Absence of Epstein-Barr virus in the brain and CSF of patients with multiple sclerosis

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ABSTRACT

Objective: Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus that becomes latent in B-lymphocytes and has been implicated in the pathogenesis of multiple sclerosis (MS). We searched for latent and active EBV infection in MS brain and CSF.

Methods: Nested and non-nested real-time PCR were used to detect cell-specific and EBV-specific transcripts in 15 fresh-frozen and 5 formalin-fixed paraffin-embedded MS plaques and in single MS CSF B-lymphocytes and plasma cells. Intrathecal anti-EBV antibody synthesis was measured by ELISA. Immunocytochemistry was used to detect binding of MS CSF and recombinant antibodies (rAbs) generated from clonally expanded plasma cells in MS CSF to EBV-infected cells.

Results: No EBV RNA was found in MS CSF B-lymphocytes or plasma cells. In active MS plaques, EBV-encoded RNA (EBER)-1 was the only and rarely detected transcript. The frequency of detected intrathecal anti-EBV antibody synthesis in patients with MS did not differ from that in non-MS inflammatory CNS disease control patients. Anti-EBV antibodies were detected in the CSF of patients with MS, but MS rAbs did not react with EBV.

Conclusions: Application of real-time PCR to multiple sclerosis brain and single B-lymphocytes in CSF did not reveal any evidence of active Epstein-Barr virus infection. *Neurology*® 2010;74:1127–1135

GLOSSARY

AI = antibody index; **EBER** = EBV-encoded RNA; **EBV** = Epstein-Barr virus; **IC** = non-MS inflammatory CNS disease; **IgG** = immunoglobulin G; **ISH** = in situ hybridization; **LFB** = Luxol fast blue; **MS** = multiple sclerosis; **OR** = odds ratio; **rAbs** = recombinant antibodies.

Epstein-Barr virus (EBV) is a common herpesvirus that is widespread in all human populations. EBV is spread orally and is the etiologic agent of infectious mononucleosis.[1] Most primary infections are asymptomatic. More than 90% of adults are positive for serum immunoglobulin G (IgG) antibodies to the EBV capsid antigen.[2] EBV becomes latent in peripheral blood B cells. EBV infection has been associated with multiple sclerosis (MS).[3] In a large meta-analysis, EBV-seropositive individuals were found to have an increased risk for MS (odds ratio [OR] = 13.5).[4] In a subsequent prospective study, a fourfold elevation in serum anti-EBV nuclear antigen (EBNA)-2 antibody titer was associated with a fourfold increased risk of developing MS.[5] Further evidence of a link between EBV and MS came from reported enhanced immunoreactivity to EBV-specific proteins BRRF2 and EBNA-1 in serum and CSF of patients with MS, and the demonstration that a small fraction of CSF oligoclonal IgG of 13% of patients with MS was removed by incubation with purified BRRF2 and EBNA-1 proteins.[6] Recently, about 90% of B-lymphocytes in active and chronic-active MS perivascular white matter lesions and about 80% of brain-infiltrating plasma cells were reported to be infected with EBV.[7] Immunohistologic detection of latent

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From the Departments of Neurology (S.A.S., A.J.S., A.M.R., M.P.B., S.A., G.P.O., D.G., J.L.B.), Microbiology (D.G.), and Ophthalmology (J.L.B.), University of Colorado Denver School of Medicine, Aurora; Department of Neurology (B.H.), Technische Universität, Munich; Universitätsmedizin der Georg-August-Universität Göttingen (C.S.), Göttingen; and Department of Pathology (S.G.), Julius-Maximilians-University, Würzburg, Germany.

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and lytic EBV proteins, together with the presence of infiltrating CD8+ cytotoxic T cells, led to the hypothesis that the immune response in active MS lesions is secondary to active EBV infection. Herein, we studied B-lymphocytes and plasma cells in MS brain and CSF using highly sensitive gene expression analyses and immunologic assays for EBV-specific RNA and antigens.

METHODS

Standard protocol approvals, registrations, and patient consents. CSF samples were collected with approval of University of Colorado School of Medicine Institutional Review Board (number 00688), and after obtaining written and informed consent.

