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Short Communication

Epstein-Barr virus-associated immune reconstitution inflammatory syndrome as possible cause of fulminant multiple sclerosis relapse after natalizumab interruption

Barbara Serafini[a], Stephanie Zandee[b], Barbara Rosicarelli[a], Eleonora Scorsi[a], Caterina Veroni[a], Catherine Larochelle[b], Sandra D'Alfonso[c], Alexandre Prat[b,d], Francesca Aloisi[a,*]

[a] Department of Neuroscience, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy
[b] Neuroimmunology Research Laboratory, Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM), 900 Rue Saint-Denis, Tour Viger, Montréal, QC H2X 0A9, Canada
[c] Department of Health Sciences, University of Eastern Piedmont, Novara, Italy
[d] Department of Neurosciences, Faculty of Medicine, Université de Montréal, Montreal, QC H3T 1J4, Canada

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ABSTRACT

Studying rebound mechanisms in MS patients after interruption of natalizumab may advance our understanding of MS pathogenesis. To verify the role of Epstein-Barr virus (EBV) infection and anti-EBV immunity in post-natalizumab rebound, we analyzed postmortem brain tissue of a case of fatal MS relapse. Using immunohistochemistry, EBV latent, early and late lytic proteins and CD8+ T cells sticking to EBV lytically-infected cells were detected in multiple inflammatory white matter lesions. Using the pentamer technology on brain sections, EBV-specific CD8+ T cells were observed perivascularly. Cell-free EBV DNA was detected in cerebrospinal fluid. These findings confirm and extend the results obtained in another case of post-natalizumab fatal MS relapse, suggesting that this condition represents an EBV-associated immune reconstitution inflammatory syndrome.

1. Introduction

Clinical and radiological rebound activity may occur in multiple sclerosis (MS) patients after discontinuing natalizumab or fingolimod, drugs that block traffic of lymphocytes to the central nervous system (CNS) (Giovannoni et al., 2017). Rare cases of fulminant rebound with many multifocal gadolinium-enhancing lesions have been reported (Lenhard et al., 2010; Rigau et al., 2012; Larochelle et al., 2017; Forci et al., 2017; Novi et al., 2017). Studies are needed to discern the mechanism of rebound relapses and prevent this condition.

Although debated (Lassmann et al., 2011), dysregulated Epstein-Barr virus (EBV) infection in the CNS is a possible mechanism of chronic neuroinflammation in MS. By studying postmortem brain of patients with progressive MS and rare cases of acute MS, we have shown that EBV-infected B cells accumulate in demyelinated lesions and meninges (Serafini et al., 2007, 2010). EBV gene and protein expression patterns in the MS brain are compatible with local activation of latent infection and, less frequently, entry into the lytic cycle (Serafini et al., 2010; Angelini et al., 2013; Veroni et al., 2018). Together, active EBV infection, predominance of CD8+ T cells in CNS immune infiltrates (Angelini et al., 2013), enrichment of EBV-specific CD8+ T cells in cerebrospinal fluid (CSF) of MS patients (Jaquiéry et al., 2010; Lossius et al., 2010; van Nierop et al., 2016), and increased frequency of EBV lytic antigen-specific CD8+ T cells in peripheral blood during active MS disease (Angelini et al., 2013), suggest that an immunopathological response toward EBV could be a major determinant of CNS damage in MS. We assumed that such an immunopathological response is halted by natalizumab but can burst when the drug is removed because the host immune system overreacts to uncontrolled intracerebral propagation of EBV infection occurring during drug treatment. To test this hypothesis, in a previous study we examined postmortem brain tissue from a patient who died during a fulminating MS relapse after natalizumab withdrawal (Rigau et al., 2012). Widespread infection and reactivation of EBV was observed in the brain of this patient, suggesting that rebound activity might represent an immune reconstitution inflammatory syndrome (IRIS) toward EBV (Serafini et al., 2017). To verify this hypothesis further, we have investigated EBV infection status and searched for EBV-specific CD8+ T cells in postmortem brain of another case of fatal MS relapse after natalizumab withdrawal (Larochelle et al., 2017).

