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Short communication

Massive intracerebral Epstein-Barr virus reactivation in lethal multiple sclerosis relapse after natalizumab withdrawal

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ABSTRACT

Rebound of disease activity in multiple sclerosis patients after natalizumab withdrawal is a potentially life-threatening event. To verify whether highly destructive inflammation after natalizumab withdrawal is associated with Epstein-Barr virus (EBV) reactivation in central nervous system infiltrating B-lineage cells and cytotoxic immunity, we analyzed post-mortem brain tissue from a patient who died during a fulminating MS relapse following natalizumab withdrawal. Numerous EBV infected B cells/plasma cells and CD8 + T cells infiltrated all white matter lesions; the highest frequency of EBV lytically infected cells and granzyme B + CD8 + T cells was observed in actively demyelinating lesions. These results may encourage switching to B-cell depleting therapy after natalizumab discontinuation.

1. Introduction

Rebound of disease activity may occur in multiple sclerosis (MS) patients after ceasing natalizumab treatment (Rasenack and Derfuss, 2016). A few cases of life-threatening or lethal exacerbations associated with numerous inflammatory demyelinating lesions on MRI, but without detection of JC virus in cerebrospinal fluid (CSF) and brain tissue, were reported (Lenhard et al., 2010; Rigau et al., 2012; Beume et al., 2015; Larochelle et al., 2016). At neuropathological examination, prominent central nervous system (CNS) infiltration by CD4 + and CD8 + T cells, B cells and plasma cells was observed (Rigau et al., 2012; Beume et al., 2015; Larochelle et al., 2016).

Our studies in post-mortem brain from patients with acute and progressive MS suggest that a deregulated Epstein-Barr virus (EBV) infection, brought into the CNS by B cells (the preferred EBV reservoir), could trigger local immune activation (Serafini et al., 2007, 2014; Angelini et al., 2013). Because infection with EBV is the strongest known risk factor for MS (Ascherio, 2013), the possibility that in susceptible individuals defective control of EBV drives a CNS-damaging immunopathological response has been raised but remains controversial (Lassmann et al., 2011). Since natalizumab interferes with CNS immune surveillance and increased VCA IgG titers in MS patients during natalizumab treatment suggest EBV reactivation (Castellazzi et al., 2015), we hypothesized that disease rebound after natalizumab discontinuation might result from a highly destructive cytotoxic immune response toward uncontrolled EBV reactivation occurring in the CNS during treatment. To test this hypothesis, we analyzed post-mortem brain tissue from a 50-year-old male who died during a fulminating MS relapse after natalizumab withdrawal (Rigau et al., 2012).

2. Material and methods

2.1. Ethics statement

Use of post-mortem human brain material was approved by the Ethics Committee of Istituto Superiore di Sanità.

2.2. Patient

Clinical history, radiological and neuropathological features of this MS patient were described previously (Rigau et al., 2012). Briefly, the patient developed highly active relapsing-remitting MS and severe neurologic impairment (EDSS 7) and was treated with natalizumab that

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reduced MRI activity and neurological disability (EDSS 4.5). Due to severe depression, the man decided to stop natalizumab after 37 infusions. Three and a half months later he was hospitalized for rapid disease worsening. Brain MRI showed more than 50 hyperintense T2-weighted lesions, mostly gadolinium-enhanced, in cerebral hemispheres and medulla oblongata. JC virus was not detected by polymerase chain reaction (PCR) in CSF, blood and urine at admission. EBV serology and EBV DNA load in CSF were not assessed. The patient rapidly worsened and, despite treatment with methylprednisolone, plasma exchange and mitoxantrone, died 17 days after hospitalization. Post-mortem neuropathological analysis confirmed characteristics of active MS rebound; PCR failed to detect JC virus in brain tissue (Rigau et al., 2012).