Patients and MS tissue donors. CSF from patients with MS and controls with non-MS inflammatory CNS disease (IC) (table e-1 on the *Neurology*® Web site at www.neurology.org) was collected at the University of Colorado School of Medicine. MS plaques (15 fresh-frozen and 5 formalin-fixed and paraffin-embedded) and EBV-infected (5 fresh-frozen and 3 formalin-fixed and paraffin-embedded, used as positive control tissues) and uninfected lymphomas (3 formalin-fixed and paraffin-embedded, used as negative control tissues) and non-MS brain tissue (2 formalin-fixed and paraffin-embedded, used as negative control tissues) were obtained from Rocky Mountain Multiple Sclerosis Center Tissue Bank, Englewood, CO; University of Colorado Hospital, Aurora; National Neurological Research Specimen Bank, Los Angeles, CA; United Kingdom MS Tissue Bank, London, UK; Department of Neuropathology, University Medical Centre Göttingen, Germany; and Department of Pathology, Julius-Maximilians-University, Würzburg, Germany. Table e-2 lists clinical features of human subjects and histology.

Luxol fast blue staining and immunohistochemistry. Frozen sections were stained with Luxol fast blue (LFB) to define plaque boundaries. The composition of inflammatory infiltrate was defined using standard staining procedures. Details are provided in appendix e-1.

cDNA preparation from single cells. Fluorescence-activated cell sorting and cDNA preparation from single MS CSF plasma cells and B-lymphocytes was conducted as described[8] using random hexamer primers. cDNA was stored at −80°C.

cDNA preparation from tissues. cDNA from fresh-frozen and formalin-fixed paraffin-embedded tissues was prepared using standard methods. Details are provided in appendix e-2.

Establishment and analysis of the EBV-infected control B-cell NC08-AJS line. The human lymphoblastoid cell line NC08-AJS was established using the B95–8 strain of EBV as described.[9] Immortalized cells were collected and sorted as described.[8,10,11] Single-cell cDNA was prepared as described above.

PCR. Nonquantitative real-time PCR was performed with and without preamplification. Preamplification was performed on cDNA from single cells and paraffin-embedded tissues due to lower yields of nucleic acid. Preamplification PCR was conducted in 50-μL volume per sample containing 50 nM of forward and reverse primers (sequences listed in table e-3) for each transcript, 15 mM Tris-HCl (pH = 8.3), 50 mM KCl, 2 mM $MgCl_2$, 100 μM dNTPs (Roche), and AmpliTaq Gold DNA polymerase (Applied Biosystems). Cycling conditions were an initial 9 minutes denaturation at 95°C followed by 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute, and a final incubation step at 72°C for 7 minutes.

Real-time TaqMan® PCR was conducted on 7500 Fast Real-Time PCR System (Applied Biosystems) in a 20-μL reaction volume containing 1× TaqMan gene expression master mix (Applied Biosystems), 900 nM forward and reverse primers (table e-3), 250 nM TaqMan probe (table e-3), and 5 μL of 1:10 diluted preamplified DNA from single-cell, formalin-fixed, paraffin-embedded tissues, or fresh-frozen tissue cDNA. Gene expression assays were custom-designed by Applied Biosystems (table e-3). Human glyceraldehyde 3-phosphate dehydrogenase, GAPDH (4326317E), and human ribosomal protein large P0, RPLP0 (4333761F), were purchased from Applied Biosystems. Detection of cellular transcripts GAPDH, RPLP0, and IgG served as positive controls for the presence of template cDNA. Nuclease-free H_2O was used as a no-template control, and samples crossing the threshold at cycle numbers lower than that for no-template control (<38) were considered positive for the target transcript.

ELISA and antibody index calculations. Paired CSF and serum/plasma samples from MS and IC patients (table e-1) were diluted in PBS to a final concentration of 5 μg/mL IgG (an IgG concentration within the linear range of antibody binding) and used in ELISA. Details are provided in appendix e-3. The anti-EBV antibody index (AI) was calculated as described.[12]

Recombinant antibody production and immunocytochemistry. Recombinant antibodies (rAbs) were prepared from single, clonally expanded MS CSF plasma cells, as described.[13] Each rAb was generated from an expanded plasma cell clone defined by identical heavy and light chain variable region sequences in 2 or more plasma cells.[8] Thirty-two rAbs, generated from 4 patients with MS (MS02-19, MS03-1, MS04-2, and MS04-3), and representing 73%–100% of clonally expanded plasma cell populations, were used in immunocytochemistry. EBV-infected B95-8 cells were spotted onto glass slides, air-dried, fixed in ice-cold acetone, and stained using standard immunocytochemistry techniques. Details are provided in appendix e-4.