* Corresponding author.
E-mail addresses: barbara.serafini@iss.it (B. Serafini), barbara.rosicarelli@iss.it (B. Rosicarelli), caterina.veroni@iss.it (C. Veroni), catherine.larochelle.chum@ssss.gouv.qc.ca (C. Larochelle), sandra.dalfonso@med.uniupo.it (S. D'Alfonso), a.prat@umontreal.ca (A. Prat), francesca.aloisi@iss.it (F. Aloisi).

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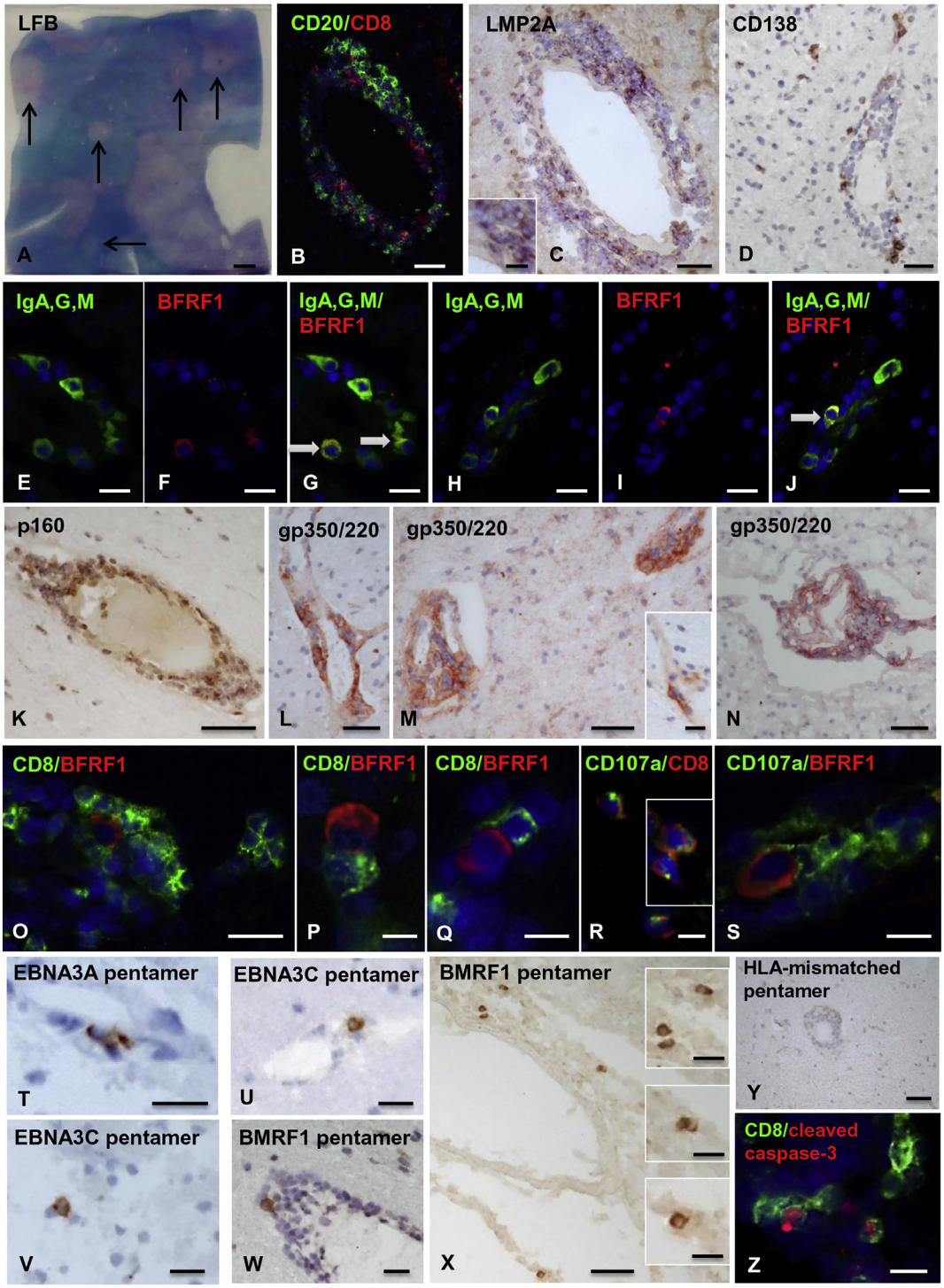

