LETTER TO THE EDITOR

Epstein Barr virus is not a characteristic feature in the central nervous system in established multiple sclerosis

Laura A. N. Peferoen,1,* Floris Lamers,1,* Lenthe N. R. Lodder,1 Wouter H. Gerritsen,1 Inge Huitinga,2 Jeroen Melief,2 Gavin Giovannoni,3,4 Ute Meier,3 Rogier Q. Hintzen,5 Georges M. G. M. Verjans,6 Gijsbert P. van Nierop,6 Wim Vos,1 Regina M. B. Peferoen-Baert,1 Jaap M. Middeldorp,1 Paul van der Valk1 and Sandra Amor1,3

1 Department of Pathology, VU University Medical Center, Amsterdam, The Netherlands
2 Netherlands Institute for Neuroscience and Netherlands Brain Bank, Amsterdam, The Netherlands
3 Neuroimmunology Unit, Queen Mary University of London, Neuroscience Centre, Barts, UK
4 Neuroimmunology Unit, Queen Mary University of London, Neuroscience Centre, Bizard Institute of Cell and Molecular Science, Barts and The London School of Medicine and Dentistry, London, UK
5 Department of Neurology, Academic Hospital, Rotterdam, The Netherlands
6 Department of Virology, Erasmus Medical Center, Rotterdam, The Netherlands

*These authors contributed equally to this work.

Correspondence to: Prof. Sandra Amor,
Department of Pathology,
VU University Medical Center,
Amsterdam,
The Netherlands
E-mail: s.amor@vumc.nl

Sir, Although recent studies have demonstrated a clear association of Epstein-Barr virus (EBV) infection with multiple sclerosis (Zaadstra et al., 2008; Lünenmann and Münz, 2009; Salvetti et al., 2009), there has been much debate if and where the virus acts in the pathogenic cascade of multiple sclerosis and whether the virus needs to gain entry to the central nervous system (CNS). A recent study reported in *Brain* by Willis et al. (2009) showed that there is little evidence for the presence of EBV in the CNS of patients with multiple sclerosis. Willis and co-workers used a variety of validated methods to determine the presence of EBV in the CNS of patients with multiple sclerosis. These methods contrast greatly with the studies by Serafini et al. (2007) that have described abundant EBV positive cells in multiple sclerosis, and the presence of extraocular B cell follicles enriched with EBV infected cells in some patients. Willis et al. did not find the presence of EBV to be a characteristic feature of multiple sclerosis. Unlike Serafini et al. (2007), Willis et al. did not find the presence of EBV to be a characteristic feature of multiple sclerosis. Clearly, the issue of whether EBV is indeed present in the CNS is crucial not only to determine the impact of the virus on the disease in the CNS, but also for diagnostic pathology in general. The studies reveal the vagaries of pathological detection methods for infectious agents such as EBV. Care in the practice and interpretation of such methodologies are of course key for correct diagnosis and proving pathogenic involvement and ultimately, to ensure correct treatment of patients with multiple sclerosis.

In our laboratory (VUMC), an *in situ* hybridization technique is widely used to detect EBV encoded RNA in various pathological specimens (Middeldorp et al., 2003) and our degree of positive samples does not differ significantly from that reported in literature. EBV encoded RNA is generally localized to the nucleus. The paper described two crucial observations. Unlike Serafini et al. (2007), Willis et al. did not find the presence of EBV to be a characteristic feature of multiple sclerosis (aptly the title of the paper). Second, ectopic follicles, suggested by Serafini et al. to harbour EBV infected B cells, were not observed, despite scrutiny of meningeal tissues where these follicles should have been present.

In our laboratory (VUMC), an *in situ* hybridization technique is widely used to detect EBV encoded RNA in various pathological specimens (Middeldorp et al., 2003) and our degree of positive samples does not differ significantly from that reported in literature. EBV encoded RNA is generally localized to the nucleus. This is the case for all EBV-associated malignancies, including Hodgkin’s disease, Burkitt’s lymphoma and brain lymphomas. EBV encoded RNA detected in the cytoplasm of cells in interphase, or in plasma cells or eosinophils are considered false positives, due to the application of over-sensitive methods (Gulley and Tang, 2008).

