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LETTER TO THE EDITOR

Detection of Epstein–Barr virus and B-cell follicles in the multiple sclerosis brain: what you find depends on how and where you look

Francesca Aloisi,[1] Barbara Serafini,[1] Roberta Magliozzi,[1,2] Owain W. Howell[2] and Richard Reynolds[2]

1 Department of Cell Biology and Neuroscience, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy
2 Centre for Neuroscience, Imperial College Faculty of Medicine, Hammersmith Hospital Campus, Burlington Danes Building, Du Cane Road, London W12 ONN, UK

Correspondence to: Dr Francesca Aloisi,
Department of Cell Biology and Neuroscience,
Istituto Superiore di Sanità,
Viale Regina Elena 299,
00161 Rome, Italy
E-mail: fos4@iss.it

Sir, The research article by Willis *et al*. (2009) and the letter to the editor by Peferoen *et al*. (2010) challenge our earlier finding that infection with Epstein–Barr virus is a common feature in the multiple sclerosis brain, provided that autopsy samples containing immune infiltrates are analysed (Serafini *et al*., 2007). Both groups also fail to detect meningeal B-cell follicles, the structures we first found in a proportion of patients with secondary progressive multiple sclerosis (Serafini *et al*., 2004; Magliozzi *et al*., 2007; Howell *et al*., 2009) and then identified as main, though not unique, intracerebral sites of Epstein–Barr virus persistence and reactivation (Serafini *et al*., 2007). Based on their inability to find follicle-like structures in 94 multiple sclerosis brain specimens collected by the Netherlands Brain Bank and in a small number of specimens obtained from the UK Multiple Sclerosis Tissue Bank, Peferoen *et al*. (2010) 'question the importance and significance of ectopic B cell follicles as a pathogenic feature of multiple sclerosis', a scenario we proposed by showing that these structures are associated with more extensive cortical pathology and aggressive clinical course (Magliozzi *et al*., 2007; Howell *et al*., 2009). Inability to detect meningeal B-cell follicles in brain tissue of the Dutch Multiple Sclerosis cohort was recently reported also by Kooi *et al*. (2009) and Torkildsen *et al*. (2010).

Given the mounting serological evidence (Ascherio and Munger, 2007; Farrell *et al*., 2009; Sundström *et al*., 2009; Levin *et al*., 2010; Lünemann *et al*., 2010) and immunological (Jilek *et al*., 2008; Lünemann and Münz, 2009; Pender, 2009; Jaquiéry *et al*., 2010) of an association between Epstein–Barr virus and multiple sclerosis, understanding the mechanisms that link this common B-lymphotropic γ herpesvirus to brain inflammation and B-cell abnormalities in multiple sclerosis is of primary importance (Salvetti *et al*., 2009). Here, we discuss several issues that may explain the differences between our results and those of recent studies that failed to detect ectopic B-cell follicles and/or Epstein–Barr virus in the multiple sclerosis brain (Kooi *et al*., 2009; Torkildsen *et al*., 2010; Willis *et al*., 2009; Peferoen *et al*., 2010; Sargsyan *et al*., 2010).

Detection of ectopic B-cell follicles

We define ectopic B-cell follicles as large B-cell aggregates localized in the sub-arachnoid space, mainly inside the cerebral sulci, that display several germinal centre-like features (i.e. presence of stromal/follicular dendritic cells expressing CXCL13, B-cell proliferation, expression of activation-induced cytidine deaminase and plasma cell differentiation) (Serafini *et al*., 2004, 2007; Magliozzi *et al*., 2007) but lack the typical structure of lymphoid follicles with a germinal centre and a mantle zone and contain mainly memory B cells (Serafini *et al*., 2010). Due to their localization and sparse distribution, a successful search for B-cell follicles in multiple sclerosis brain samples requires optimal preservation of the meninges during tissue processing, gentle cutting and handling of the sections during the staining procedures, as well as extensive sampling in terms not only of number of cases but also of blocks per case and sections per block analysed. We used mainly paraformaldehyde-fixed and snap-frozen brain samples of the UK Multiple Sclerosis Tissue Bank collection and observed that

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the processing of paraffin-embedded sections often results in the loss of the cellular content of the meninges (O. Howell and R. Reynolds, personal communication). Thus, it is possible that inability to detect B-cell follicles in paraffin-embedded (Kooi *et al*., 2010; Peferoen *et al*., 2010) and snap-frozen (Torkildsen *et al*., 2010) brain samples of the Dutch Multiple Sclerosis cohort is due to different procedures of tissue processing, cutting and staining.