RESULTS

MS CSF B-lymphocytes and plasma cells are devoid of EBER-1 transcript. Nested real-time PCR detected EBER-1 transcript in all NC08-AJS EBV-infected B cells, but not in any single CD138+ plasma cell or single CD19+ B-lymphocyte in MS CSF (table 1). The cell-specific transcript RPLP0 was detected in NC08-AJS infected cells as well as in plasma cells and B-lymphocytes in MS CSF. Occasionally, single plasma cells and B-lymphocytes are recovered from MS CSF that fail to amplify variable region immunoglobulin sequences.[8,11] To exclude the possibility that EBV infection might be limited to plasma cells with abrogated IgG transcription,[14,15]

Table 1 Expression of cell-specific and EBV-specific transcripts in sorted single cells from MS CSF and sorted single EBV-infected cells in tissue culture

Single cells	Cellular transcript: RPLP0	EBV-specific transcript: EBER-1
EBV-infected control B cells (n = 25)	25/25[a]	25/25
Plasma cells (CD138+)		
Successful IgG amplification		
MS04-2 (n = 53)	53/53	0/53
MS05-6 (n = 16)	16/16	0/16
MS05-9 (n = 71)	71/71	0/71
MS06-6 (n = 77)	77/77	0/77
Unsuccessful IgG amplification		
MS04-2 (n = 18)	18/18	0/18
MS05-6 (n = 15)	15/15	0/15
MS05-9 (n = 12)	12/12	0/12
MS06-6 (n = 12)	12/12	0/12
B cells (CD19+ CD138−)		
MS04-2 (n = 27)	27/27	0/27
MS05-6 (n = 38)	38/38	0/38
MS06-6 (n = 2)	2/2	0/2

Abbreviations: EBER-1 = EBV-encoded RNA-1; EBV = Epstein-Barr virus; IgG = immunoglobulin G; MS = multiple sclerosis; RPLP0 = ribosomal protein large P0.
[a]Number of positive/number of total cells.

single plasma cells in which variable region heavy chain did not amplify were also examined for EBER-1 transcript; again, while the RPLP0 transcript was readily identified in these single plasma cells, EBER-1 transcript was absent.

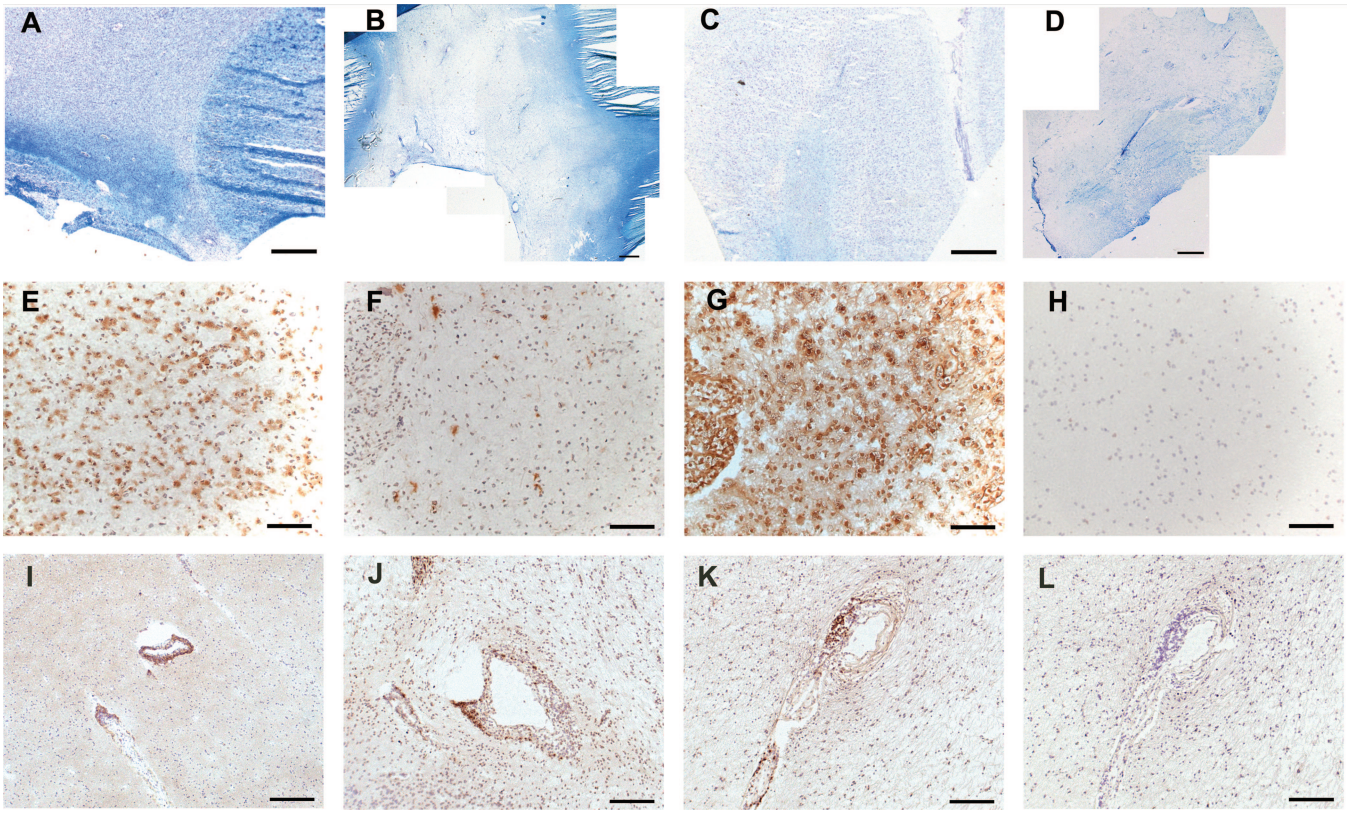
Active demyelinating MS plaques are devoid of EBV. MS brain sections were stained with LFB to detect demyelination. Cellular infiltrates were characterized by immunohistochemistry for markers indicative of macrophages, B-lymphocytes, and plasma cells (table e-2). Plaques were infiltrated with all 3 cell types; IgG deposition was extensive (figure 1).