Received September 17, 2009. Revised October 6, 2009. Accepted October 6, 2009. Advance Access publication November 16, 2009
© The Author (2009). Published by Oxford University Press on behalf of the Guarantors of Brain. All rights reserved.
For Permissions, please email: journals.permissions@oxfordjournals.org
As a consequence of the report by Serafini et al. (2007), many laboratories have been keen to examine whether their detection methods allowed them to detect EBV in the CNS of people with multiple sclerosis. Together with many collaborators in the Netherlands and UK, we have screened 632 CNS specimens from 94 multiple sclerosis patients collected by the Netherlands Brain Bank. All samples were carefully processed with short fixation times to optimize RNA quality and subsequently subjected to EBV encoded RNA staining using an optimized EBV encoded RNA PNA-based (Dako, Denmark) protocol. In addition, 12 blocks of tissue samples from 12 patients documented in the Serafini et al. (2007) paper were obtained from the Multiple Sclerosis Society Brain Bank in London, UK, and taken from the very same tissue blocks used in that study. All the UK samples on which we performed the EBV encoded RNA technique were negative. We also performed immunohistochemistry for EBV lytic (BZLF1, BMRF1, BFRF3 and BLLF1) and latent (LMP1) proteins. One of us (J.M.M.) has produced many EBV antibodies including those used in the Serafini et al. paper, and has developed sensitive methods for EBV detection in pathological tissues. All tissue blocks available were screened for ectopic lymphoid follicles and the presence of infiltrates, B cells and plasma cells. In the majority of tissue samples, meningeal tissues were present.

Given the claim that ectopic follicles are associated with early onset multiple sclerosis and death in the secondary progressive phase (Serafini et al., 2007), we specially studied such a group of 11 patients (76 blocks), who all died before the age of 50. Ectopic B-cell follicles, as defined by the presence of CXCL13 or podoplanin (Marsee et al., 2009), were not observed in this cohort. Likewise, we examined 60 tissue blocks from 16 patients exhibiting prominent B cell infiltrates (Table 1). Again, however, ectopic follicles or follicular-like structures were not observed. Two of the 50 blocks containing B cells in perivascular infiltrates show only diffuse staining for CXCL-13.

However, the absence of ectopic follicles cannot immediately be taken to signify the absence of EBV-infected B cells. To examine the issue more closely, B cell-rich areas were screened not only for EBV encoded RNA but also for viral lytic and latent proteins. As positive controls, samples of EBV-associated tumours including CNS lymphoma all revealed clear nuclear staining of EBV encoded RNA. LMP1 was positive in tumours with EBV latency-II and in oral hairy leukoplakia (Webster-Cyriaque et al., 2000), the latter also being abundantly positive for EBV lytic gene expression. In contrast, nuclear EBV encoded RNA was found in only one tissue specimen from a single multiple sclerosis patient (Fig. 1B). All other specimens, including those examined by Serafini et al. (2007), were negative in our study. Staining for lytic and latent antigens using a wide range of antibodies also produced only negative results, apart from a single sample that was EBV encoded RNA negative, but positive for multiple EBV lytic cycle markers (BZLF1, BMRF1, BLLF1), supporting the possibility of lytic infection in this lesion. Appropriate positive controls were all positive and brain tissues from healthy controls were all negative.