In a first analysis of a random sample of 36 multiple sclerosis cases (29 with secondary progressive multiple sclerosis and 7 with primary progressive multiple sclerosis; Serafini *et al*., 2004, 2007; Magliozzi *et al*., 2007), and in a more recent analysis of 126 progressive multiple sclerosis cases with a wide age range (27–83 years) (Howell *et al*., 2009 and unpublished data), 50 cases have been detailed (40%) that harbour follicle-like structures in the meninges. The frequency of blocks containing follicle-like structures is highly variable (range = 5–65%, median = 21%) (Magliozzi *et al*., 2007; Howell *et al*., 2009 and unpublished data) and sampling of 20 blocks is required in many cases, thus raising the possibility that we have actually underestimated the number of cases displaying this pathological feature.

Another critical determinant in the detection of B-cell follicles is the multiple sclerosis cohort analysed. Only a few of the multiple sclerosis cases analysed by Kooi *et al*. (2009), Torkildsen *et al*. (2010) and Peferoen *et al*. (2010) show features (secondary progressive phase of disease, age at death <50 years, B-cell-rich case) comparable to those of the multiple sclerosis cases with follicles characterized in our studies (Serafini *et al*., 2004, 2007, 2010; Magliozzi *et al*., 2007; Howell *et al*., 2009). It remains possible that the cohort of multiple sclerosis cases in the UK Multiple Sclerosis Tissue Bank present a more active inflammatory progressive disease than those used by others.

Detection of Epstein–Barr virus transcripts by *in situ* hybridization

In situ hybridization for Epstein–Barr virus-encoded small RNA (EBER) is considered the best method for detecting and localizing Epstein–Barr virus in tissue samples and is widely used for the diagnosis of Epstein–Barr virus-associated malignancies (Gulley and Tang, 2008). Nevertheless, several pitfalls in technique and interpretation have been highlighted. For example, cytoplasmic EBER staining, as opposed to nuclear EBER staining, is considered to be non-specific in tumour cells, whereas false-negative EBER results may be a consequence of RNA degradation. The latter issue is particularly critical when analysing autopsy and formalin-fixed tissue as well as snap-frozen tissue not processed *ad hoc* for RNA analysis (Niedobitek and Herbst, 2006). In our study, a commercial peptic nucleic acid probe for EBERs (EBER1 and EBER2) and a peptide nucleic acid *in situ* hybridization detection kit (both from Dako, Denmark) allowed the detection of Epstein–Barr virus infected cells in brain samples from 19 of 22 multiple sclerosis cases analysed (12 paraformaldehyde-fixed frozen, 4 snap-frozen and 3 paraffin-embedded samples), independently of the disease course (Serafini *et al*., 2007). No EBER signals were detected in a brain sample devoid of immune infiltrates and in paraffin-embedded archival brain samples from two cases with acute multiple sclerosis, which stained positively for viral latent and lytic proteins (Serafini *et al*., 2007). In the latter cases, GAPDH RNA was also undetectable by *in situ* hybridization (Serafini, unpublished data), highlighting how tissue processing is critical for *in situ* RNA detection.

In our study, the frequency of EBER$^+$ cells detected in different multiple sclerosis brain samples correlated with the extent of immune infiltration and number of CD20$^+$ B cells. Double immunostaining for EBER and the B-cell marker CD20 or the plasma cell marker CD138 indicated that a high proportion of brain-infiltrating B cells and plasma cells (up to 80–90%) are EBER$^+$ (Serafini *et al*., 2007). Although EBER signals were generally localized in the nuclei, a cytoplasmic localization was also observed in a variable proportion of EBER$^+$ cells (up to 25%), predominantly in cells with a plasma cell morphology (Serafini and Aloisi, personal communication). We have excluded that our *in situ* hybridization protocol yields non-specific EBER signals by analysing non-pathological (lymph node, spleen and thymus) and pathological (tonsil, lymphoblastic leukaemia, Epstein–Barr virus-negative lymphomas) lymphoid tissues (Serafini *et al*., 2007, 2010 and unpublished data; Cavalcante *et al*., 2010) and highly infiltrated brain samples from cases with acute inflammatory neurological diseases, mostly of infectious origin (Serafini *et al*., 2007, 2010). Among the latter, rare nuclear EBER signals were detected in only 2 samples out of 12 analysed (1 luetic meningitis and 1 tuberculous meningoencephalitis), indicating that abundant Epstein–Barr virus infection in the CNS might be specific to multiple sclerosis. Because EBER was recently shown to be released from Epstein–Barr virus infected cells (Iwakiri *et al*., 2009), EBER mislocalization in the multiple sclerosis brain could be associated with a chronically dysregulated Epstein–Barr virus infection.