Fig. 1. Detection of EBV infection markers and EBV antigen-specific CD8 T cells in fresh frozen brain tissue obtained at autopsy 5 months after NTZ withdrawal. Numerous actively demyelinating white matter lesions (arrows) are visualized in the frontal lobe by Luxol fast blue stain (A). Serial brain sections were immunostained for CD20 and CD8 (B) and for the EBV latent protein LMP2A (C): one large B-cell rich perivascular cuff containing many LMP2A+ cells in an active white matter lesion is shown. CD138 immunostaining shows infiltrating plasma cells (D). Double immunofluorescence staining for IgA, G, M and the EBV early lytic protein BFRF1 (E–J) shows that EBV reactivates in a fraction of Ig-producing plasma cells (arrows in G, J). Immunoreactivity for proteins of the EBV capsid (p160) (K) and envelope (gp350/220) (L–N) is present in numerous perivascular lymphocytic cells in cerebellar (K–M) and cerebral (N) white matter lesions; a gp350/220+ endothelial-like cell in an active lesion is also shown (inset in M). Double immunostaining for CD8 and BFRF1 reveals CD8+ cells surrounding and sticking to single EBV lytically infected cells (O–Q). Double immunostainings for CD107a and CD8 or BFRF1 reveal surface localization of the degranulation marker CD107a on CD8+ cells (R) and a BFRF1+ cell embraced by a CD107a + cell (S). Detection of EBV-specific CD8+ T cells (T-X): cells binding HLA-B*0702/EBNA3A 247-255 (T), HLA-B*0702/EBNA3C 881-889 (U, V) and HLA-B*0702/BMRF1 116–128 (W, X) pentamers are present in perivascular immune infiltrates in cerebral (T, U) and cerebellar (V–X) white matter lesions. Absence of staining with HLA-A*0201/LMP2A 356-364 pentamer (Y). Apoptotic CD8+ cells are visualized by double immunofluorescence staining for CD8 and cleaved caspase-3 (Z). Nuclei were stained with hematoxylin (C, D, K–N, T–W and Y) or DAPI (B, E-J, O-S and Z). Bars: 2 mm in A; 50 μm in B–D, K–N, X and Y; 20 μm in E-J, O, T-W, Z, and insets in C, M and X; 10 μm in P-S.

2. Materials and methods

2.1. Ethics statement

The patient provided written informed consent for postmortem CNS donation to research (Centre Hospitalier de l'Université de Montréal research ethics committee approval number BH.07.001) and use of CSF samples (approval numbers SL05.022 and 023, and BH07.001). Study of post-mortem human brain was approved by Istituto Superiore di Sanità ethics committee (protocol CE12/356).

2.2. Patient

A 32-year-old female was diagnosed relapsing-remitting MS in 2005 and received interferon-β for 1 year and glatiramer acetate for 3 years as disease-modifying drugs before natalizumab was started because of continuing relapses; clinical history, radiological, neuropathological and immunological features of this patient were described previously (Larochelle et al., 2017). After two years on natalizumab, the patient was clinically and radiologically stable but, due to positive JC virus (JCV) serology, agreed to stop natalizumab. Four months later, while on glatiramer therapy, she was hospitalized due to marked clinical and radiological disease worsening. The patient rapidly worsened and, despite several courses of steroids, died one month after hospitalization. Autopsy was performed within 1 h of death; postmortem neuropathological analysis confirmed exacerbating MS pathology. PCR failed to detect JCV in CSF and CNS tissue samples; PCR, serology or immunohistochemistry for other neurotropic and non-neurotropic pathogens were negative (Larochelle et al., 2017).