2.3. Immunohistochemistry

Three-μm sections were cut from paraffin-embedded brain tissue blocks [frontal lobe ($n = 2$), medulla oblongata ($n = 2$)] and analyzed for demyelination and inflammation using monoclonal antibodies (mAbs) against myelin oligodendrocyte glycoprotein (MOG; Z12, kind gift of Dr. S. Piddlesden, Cardiff), MHC class II (CD3/43, Dako) and CD68 (KP1, Dako). Single and double immunostainings were performed using anti-CD20 mAb (L26), anti-CD8 polyclonal Ab (pAb), anti-Ig A, G, M pAb, anti-granzyme B mAb (GrB-7) and anti-laminin pAb (all from Dako), as described (Serafini et al., 2007; Angelini et al., 2013). EBV infection was investigated with mAbs specific for EBV latent membrane protein 2A (LMP2A) (15F9, Serotec) and EBV lytic proteins BZLF1 (BZ-1), p160 (OT10) and gp350/220 (OT 6.2) (kindly provided by Dr. J. Middeldorp, VUMC, Amsterdam, Netherlands), and with a pAb specific for EBV lytic protein BFRF1 (kind gift of Dr. A. Faggioni, University of Rome La Sapienza, Italy) (Serafini et al., 2007; Angelini et al., 2013). Sections were analyzed and images acquired with Axiophot microscope (Carl Zeiss, Jena) or digital epifluorescence microscope (Leica Microsystem, Wetzlar).

2.4. In situ hybridization for EBV-encoded small RNA (EBER)

In situ hybridization was performed using EBER PNA Probe/Fluorescein and the PNA ISH detection kit (Dako), following the manufacturer's instructions.

2.5. Cell counts

EBER + cells and cells immunoreactive for EBV proteins were counted in serial sections. Cell density was calculated relatively to the area of perivascular immune infiltration assessed with Axiovision 4 AC software, and expressed as mean number ± SE of stained cells per 0.1 mm^2 of infiltrated area. Differences in stained cell densities among white matter lesions were checked using Kruskal Wallis test followed by Dunn-Bonferroni *post-hoc* method for pairwise comparisons.

3. Results

3.1. Neuropathological findings

Large white matter lesions were present in sections from the frontal lobe (3 chronic inactive lesions with scarce perivascular leukocyte infiltration) and the medulla oblongata (3 chronic active and 3 actively demyelinating lesions with prominent perivascular and intraparenchymal leukocyte infiltration) (Fig. 1 A, B). B cells (CD20 +) and T cells (CD3 +), 70–80% of which were CD8 +, were the most abundant immune cell types in all lesions (Fig. 1 C, D). Ig-producing plasma cells were 5- to 25-fold less numerous than B cells and more frequent in chronic active and actively demyelinating lesions (Fig. 1 E).

3.2. EBV infection status and CD8 + T-cell infiltration

Markers of EBV infection were detected in all lesions and most (85%) perivascular cuffs analyzed. The density of cells stained for EBER, the non-translated EBV RNA expressed during all phases of latent infection, was similar in inactive, chronic active and actively demyelinating lesions (Fig. 1 F–I, P). Cells expressing LMP2A, a viral protein associated with latency activation (latency programs III and II), were more numerous in chronic active lesions (Fig. 1 J,P), while cells expressing viral proteins associated with the immediate early (BZLF1), early (BFRF1) and late [p160 (viral capsid), gp350/220 (viral envelope)] phases of the EBV lytic cycle were more frequent in actively demyelinating lesions (Fig. 1 K-M,P). Consistent with the B-cell tropism of EBV and knowledge of EBV life cycle (Taylor et al., 2015), latently and lytically infected cells were identified as B cells and plasma cells, respectively (Fig. 1 N,O). Only in actively demyelinating lesions, gp350/220 immunoreactivity was associated with endothelial-like cells in about 15% of small caliber blood vessels (Fig. 1 Q).

The lytic enzyme granzyme B was detected in a high percentage of CD8 + T cells in chronic active and actively demyelinating lesions (median 51%, range 37–63) (Fig. 1 R), and to a lesser extent in inactive lesions (median 20%, range 5–24). Immune synapse-like contacts between CD8 + T cells and B cells and between granzyme B + cells and BFRF1 + lytically infected cells strongly suggest ongoing cytotoxic activity (Fig. 1 S, T).

4. Discussion

Analysis of post-mortem brain tissue from a MS patient dying from a fulminating neurologic episode after natalizumab withdrawal indicates extensive demyelination and inflammation in the white matter and a widespread EBV latent and productive infection of infiltrating B-lineage cells. The higher frequency of EBV lytically infected cells, including some cerebrovascular endothelia, and granzyme B + CD8 + T cells in immunologically active lesions suggests a link between *in situ* EBV reactivation, cytotoxic activity and highly destructive inflammation.

