	14/36
Age at onset, years*	33 ± 9
Symptoms at onset	
ON, *n* (%)	16 (32)
Sensory, *n* (%)	12 (24)
Motor/sensory motor, *n* (%)	6 (12)
Brainstem/cerebellum, *n* (%)	13 (26)
Other, *n* (%)	3 (6)
Spinal onset, yes/no	18/32
EDSS at onset*	2.3 ± 1
Complete recovery, *n* (%)	41 (82)
Baseline brain MRI	
0 lesions, *n* (%)	4 (8)
1–2 lesions, *n* (%)	7 (14)
>2lesions, *n* (%)	39 (78)
Presence of infratentorial lesions, *n* (%)	25 (50)
Presence of CE, *n* (%)	26 (54)
Baseline spinal cord MRI (available in 26 patients)	
Positive (at least one lesion), *n* (%)	20 (77)
Total CSF protein* (normal value 15–45 mg/dl)	38.5 ± 12
Patients with elevated CSF protein, *n* (%)	13 (28)
Cells* (normal value <4/mm3)	7.2 ± 8
Patients with elevated CSF cells, *n* (%)	30 (63)
CSF/serum albumin* (normal value <6.5)	4.1 ± 1.3
Patients with elevated CSF serum/albumin, *n* (%)	3 (6)
IgG index* (normal value <0.7)	1.2 ± 0.5
Patients with elevated IgG index, *n* (%)	45 (92)

ON optic neuritis, *CE* contrast enhancement

*Values expressed as mean ± SD

Correlations between EBV-specific IgG/IgM OCB and clinical, MRI, and CSF parameters in CIS patients

There were no differences in baseline clinical, MRI, and CSF variables between EBV-specific IgG OCB positive and negative patients, except for a higher mean number of CSF cells in EBV-specific IgG OCB-negative patients; there were no differences in follow-up data (Table 3). EBV-specific IgM OCB positive patients did not differ from EBV-specific IgM OCB-negative patients with regard to baseline and follow-up data (data not shown).

Table 2 Demographic and CSF characteristics of GBS patients (nr = 27)

Sex M/F	18/9
Age at onset, years*	54 ± 17
Total CSF protein* (normal value 15–45 mg/dl)	91 ± 49
Patients with elevated CSF protein, *n* (%)	23 (85)
Cells* (normal value <4/mm3)	32 ± 124
Patients with elevated CSF cells, *n* (%)	7 (26)
CSF/serum albumin* (normal value <6.5)	14.1 ± 9.7
Patients with elevated CSF serum/albumin, *n* (%)	23 (85)

*Values expressed as mean ± SD

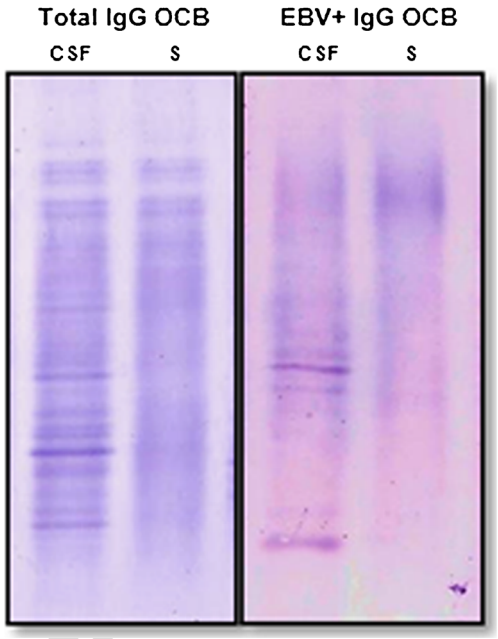

Fig. 1 EBV-specific IgG OCB in a CIS patient. Identical amounts of serum (*S*) and cerebrospinal fluid (*CSF*) IgG were isoelectrically focused and transferred to uncoated membrane (*left*) or membrane coated with EBV viral antigens (*right*). The figure shows a CIS patient with positivity for CSF-restricted IgG OCB (*left*) and for EBV-specific IgG OCB (*right*)

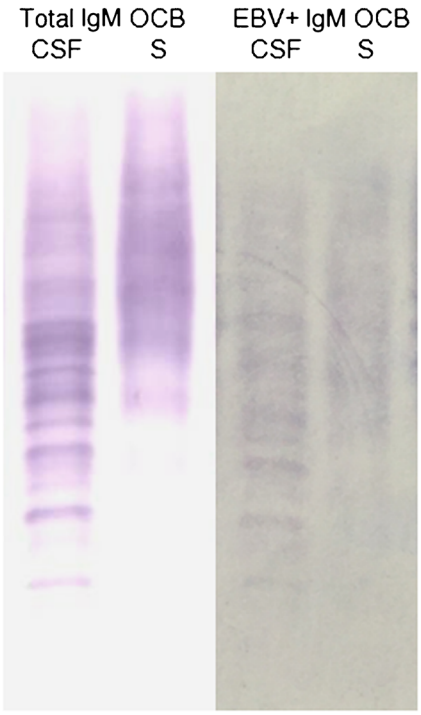

Fig. 2 EBV-specific IgM OCB in a CIS patient. The figure shows the CSF and serum of one CIS patient with positivity for CSF-restricted IgM OCB (*left*) and EBV-specific IgM OCB (*right*)

Table 3 Demographic, clinical, MRI, CSF, and follow-up data of EBV-specific IgG OCB positive and negative CIS patients. Significant results in bold.