Because the absence of EBV in single CSF B-lymphocytes and plasma cells may be due to a difference in frequency of EBV infection between CSF and tissue lymphoid cells, cDNA was generated from total RNA of sections from fresh-frozen active and chronic-active MS plaques from patients with acute, relapsing-remitting, primary progressive, progressive relapsing, and secondary progressive disease. Samples included 7 plaques from 6 MS patients from US tissue banks, and 8 plaques from 8 patients from UK MS tissue bank. Of 15 plaques, 4 contained perivascular cuffs of B-lymphocytes (figure 1; table e-2) and 5 were from patients previously reported to have plaques with high EBV load (patients MS079, MS121, MS153, MS154, and MS160).[7] Tissue sections from fresh-frozen EBV-infected lymphomas served as controls. While 3 cellular transcripts (IgG, RPLP0, and GAPDH) were readily detected in all plaques and controls, EBV-specific transcripts (EBER-1, EBNA-2, LMP-1, and BFRF-1) were not found in any of 15 plaques (table 2). The same cellular- and EBV-specific transcripts were found in all 5 EBV-infected Hodgkin and B-cell lymphomas (table 2), although LMP-1 was not found in 3 of 5 EBV-infected lymphoma samples, possibly due to sequence variations in the LMP-1 gene.[16] Overall, EBER-1 transcript was not found in single plasma cells or B-lymphocytes in MS CSF, or in B-lymphocytes infiltrating actively demyelinating MS plaques.

Previously, EBV DNA was detected in ~10% of MS plaques, although EBV gene expression in those MS plaques was not analyzed.[17] To further investigate this finding, we obtained additional MS plaques prescreened by PCR for EBV DNA. We examined 5 of these MS plaques, including the 3 that had tested positive for EBV DNA, as well as control samples of EBV-positive and EBV-negative lymphomas and non-MS brain tissues. Although all tissue samples had been formalin-fixed and paraffin-embedded, they still contained amplifiable RNA and were positive for endogenous, cell-specific transcripts (table 3). We detected EBER-1, EBNA-2, and BFRF-1 transcripts in EBV-positive lymphomas, but no EBV-specific transcripts were found in EBV-negative lymphomas or non-MS brain tissues. We confirmed the presence of EBER-1 RNA, but not the EBV-specific EBNA-2, LMP-1, and BFRF-1 transcripts in the 3 MS brain plaques (DE03, DE04, and DE07) that had previously tested positive for EBV DNA (table 3).

Absence of intrathecal anti-EBV antibody production in MS. Paired plasma-serum and CSF samples from 20 MS and 5 non-MS inflammatory CNS disease control patients were tested for anti-EBV antibodies. Of 20 patients with MS, 17 (85%) had an IgG index >0.7, as did 3/5 (60%) controls (table e-1), indicating intrathecal synthesis of total IgG.[12] Intrathecal synthesis of anti-EBV IgG antibody (AI ≥1.5, figure 2A) was found in 3/20 (15%) patients with MS and in 2/5 (40%) controls. No significant difference between MS and control patients was found in the frequency of intrathecal

Figure 1 Histology of active multiple sclerosis (MS) plaques used to screen for Epstein-Barr virus infection



Luxol fast blue–stained white matter lesions of MS1987 (A), MS160 (B), MS2180 (C), and MS03-A1.C1 (D). MS03-A1.C1 stained with anti-CD68 antibody (E), anti-CD138 antibody (F), and anti-human-immunoglobulin G (IgG) antibody (G). Normal human brain white matter stained with anti-human IgG (H). Perivascular B-lymphocyte cuffs in chronic-active lesions of MS121 (I) and MS160 (J and K) stained with anti-CD20 antibody. An adjacent section of a lesion from MS160 stained with secondary antibody alone (L). Scale bars: A–D = 1 mm, E–H = 100 μm, I–L = 200 μm.