In contrast with our findings, Willis *et al*. (2009) and Peferoen *et al*. (2010) did not detect EBER signals in any of the multiple sclerosis brain samples from the UK Multiple Sclerosis Tissue Bank. EBER *in situ* hybridization also yielded negative results in a separate series of paraffin-embedded brain samples analysed by Willis *et al*. (2009) and in all paraffin-embedded brain samples, except one, of the Vrije Universiteit Medical Center Dutch Multiple Sclerosis cohort (Amsterdam) analysed by Peferoen *et al*. (2010). Although demonstration of EBER nuclear signals in Epstein–Barr virus$^+$ lymphomas confirms the specificity of the *in situ* hybridization techniques used in our study and in the studies of Willis *et al*. (2009) and Perefoen *et al*. (2009), direct comparison of the results obtained is difficult at present due to use of different probes and protocols in differently processed tissue samples. However, the fact that Peferoen *et al*. (2010) did not find EBER signals in one multiple sclerosis sample in which Epstein–Barr virus lytic proteins were detected with immunohistochemistry and Willis *et al*. (2009) did not detect EBER by *in situ* hybridization in two samples, where low levels of Epstein–Barr virus nucleic acids were detected with polymerase chain reaction (PCR) methods, raises the suspicion that RNA preservation in the tissues analysed was not optimal and/or that the EBER *in situ* hybridization protocols used by these groups

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are not sensitive enough to detect Epstein–Barr virus in the multiple sclerosis brain.

Immunohistochemistry for Epstein–Barr virus proteins

Because of pitfalls of EBER *in situ* hybridization, immunohistochemistry for Epstein–Barr virus proteins is considered as a useful complement to detect Epstein–Barr virus and assess the status of viral infection in pathological tissues (Gulley *et al*., 2002). In our study, several antibodies directed against Epstein–Barr virus latent (EBNA2, LMP1 and LMP2A), early lytic (BFRF1, BMRF1) and late lytic (p160, gp350/220) proteins were used to stain fixed-frozen and, to a lesser extent, snap-frozen tissues and for each antigen and type of tissue the optimal staining protocol was established (Serafini *et al*., 2007, 2010 and unpublished data). Stainings in paraffin-embedded tissues were less convincing and reproducible. Using the whole panel of the above antibodies for each multiple sclerosis case analysed, we have shown that the latency II programme of Epstein–Barr virus infection (abundance of $LMP1^+$, $LMP2A^+$ cells, but low frequency of $EBNA2^+$ cells) predominates in the multiple sclerosis brain, whereas viral reactivation appears restricted to plasma cells in acute lesions and meningeal B-cell follicles.

In contrast to our findings, EBNA2 and LMP1 were not detected in any of 12 paraffin-embedded autopsy and biopsy samples analysed by Willis *et al*. (2009) and of six snap-frozen autopsy samples analysed by Torkildsen *et al*. (2010). Perefoen *et al*. (2010) also report absence of LMP1 immunoreactivity in all paraffin-embedded multiple sclerosis brain samples analysed and positivity for several Epstein–Barr virus lytic proteins (BZLF1, BMRF1 and BLLF1) in one sample only. At present, a direct comparison of the immunohistochemical data for Epstein–Barr virus proteins is hampered by differences in tissue processing, staining protocols, use of antibodies recognizing different Epstein–Barr virus proteins and different antibody sources. Precise characterization of inflammatory infiltrates and lesion type in multiple sclerosis brain samples is, however, necessary for correct interpretation of immunohistochemical data. For example, absence of immunoreactivity for Epstein–Barr virus lytic proteins in most multiple sclerosis brain samples analysed by Peferoen *et al*. (2010) could be due to the fact that these do not contain ectopic follicles and acute lesions, the sites where we have found evidence of Epstein–Barr virus reactivation (Serafini *et al*., 2007).