Variable	Presence of EBV-specific CSF-restricted OCB (14 patients)	Absence of EBV-specific CSF-restricted OCB (36 patients)	*p* value
Sex M/F	1/13	13/23	0.07
Age at onset, years*	32 ± 8	33 ± 10	0.74
Spinal onset, yes/no	5/9	13/23	1
EDSS at onset*	1.9 ± 0.6	2.5 ± 1.1	**0.03**
Complete recovery, yes/no	13/1	8/28	0.21
Baseline brain MRI			
At least one lesion, yes/no	13/1	33/3	0.7
Presence of infratentorial lesions, yes/no	7/7	18/18	1
Presence of CE, yes/no	6/7	20/15	0.36
Baseline spinal cord MRI			
Positive (at least one lesion), yes/no	6/7	20/15	0.4
Total CSF protein* (normal value 15–45 mg/dl)	35 ± 9	40 ± 13	0.34
Elevated CSF protein yes/no, yes/no	3/10	10/23	0.46
Cells* (normal value <4/mm3)	3.9 ± 2.6	8.4 ± 8.8	**0.04**
Elevated CSF cells yes/no, yes/no	6/7	24/11	0.14
CSF/serum albumin* (normal value <6.5)	3.8 ± 1.1	4.2 ± 1.4	0.21
Elevated CSF serum/albumin, yes/no	1/13	2/32	0.65
IgG index* (normal value <0.7)	1.1 ± 0.5	1.2 ± 0.6	0.97
Elevated IgG index yes/no, yes/no	11/3	34/1	0.07
CDMS diagnosis at 1 year, yes/no	7/7	13/23	0.3
CDMS diagnosis at 3 years, yes/no	11/3	22/14	0.2
EDSS at 3 years*	1.1 ± 1.2	1.5 ± 1.3	0.42
EDSS annual progression*	0.19 ± 0.35	0.25 ± 0.37	0.52

CE contrast enhancement

*Values expressed as mean ± SD

Patients with EBV-specific IgG and IgM OCB did not have a higher risk of conversion to CDMS at 1 or 3 years or of a higher disability at 3 years at logistic regression analysis (data not shown).

Discussion

In the present study, we found no differences in the proportion of CIS and GB patients with positivity for anti-EBV-specific IgG or IgM OCB. EBV-specific CSF OCB were reported in a variable proportion of MS patients (Cepok et al. 2005; Franciotta et al. 2011; Rand et al. 2000; Virtanen et al. 2014), though, in part, they were not CSF-restricted but present in both serum and CSF (Franciotta et al. 2011; Rand et al. 2000; Virtanen et al. 2014).

With regard to EBV-specific intrathecal IgG synthesis, as measured by CSF-to-serum antibody indices, some studies found no evidence for increased frequency of intrathecal antibody production against EBV in MS patients compared to controls (Jafari et al. 2010), or compared to the response to other viruses (Pohl et al. 2010), while another found such evidence only early in the disease course, i.e., within 1 year from onset (Jaquiéry et al. 2010).

The present study, carried out early in the disease course, at the time of the first demyelinating event (median interval between clinical onset and spinal tap, 2 weeks; range, 0–5 months), shows that the intrathecal synthesis of EBV-specific IgG OCB is present only in a proportion of patients with CIS and the frequency of EBV-specific IgG OCB in the CSF of CIS patients does not differ from that of control subjects with GBS. Clearly, CSF anti-EBV OCB in CIS patients differ from those found in GBS patients, since they are CSF-restricted in CIS patients, and present in "a mirror pattern" (with identical bands in serum, indicating passive transfer from serum to CSF) in GBS patients. This is, however, what we would expect in case of an intrathecal antibody production against a target antigen within the CNS in CIS patients, as opposed to a systemic response against a target antigen in the peripheral nervous system in GBS patients.

In order to increase the probability of finding an intrathecal EBV-specific immune response, we also sought to examine whether an intrathecal production of EBV-specific IgM OCB may be present in CIS patients, since a recent study (Beltrán

et al. 2014) showed that there were no clonal overlaps between the IgG and the IgM CSF repertoires, suggesting that IgM- and IgG-producing B cells independently enter the intrathecal compartment, and that they further mature and expand independently of each other in the CSF without intrathecal isotype switching from IgM to IgG. Furthermore, the majority of CSF IgM OCB (in particular those directed against lipids and which are thought to be secreted by CD5+ B cells) are persistent and do not represent a transient primary immune response (L. Villar et al. 2008). IgM OCB are present in approximately 40% of MS patients and 20% of patients with CIS (Boscá et al. 2010; Ferraro et al. 2013). To our knowledge, however, this is the first study assessing the presence of EBV-specific IgM OCB in MS patients. Only a very small proportion of patients, however, showed a positivity for EBV-specific IgM OCB (6%), and, again, the frequency did not differ from that of EBV-specific IgM OCB in control subjects with GBS.

Finally, data on high serum levels of anti-EBNA-1 or anti-EBV-VCA IgG increasing the risk of developing MS in CIS patients (Lünemann et al. 2010) and correlating with MRI activity (Farrell et al. 2009; Lünemann et al. 2010), disability (Lünemann et al. 2010) and brain atrophy (Zivadinov et al. 2009) in MS patients, prompted us to evaluate whether CIS patients with positivity for EBV-specific IgG/IgM OCB showed different clinical/CSF/MRI parameters or if they had a worse prognosis in terms of conversion to MS and disability during a 3-year follow-up, but our data did not support this hypothesis.

Conclusion

There was no difference in the proportion of CIS and GB patients with positivity for CSF anti-EBV-specific IgG or IgM OCB. Furthermore, there were no correlations between EBV-specific IgG/IgM OCB and CIS patients' clinical, MRI, and CSF parameters, nor did we find evidence for a prognostic role of EBV-specific IgG/IgM OCB in CIS patients.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Q3 We obtained the approval of the Modena Ethics Committee (protocol nr. 116/09).

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AUTHOR QUERIES

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