anti-EBV antibody synthesis ($p = 0.25$, Fisher exact test, figure 2A).

rAbs from MS CSF do not bind EBV antigens. Because most oligoclonal bands and antibodies from expanded plasma cell clones in inflammatory and infectious CNS disorders are directed against disease-relevant agents,[18] we tested whether rAbs generated from clonally expanded MS CSF plasma cells bind to EBV antigens. B95–8 B-lymphocytes infected with EBV were used in immunostaining with MS CSF and with rAbs generated from the same patients with MS. While B95–8 cells readily stained with control anti-EBV-gp125 antibody and with MS CSF (figure 2B, a–d, f), none of 32 rAbs from patients with relapsing or progressive MS bound to EBV-infected cells (figure 2B, g–i).

DISCUSSION We showed that most active and chronic-active MS plaques replete with perivascular B-lymphocyte cuffs and single MS CSF B-lymphocytes and plasma cells do not contain EBV-specific transcripts. EBV-specific transcripts examined were as follows: EBER-1, the most abundant transcript in latently infected cells[19]; latent transcripts EBNA-2 and LMP-1; and lytic state transcript BFRF-1.[20] Unlike other human herpesviruses, EBV is associated with multiple types of latency. Type 0 latency, in which only the EBER-1 transcript is found, is seen in circulating nondividing B-lymphocytes of healthy seropositive individuals, while types I, IIa, IIb, and III, in which EBER-1 and other EBV transcripts are found, is seen in Burkitt and Hodgkin lymphomas, chronic lymphocytic B-cell leukemia, and posttransplant lymphoproliferative disease.[21,22] EBER-1 transcript is present in all forms of EBV latency.

Real-time PCR did not detect EBER-1 transcript in any single B-lymphocyte or plasma cell examined in MS CSF, and an extensive analysis of 15 acute, active, and chronic-active MS plaques revealed no EBER-1 transcripts. Additional EBV gene expression analysis of 3 plaques known to be EBV DNA-positive identified EBER-1 as the only EBV-specific transcript present. This transcription profile is characteristic of type 0 EBV latency, indicating that B-lymphocytes in the few plaques containing EBV

Table 2 Expression of cellular and EBV-specific transcripts in fresh-frozen active and chronic-active MS plaques as well as in fresh-frozen control tissue samples

	Cellular transcripts			EBV-specific transcripts			
Tissue samples	IgG	RPLP0	GAPDH	EBER-1	EBNA-2	LMP-1	BFRF-1
MS plaques							
MS03-A1.C1[a]	+	+	+	–	–	–	–
MS03-A1.C2[b]	+	+	+	–	–	–	–
MS1897	+	+	+	–	–	–	–
MS1934	+	+	+	–	–	–	–
MS2180	+	+	+	–	–	–	–
MSRH	+	+	+	–	–	–	–
MSPN	+	+	+	–	–	–	–
MS058	+	+	+	–	–	–	–
MS079[c]	+	+	+	–	–	–	–
MS090	+	+	+	–	–	–	–
MS094	+	+	+	–	–	–	–
MS121[c]	+	+	+	–	–	–	–
MS153[c]	+	+	+	–	–	–	–
MS154[c]	+	+	+	–	–	–	–
MS160[c]	+	+	+	–	–	–	–
EBV-positive lymphomas							
H1579	+	+	+	+	+	–	+
H7459	+	+	+	+	+	–	+
H27258	+	+	+	+	+	–	+
L1529	+	+	+	+	+	+	+
L29493	+	+	+	+	+	+	+

Abbreviations: BFRF-1 = BamH1 F rightward reading frame-1; EBER-1 = EBV-encoded RNA-1; EBNA-2 = EBV nuclear antigen-2; EBV = Epstein-Barr virus; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; IgG = immunoglobulin G; LMP-1 = latent membrane protein-1; MS = multiple sclerosis; RPLP0 = ribosomal protein large P0.
[a]Plaque C1 of MS03-A1.
[b]Plaque C2 of MS03-A1.
[c]Plaques from these patients were reported to have high EBV load.[7]

are of the same latency type as that found in circulating nondividing B-lymphocytes of healthy seropositive individuals.