Compared to findings in post-mortem tissue from chronic stages of MS (mainly secondary progressive MS) (Serafini et al., 2007; Angelini et al., 2013), both inflammatory status and EBV reactivation are definitely more pronounced in this patient. Typically, only B-lineage cells expressing proteins associated with the latent-to-lytic switch (BZLF1) and the early (BFRF1) but not late (gp350/220, p160) phases of EBV replication were detected in the rare active lesions of chronic MS and at a much lower frequency than in the present study (up to 12-fold relatively to the infiltrated area). The inflammatory status and high density of cells expressing EBV early lytic proteins in the white matter of the patient analyzed here resemble more findings in two MS patients with an acute clinical course (23 days and 10 months, respectively) described previously (Serafini et al., 2007). These observations suggest that widespread EBV reactivation in MS white matter lesions is associated with dramatic inflammatory tissue destruction and fast neurological deterioration.

The present findings could be explained by *de novo* migration of EBV infected B cells from the peripheral blood, as part of immune cell reentry in the CNS after natalizumab withdrawal. Natalizumab increases the number of circulating B cells (Krumbholz et al., 2008) but it is not known if it affects peripheral EBV DNA load, which is generally low and not significantly different between MS patients and healthy individuals (Lünemann and Münz, 2009). Alternatively, massive reactivation of a pre-existing EBV infection in the CNS during natalizumab treatment could have triggered a very aggressive cytotoxic immune response after drug discontinuation, causing the extensive pathology observed here. This interpretation is consistent with the knowledge that EBV proteins, mainly those associated with the immediate early and early lytic cycle, induce potent CD8 + T cell responses and that CD8 + T cells are the main drivers of bystander tissue damage in EBV-associated

immunopathologic diseases (Taylor et al., 2015). Most importantly, the hypothesized scenario is supported by several studies showing selective enrichment of EBV-specific (including lytic antigen-specific) CD8 + T cells in the CSF of MS patients (Jaquiéry et al., 2010; Lossius et al., 2010, van Nierop et al., 2016).

In conclusion, it is proposed that the hyper-inflammatory CD8 + T-cell dominated response observed after natalizumab discontinuation in the absence of a detectable JC virus infection in the CNS (this study; Larochelle et al., 2016) could represent an immune reconstitution inflammatory syndrome directed against EBV, possibly amplified by accumulation of EBV-specific CD8 + T cells in the blood of natalizumab-treated MS patients (Jilek et al., 2010; Angelini et al., 2013). Considering that B cells are the main reservoir of EBV infection and EBV deregulation in MS can involve the CNS as well as cervical lymph nodes (Serafini et al., 2014), B-cell depletion could lower EBV load and inhibit antiviral immunity induction in the periphery, thereby decreasing the CNS