Detection of Epstein–Barr virus nucleic acids using PCR techniques

Analysis of viral nucleic acids represents an additional approach to search for Epstein–Barr virus in biological samples. Using very sensitive, non-quantitative PCR and reverse transcriptase (RT)–PCR techniques able to detect Epstein–Barr virus DNA and RNA, respectively, in single Epstein–Barr $virus^+$ lymphoblastoid line cells, Perefoen *et al*. (2010) failed to detect Epstein–Barr virus nucleic acids in all multiple sclerosis brain samples analysed (five tissue blocks from three multiple sclerosis cases); while Willis *et al*. (2009) and Sargsyan *et al*. (2010) found Epstein–Barr virus DNA and/or EBER1 only in a minority of samples. Other Epstein–Barr virus transcripts, like EBNA1, EBNA2, LMP1, LMP2, BFRF1 and BZLF1, were not found in any of the multiple sclerosis brain samples analysed by Torkildsen *et al*. (2010) and Sargsyan *et al*. (2010). These largely negative findings raise concerns on assessment of PCR sensitivity relatively to the B cell and Epstein–Barr virus content of the brain material analysed. First, the content and quality of RNA extracted from an autopsy tissue sample cannot be compared with that of viable, highly replicating lymphoblastoid cells that contain multiple copies of Epstein–Barr virus genomes and probably display high transcriptional activity. Moreover, if one considers that Epstein–Barr virus infected B cells represent only a tiny fraction of the total brain and inflammatory cell population, it should be suspected that detectability of viral nucleic acids in whole sections of autopsy brain tissue might be well below the sensitivity of conventional real-time PCR techniques, except perhaps for the most infiltrated tissue samples. Consistent with this, we could detect Epstein–Barr virus transcripts (EBNA1 and LMP2A) in multiple sclerosis brain sections from three out of four highly infiltrated multiple sclerosis cases analysed only after selective cDNA pre-amplification (Serafini *et al*., 2010). Similarly, Sargsyan *et al*. (2010) found EBER1 in three out of five paraffin-embedded multiple sclerosis brain samples that were analysed using pre-amplification RT–PCR, but in none of the 15 fresh frozen samples analysed without pre-amplification.

If the inability of PCR techniques to detect Epstein–Barr virus nucleic acids in multiple sclerosis brain sections was due to the presence of small numbers of infected cells within a heterogeneous cell population, different strategies should be applied to increase the sensitivity of the techniques. Because successful detection of infectious agents in cellular subsets within normal and pathological brain tissues was recently achieved by combining laser capture microdissection with quantitative real-time PCR or RT–PCR (Perez-Liz *et al*., 2008; Wilkinson *et al*., 2009), we tested the sensitivity of this combination to detect Epstein–Barr virus latency transcripts (EBNA1, LMP2A) in the multiple sclerosis brain. We laser-cut B cell containing immune infiltrates (perivascular cuffs in white matter lesions, sparse inflammatory cell infiltrates and ectopic B-cell follicles in the meninges) and adjacent non infiltrated brain parenchyma and analysed expression of B cell (CD19) and Epstein–Barr virus genes in these distinct brain regions using real-time RT–PCR, both with and without selective cDNA pre-amplification. Using this strategy, we could detect and quantify CD19 as well as viral transcripts in all multiple sclerosis brain regions containing B-cell infiltrates, but not in those devoid of immune infiltrates. Importantly, we found that expression of LMP2A and/or EBNA1 genes was comparable to or even higher than that of the B-cell associated gene CD19. These findings are consistent with the observation that a high percentage of multiple sclerosis brain-infiltrating B cells are latently infected with Epstein–Barr virus. We concluded that without the sensitivity of laser capture microdissection and pre-amplification real-time PCR

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techniques, Epstein–Barr virus detection would be unattainable in multiple sclerosis brain samples.

Conclusions

Searching for a herpes virus that establishes a predominantly latent infection in B cells and uses several strategies to escape immune surveillance is extremely challenging. For this reason, technical issues related to Epstein–Barr virus and B-cell detection in the multiple sclerosis brain should be rigorously assessed and compared between different laboratories. Based on their inability to detect Epstein–Barr virus in most multiple sclerosis brain samples analysed, Willis *et al*. (2009) and Sargsyan *et al*. (2010) propose that mechanisms other than dysregulated intracerebral Epstein–Barr virus infection (e.g. molecular mimicry) may link Epstein–Barr virus to multiple sclerosis pathology, whereas Perefoen *et al*. (2010) suggest that Epstein–Barr virus infection in the central nervous system may contribute to the early rather than the later stages of multiple sclerosis. Based on our observations that Epstein–Barr virus infected B cells can be detected in the brain of cases with acute, relapsing remitting and progressive multiple sclerosis and appear to be targeted by cytotoxic $CD8^+$ T cells (Serafini *et al*., 2007), we favour the idea that persistent Epstein–Barr virus infection in the brain contributes to sustained intrathecal B-cell dysregulation (due to the B-cell activating properties of the virus) and an immunopathological response driven by the sequestered viral deposits (Salvetti *et al*., 2009). This scenario is also supported by the recent finding that Epstein–Barr virus-specific, $CD8^+$ cytotoxic T cells accumulate in the cerebrospinal fluid of early multiple sclerosis patients (Jaquiéry *et al*., 2010). Transition from a more florid inflammatory response toward a weaker inflammatory response during disease progression could be determined by quantitative and, possibly, qualitative changes in the immune response to Epstein–Barr virus as a consequence of chronically dysregulated viral infection (so called immune exhaustion), as suggested by the recent work of Jilek *et al*. (2008).

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