ELISA and immunostaining detected anti-EBV antibodies in CSF of MS and control patients with non-MS inflammatory CNS diseases. The anti-EBV AI, an indicator of intrathecal anti-EBV antibody production at AI values greater than 1.5,[12] identified intrathecal synthesis in a few patients with MS and controls, but there was no significant difference between the patient groups in the frequency of anti-EBV IgG intrathecal synthesis. Anti-EBV oligoclonal IgG, reflecting substantial intrathecal anti-EBV antibody synthesis, is rare in MS.[23,6,7] For example, about 13% of 132 patients with MS, and none of 125 control patients with noninflammatory neurologic disease, had oligoclonal IgG in their CSF to anti-EBNA-1 and anti-BRRF2 peptides.[6] In another study, the CSF in 7 of 16 patients with MS contained EBV-specific oligoclonal IgG, although in 6 of these 7 patients with MS, anti-EBV bands were few and faint.[7] It remains to be determined whether a subset of patients with other CNS inflammatory diseases whose CSF contains oligoclonal IgG also contains any EBV-specific oligoclonal IgG. Meanwhile, the rare intrathecal humoral immune response against EBV in MS CSF indicates that the virus is unlikely to be linked with disease pathogenesis. Besides anti-EBV antibodies, antibodies to measles, rubella, varicella zoster virus, and herpes simplex virus are also synthesized intrathecally in some patients with MS and patients with CNS infections,[24,25] a finding that has been explained by infiltration of antibody-secreting B cells into the CNS of patients with infectious and inflammatory CNS diseases. Patients with high levels of intrathecal IgG production are more likely to have any one of the antiviral antibodies in their CSF[25] than patients with low intrathecal IgG synthesis. Polyclonal activation of long-lived memory B cells has been observed after secondary immunization[26,27] and could explain the infrequent detection of intrathecal antiviral antibodies, particularly in chronic inflammatory CNS disease. Thus, the presence of antibodies to diverse viruses in MS CSF and patients with other infectious and inflammatory diseases of the CNS represents an immune reaction common to infectious and inflammatory CNS diseases.

Most importantly, rAbs generated from clonally expanded plasma cells in MS CSF did not bind to EBV. CSF plasma cells, absent in healthy human CSF, are unique to the CSF of patients with infectious or inflammatory CNS diseases, and the antibodies they produce are directed against specific antigens involved in the pathogenesis of disease. Clonally expanded MS CSF plasma cells are generated through an antigen-driven germinal center-like maturation and differentiation process,[8] and are the source of intrathecally synthesized oligoclonal bands found in MS CSF.[28] The specificity of MS CSF rAbs likely duplicates the specificity of MS CSF oligoclonal bands that are thought to be directed against disease-relevant antigens.[18] For example, most rAbs derived from brain and CSF plasma cell clones in subacute sclerosing panencephalitis bind to measles virus, the cause of subacute sclerosing panencephalitis, and most rAbs derived from CSF plasma cell clones in neuromyelitis optica bind to the disease-associated aquaporin-4 water channel.[29-31] Additionally, oligoclonal IgG in CSF of patients with varicella

Table 3 Expression of cellular and EBV-specific transcripts in formalin- fixed, paraffin-embedded active, and chronic-active MS plaques as well as in formalin-fixed, paraffin-embedded control tissue samples

	Cellular transcripts			EBV-specific transcripts			
Tissue samples	IgG	RPLP0	GAPDH	EBER-1	EBNA-2	LMP-1	BFRF-1
MS plaques							
DE01	+	+	+	–	–	–	–
DE03[a]	+	+	+	+	–	–	–
DE04[a]	+	+	–	+	–	–	–
DE05	+	+	+	–	–	–	–
DE07[a]	+	+	–	+	–	–	–
EBV-positive lymphomas							
DE08	+	+	+	+	+	–	+
DE09	+	+	+	+	+	–	+
DE10	+	+	–	+	+	–	+
EBV-negative lymphomas							
DE11	+	+	+	–	–	–	–
DE12	+	+	+	–	–	–	–
DE13	+	+	+	–	–	–	–
Non-MS control brain							
DE02	+	+	+	–	–	–	–
DE06	+	+	+	–	–	–	–

Abbreviations: BFRF-1 = BamH1 F rightward reading frame-1; EBER-1 = EBV-encoded RNA-1; EBNA-2 = EBV nuclear antigen-2; EBV = Epstein-Barr virus; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; IgG = immunoglobulin G; LMP-1 = latent membrane protein-1; MS = multiple sclerosis; RPLP0 = ribosomal protein large P0.
[a]EBV DNA detected by PCR.