2.3. Immunohistochemistry

Seven-μm sections were cut from fresh frozen brain tissue blocks [frontal lobe (n = 3), cerebellum (n = 2)], and demyelination and inflammation were analyzed (Larochelle et al., 2017).

Immunostainings were performed and analyzed as described (Serafini et al., 2007; Angelini et al., 2013). Primary antibodies (Abs) used were: anti-CD20 and anti-CD8 mAbs (L26 and C8/144B, DakoCytomation), anti-IgA,G,M pAb (DakoCytomation), anti-CD8 pAb (ThermoScientific), anti-CD138 mAb (B-B4, Serotec), anti-cleaved caspase 3 pAb (Cell Signaling Technology), anti-CD107a mAb (H4A3, BD Pharmingen), anti R-PE pAb (GeneTex), anti-latent membrane protein (LMP)2A mAb (4E11, Ascenion), anti-EBV early lytic protein BFRF1 pAb (kind gift of Prof. A. Faggioni, Rome, Italy), anti-EBV late lytic protein p160 (OT10) and gp350/220 (OT 6.2) mAbs (kind gift of Prof. J. Middeldorp, Amsterdam, Netherlands).

2.4. In situ pentamer binding

HLA typing performed with brain-derived DNA revealed that the patient was HLA-B*0702 positive. As EBV peptide epitopes restricted to HLA-B*0702 have been characterized, EBV-specific CD8+ T cells were investigated by staining fresh frozen brain sections with R-phycoerythrin-labeled HLA-B*0702/EBV EBNA3A 247-255 (RPPIFIRRL), HLA-B*0702/EBV EBNA3C 881-889 (QPRAPIRPI) and HLA-B*0702/EBV BMRF1 116–128 (RPQGGSRPEFVKL) peptide pentamers (ProImmune; final concentration 10 μg/ml), following the manufacturer's instructions. HLA-B*0702/HCMV pp65 341-349 (QYDPVAALF) and HLA-A*0201/EBV LMP2A 356-364 (FLYALALLL) peptide pentamers (ProImmune) were used as controls.

2.5. EBV DNA load in CSF

DNA was extracted from 0.6 ml of cell-free CSF using QIAmp Mini Kit (Qiagen, Valencia, CA). Quantitative analysis of EBV DNA was performed for the *Bam*HI W fragment of the EBV genome using Taqman primers (Life Technologies, Grand Island, NY) (Chijioke et al., 2013). Droplet Digital PCR was performed using the QX200 ddPCR system (Bio-Rad Laboratories, Pleasanton, CA). Data are expressed as number of EBV DNA copies per ml of CSF (Berger et al., 2001).

3. Results

As reported (Larochelle et al., 2017), many abundantly infiltrated active and chronic active lesions were present in the cerebral and cerebellar white matter of this post-natalizumab fatal case (Fig. 1A). Inflammatory cell infiltrates were dominated by CD20+ B cells and/or CD8+ T cells (Fig. 1B), with relatively fewer CD138+ plasma cells (Fig. 1D). In one cerebral and one cerebellar tissue block with the highest degree of B-cell infiltration, 50 to 80% of the perivascular cuffs contained numerous cells expressing LMP2A, an EBV protein associated with viral latency activation (Thorley-Lawson, 2015) (Fig. 1C). In all tissue blocks examined, the EBV early lytic protein BFRF1 (Serafini et al., 2007) was expressed in 20–30% of Ig-producing plasma cells, the B-lineage cells in which EBV reactivates (Fig. 1E–J). Lymphocyte-like cells expressing proteins of the EBV capside (p160) and envelope (gp350/220) were detected perivascularly in sections of 1 and 4 out of 5 tissue blocks, respectively (Fig. 1K–N). In some small caliber blood vessels, gp350/220 immunoreactivity was also present in endothelial-like cells (inset in M). The percentage of perivascular cuffs containing gp350/220+ cells ranged from 7 to 16% and reached 60% in one cerebellar tissue block.