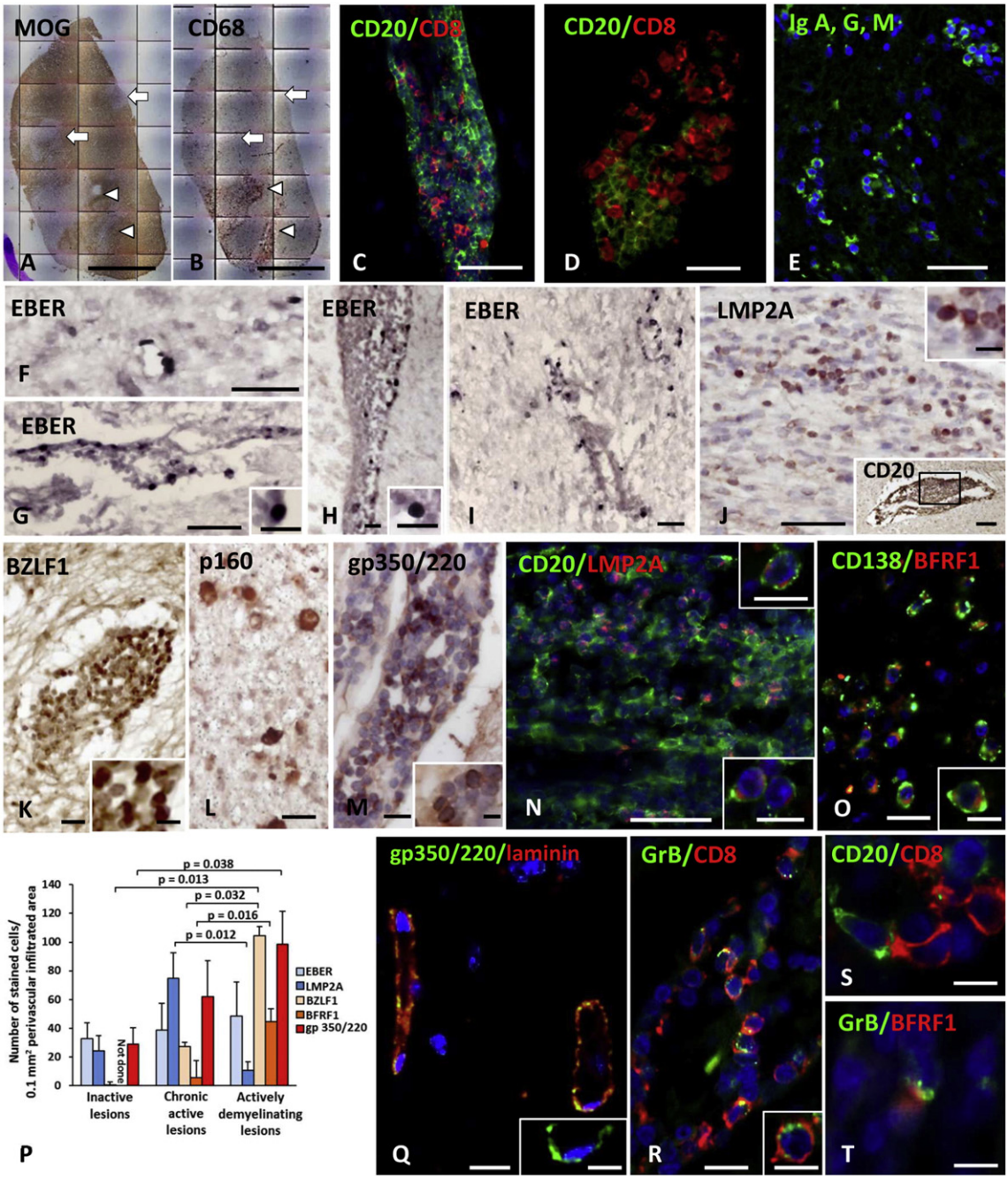

Fig. 1. Neuropathological analysis and detection of EBV infection in brain tissue obtained at autopsy 4 months after natalizumab withdrawal. Immunohistochemistry for MOG (A) and CD68 (B) shows white matter lesions [2 chronic active (arrows) and 2 actively demyelinating (arrowheads)] in the medulla oblongata. Double staining for CD20 and CD8 (C, D) and staining for Ig A, G, M (E) show high density of perivascular CD20 + B cells, CD8 + T cells and plasma cells in an active lesion. EBER *in situ* hybridization reveals numerous EBV latently infected cells (EBER + blue nuclei) in inactive (F), chronic active (G, H) and actively demyelinating (I) lesions. EBV protein-expressing cells in chronic active (J) and active lesions (K–M) are visualized by immunostainings for the latent protein LMP2A and for the lytic proteins BZLF1, p160 and gp350/220 (J–M). CD20 + B cells co-expressing LMP2A (N) and CD138 + plasma cells co-expressing the lytic protein BFRF1 (O) are shown. Statistically significant differences in the density of perivascular cells expressing the indicated EBV markers among lesions were evaluated (P). Double immunostaining for gp350/220 and laminin shows gp350/220 in endothelial-like cells in an active lesion (Q). Double immunostainings show expression of granzyme B (GrB-7 mAb, Dako) in CD8 + T cells (R), a lymphoblastoid CD8 + T cell sticking to a CD20 + B cell (S) and polarization of granzyme B + granules toward a BFRF1-expressing cell (T) in active lesions, supporting cytotoxic T-cell-mediated attack against EBV infected cells. Bars: 2.5 mm in A, B; 100 μm in C, lower inset in J; 50 μm in D–G, I, J, N; 20 μm in H, K–M, O–R; 10 μm in S, T, insets.

inflammatory burden. Hence, these results suggest a rationale for the switch to B-cell-depleting therapies in patients who stop natalizumab due to JC-virus antibody positivity or other reasons (Alping et al., 2016).

Author contribution

BS, ES, BR performed the experiments. BS acquired and analyzed the data. VR, ET provided the samples and collected clinical data. FA conceived the study, analyzed the data and wrote the manuscript. BS, ET, FA revised the article. All authors approved the final version of the manuscript.

Competing interests

BS, ES, BR, VR declare that they have no competing interests. ET received honoraria from Biogen-Idec, Genzyme, Merck-Serono, Novartis, Sanofi-Aventis and Teva. FA received research grant from Glaxo-Smith Kline China.

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