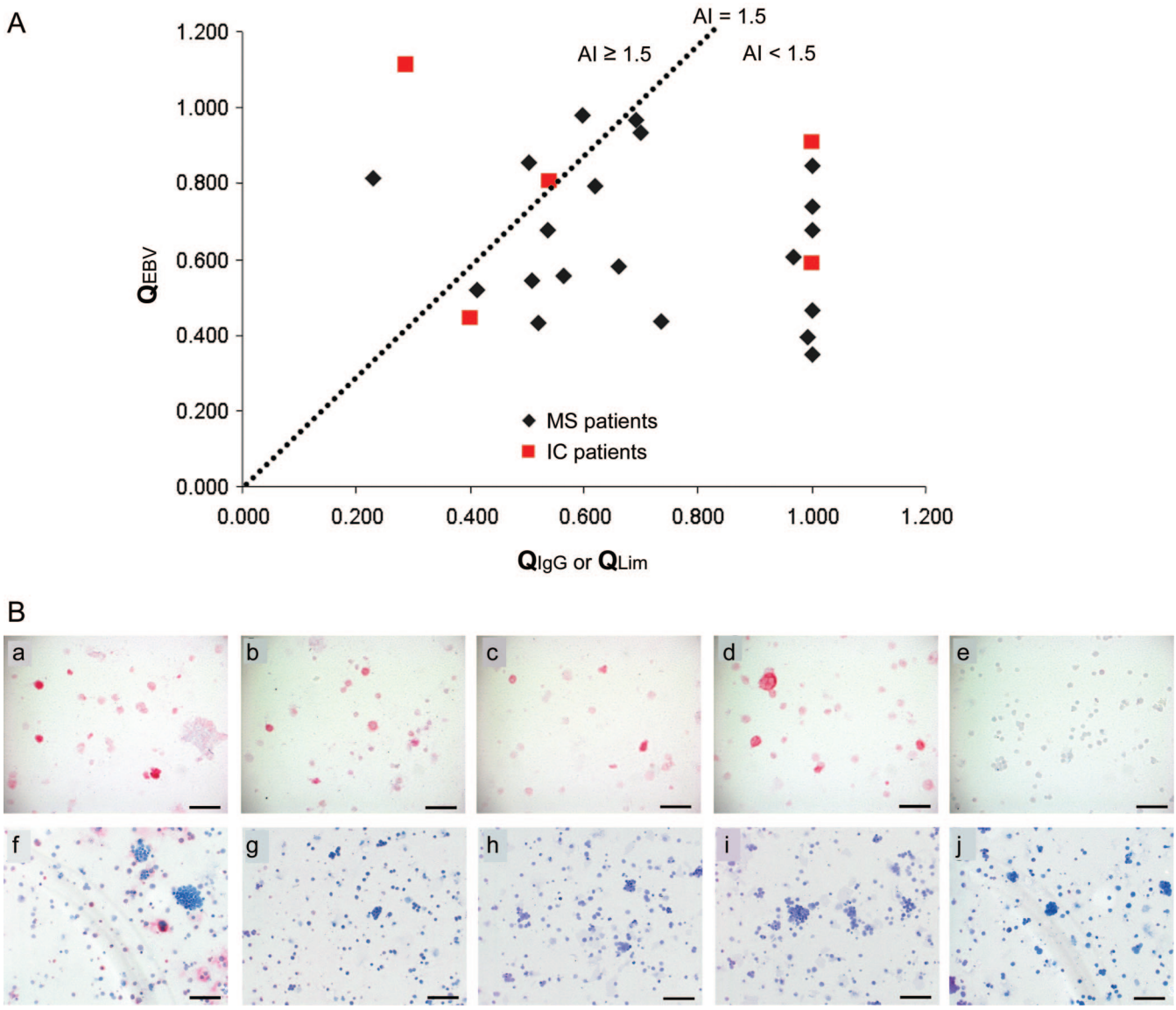
zoster virus vasculopathy is directed against varicella zoster virus antigens.[32] Although occasional plasma cells producing anti-EBV antibodies could be present in MS CSF, the absence of anti-EBV reactivity by MS rAbs examined here, which collectively represent the majority of MS patients' clonally expanded plasma cell populations, argues against the role of EBV in MS brain pathogenesis.