CD8+ T cells surrounding and sticking to BFRF1+ lytically infected cells were frequently noted in the sections (Fig. 1O–Q). Surface immunostaining for CD107a, a marker of cytotoxic granule exocytosis, was detected in 11–14% of CD8+ cells and CD107a + cells sticking to BFRF1+ cells were observed (Fig. 1R,S), suggesting ongoing cytotoxic activity toward EBV-lytically infected cells.

Using *in situ* pentamer staining, CD8+ T cells recognizing EBV latent (EBNA3A, EBNA3C) and early lytic (BMRF1) proteins were detected in sections of one cerebral and two cerebellar tissue blocks (Fig. 1T–X). Conversely, no cells were stained by CMV pentamer (not shown) or HLA-mismatched pentamer (Fig. 1Y). The fraction of CD8+ T cells specific for each EBV antigen was low (0.2–1.2%), as assessed by counting total pentamer+ cells and CD8+ cells in serial sections. A possible explanation is that EBV-specific CD8+ T cells undergo apoptosis *in situ* after encounter and killing of infected cells. Accordingly, within white matter perivascular cuffs a large proportion of active caspase-3+ cells (apoptotic cells) were CD8+ (median value 60%, range 42–75%). Moreover, 16% (median value; range 10–18%) of CD8+ cells, but only 2.0–2.5% of CD20+ B cells, were apoptotic (Fig. 1Z).

EBV DNA was detected in cell-free CSF collected one week before and at the time of death (7.2 and 8.5 copies/ml, respectively).

4. Discussion

This study shows that overwhelming inflammation is associated with widespread EBV infection and reactivation in the brain of a MS patient dying from a lethal relapse after natalizumab withdrawal, confirming findings obtained in another fulminant post-natalizumab case (Serafini et al., 2017). Both patients were negative for JCV in CSF and brain tissue and had no pathological features of progressive multifocal leukoencephalopathy (Rigau et al., 2012; Larochelle et al., 2017). The presence of major glycoproteins of the EBV capside and envelope in the brain of post-natalizumab fatal cases contrasts with absence/rarity of these proteins in brain samples of chronic and acute MS cases. In the latter, mostly EBV latent proteins and to a lesser extent immediate early and early lytic proteins were detected, suggesting abortive EBV reactivation (Serafini et al., 2007; Angelini et al., 2013). Collectively, these results indicate that the hallmark of post-natalizumab fatal rebound is EBV spread within the CNS with production of

viral particles, likely due to lack of immune surveillance during prior natalizumab treatment. Presence of cell-free EBV DNA in the CSF of the patient analyzed here corroborates this scenario and suggests that EBV should be also measured in CSF drawn at post-natalizumab rebound. Of interest, a recent study has shown an increase in VCA IgG titers, which suggests EBV reactivation, in MS patients treated with natalizumab (Castellazzi et al., 2015).

A pathogenetic model of MS in which both EBV-infected B cells and EBV-specific CD8+ T cells are present in the CNS may explain bystander damage (Taylor et al., 2015). The frequent contacts between actively degranulating or granzyme B+ CD8+ cells and EBV lytically infected cells observed in both fatal rebounds, and the presence of CNS-infiltrating EBV-specific CD8+ T cells observed for the first time in this study, strongly suggest that post-natalizumab rebound activity represents an IRIS mediated by cytotoxic T cells that re-enter the brain after drug clearance and kill EBV-infected cells causing devastating inflammation.

Besides supporting the switch to B-cell depleting therapies after natalizumab discontinuation (Alping et al., 2016), these results may foster studies aimed at evaluating the efficacy of anti-herpesvirus treatment in patients stopping natalizumab or with highly active MS (Lycke, 2017).

Author contribution

BS, ES, BR performed immunohistochemistry and *in situ* pentamer binding. BS acquired and analyzed immunohistochemical data. CV performed digital PCR and analyzed the data. SD performed HLA typing. SZ, CL, AP provided the samples and collected clinical and neuropathological data. FA conceived the study, analyzed the data and wrote the manuscript. BS, AP, FA revised the article. All authors approved the final version of the manuscript.

Competing interests

The authors declare no conflict of interest.

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