Our findings on the rarity of EBV in MS plaques agree with most published studies examining EBV in MS brain. Using PCR amplification, one study detected EBV DNA in about 10% of MS plaques,[17] and another in less than 10%.[33] Additionally, a higher percentage of EBV-positive samples was present in nonneurologic disease brains (46%) and Parkinson disease brains (42%) as compared to MS (27%) and Alzheimer (25%) brains.[17]

Multiple studies to detect EBV-specific RNA in MS brains have also been conducted. In situ hybridization (ISH) did not reveal EBV RNA in MS brain,[34,35] and another ISH study failed to detect EBV RNA in MS or control tissues, although the authors acknowledged that they were hampered by the poor quality of RNA obtained from the stored brain tissues.[36] In contrast, ISH reportedly revealed an abundant presence of EBV RNA in active and chronic-active MS plaques.[7] In the latter and the present studies, plaques from patients with primary progressive MS, secondary progressive MS, relapsing-remitting MS, and acute MS were examined (15 plaques by our group and 21 plaques by Serafini et al.[7]). Five active and chronic-active plaques analyzed in our study were from 5 patients whose active and chronic-active plaques were reported to contain a high load of EBV.[7] These patients (MS079, MS121, MS153, MS154, and MS160) are identified in table 2 and in table S1 in reference 7. Although our group and Serafini et al.[7] studied different plaques from these same patients, the plaques examined by both groups contained perivascular cuffs of B-lymphocytes (figure 1, table e-2, and table S1 in Serafini et al.[7]). Thus, tissue differences are not likely to account for the different results. A more likely explanation for this discrepancy lies in different methods of detection. We used PCR amplification of EBV RNA because of its higher sensitivity and specificity compared to ISH, consistent with a recent study showing that EBV RNA was readily detected by PCR in the same MS samples that were negative by ISH.[33] Additionally, PCR amplification allows sequencing of the amplicon and confirmation of specific amplification of the target transcript. Unlike ISH, PCR is highly sensitive, as evidenced by our detection of EBV-specific transcripts in single EBV-infected B cells (including EBER-1 and less abundant EBNA-2, LMP-1, and BFRF-1 transcripts, data not shown) and EBV-positive control tissue.

Our results indicate that the demyelinating events in MS occur largely in the absence of latent or productive EBV infection of B-lymphocytes or plasma cells in MS CNS, and that there is no robust intrathecal anti-EBV antibody production exclusive to MS. Meanwhile, because we did not examine the titer of anti-EBV antibody or the cytotoxic immune response to EBV in MS or control patients, our findings do not contradict the considerable seroepidemiologic data that link EBV infection with an increased risk of developing MS,[3-5] or an association of the peripheral anti-EBV antibody titer and HLA-DRB1*15 haplotype with the MRI lesion load and MS brain atrophy.[37,38] Overall, latent or productive EBV infection is not present in MS brain or CSF, and there is no specific intrathecal anti-EBV antibody response in MS.

Figure 2 Anti-Epstein-Barr virus (EBV) antibody detection in multiple sclerosis (MS) CSF



(A) Intrathecal anti-EBV antibody production in the CSF of MS and non-MS inflammatory disease control (IC) patients. The anti-EBV immunoglobulin G (IgG) antibody index (AI) was calculated based on 20 MS and 5 IC patients' CSF and plasma-serum: if $Q_{IgG} < Q_{Lim}$, then $AI = Q_{EBV}/Q_{IgG}$; otherwise, $AI = Q_{EBV}/Q_{Lim}$. The distribution of each patient's AI is presented relative to the diagonal line (AI = 1.5). Three of 20 MS and 2 of 5 IC patients demonstrated intrathecal anti-EBV IgG antibody synthesis (AI ≥1.5). The frequency of intrathecal anti-EBV antibody synthesis did not differ between MS and IC patients ($p = 0.25$, Fisher exact test). (B) EBV-infected B95-8 cells stained with MS CSF and with rAbs generated from clonally expanded plasma cells of MS CSF. B95-8 cells stained with mouse anti-EBV-gp125 antibody (a) and (f) followed by alkaline-phosphatase conjugated anti-mouse IgG; B95-8 cells stained with CSF of MS subjects MS02-19 (b), MS03-1 (c), MS04-2 (d) followed by alkaline phosphatase-conjugated anti-human IgG; B95-8 cells stained with alkaline phosphatase-conjugated anti-human IgG alone (e); B95-8 cells stained with MS recombinant antibodies (rAbs) MS02-19 rAb 11 (g), MS03-1 rAb 15 (h), MS04-2 rAb 13 (i) followed by mouse anti-flag IgG and alkaline phosphatase-conjugated anti-mouse IgG; B95-8 cells stained with mouse anti-flag IgG and alkaline phosphatase-conjugated anti-mouse IgG (j). Immunostainings of B95-8 cells with CSF or rAbs obtained from the same patient with MS are shown in pairs: (b) and (g) represent the CSF and rAb from MS02-19; (c) and (h) represent the CSF and rAb from MS03-1; and (d) and (i) represent the CSF and rAb from MS04-2. Scale = 100 μm.

AUTHOR CONTRIBUTIONS

Statistical analysis was conducted by Dr. Siranush A. Sargsyan.

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