In interpreting these data, the sensitivity of the EBV encoded RNA-staining method is of crucial concern. We therefore additionally used a very sensitive real-time PCR (Stevens et al., 2005a) and

| Table 1 Extent of CNS B-cell infiltration in the VUMC multiple sclerosis cohort |
|---------------------------|-----------------|-----------------|-----------------|-----------------|
| Multiple sclerosis type   | Age at death (years) | B-cell-rich cases | B-cell medium, poor or absent cases | Total number of cases |
| Relapsing remitting       | <50             | 0               | 1               | 1               |
|                          | ≥50             | 0               | 1               | 1               |
| Secondary progressive     | <50             | 1               | 10              | 11              |
|                          | ≥50             | 2               | 14              | 16              |
| Primary progressive       | <50             | 0               | 10              | 10              |
|                          | ≥50             | 4               | 15              | 19              |
| Undefined                 | <50             | 2               | 6               | 8               |
|                          | ≥50             | 7               | 21              | 28              |
| Total                     | 16              | 78              | 94              |

a Multiple sclerosis tissue blocks were screened from 94 multiple sclerosis cases (632 blocks) for the presence of B cells. In total, 60 tissue blocks from the 16 B cell-rich cases were screened. The extent of B cell infiltration was defined subjectively as the number of perivascular infiltrates containing CD20 positive cells as well as the number of B cells in the infiltrate and the meninges.

Figure 1  B-cell-rich area in multiple sclerosis Case 1 as defined by a high number of CD20 positive cells in perivascular lesion (A). This area was negative for EBV encoded RNA. In Case 2 (B) EBV encoded RNA positive nuclei (red) in a perivascular infiltrate of a 53-year-old female with fast progressive disease with a disease duration of 16 years.
reverse transcription–PCR method (Stevens et al., 2005b) to search for EBV genomes and encoded RNAs, respectively, in five tissue blocks containing B cell-rich areas from three patients. Using this method, one positive cell in 100,000 cells can be detected. None of the multiple sclerosis tissues examined revealed the presence of EBV DNA or RNA while all EBV-associated tumour samples and the EBV-positive cell line JY (1/100,000) were all positive.

Why is there such a contrast between the findings reported by Serafini et al. (2007) compared to those of Willis et al. (2009) and our data? As discussed by Willis (2009), the age of the cohort of patients could be a confounding factor, given that follicles were suggested to be associated with more aggressive disease and early death. In our cohort, we tried to examine patients fitting these criteria, including secondary progressive, primary progressive or undefined clinical types. Yet, none of the samples from these cases revealed the presence of ectopic follicles. Of all the B cell-rich cases that we found, only three patients died before the age of 50 (Table 1) while all of the 13 others had longstanding disease.

Another factor could be the sensitivity and specificity of the assay(s) used. In establishing a sensitive and reliable assay for the detection of EBV encoded RNA, several probes have been examined by the diagnostic department at the VUMC, Amsterdam. The use of some probes produced non-specific cytoplasmatic staining of plasma cells and some B cells. Such cytoplasmatic staining was also occasionally observed in B cell areas in the studies reported by Serafini et al. (2007), while B cell-rich areas from patients with bacterial meningitis did not show such staining. The possibility remains that ultra-sensitive detection methods may reveal a form of EBV that is specific for multiple sclerosis, and that may not necessarily be present in other EBV-positive samples, such as the ones used as positive controls. Moreover, since it is currently not possible to scrutinize the entire CNS, EBV-infected B cells may have resided in discrete areas that consistently happened not to have been included in our screen, although this is highly unlikely considering the number of specimens examined. It is relevant to note a recent report showing that EBV encoded RNA bound to the cellular La protein may be released by EBV-infected cells and taken-up in the cytoplasm of bystander cells through TLR3, thus triggering inflammation (Iwakiri et al., 2009). Indirect routes of (latent) EBV involvement in multiple sclerosis have been proposed (Lünenmann and Münz, 2009) and are supported by the increase of EBNA1- but not VCA-specific antibodies frequently observed in multiple sclerosis patients.

Nevertheless, our findings support those reported by Willis et al. (2009) and indicate that the detection of EBV in the brains of multiple sclerosis patients with established chronic multiple sclerosis is rare. Abundant EBV infection of the CNS is unlikely to contribute to the later stages of the pathogenesis of multiple sclerosis. Whether or not EBV acts earlier in the course of disease, perhaps as a trigger, or activating and perpetuating inflammation cannot be excluded by our data. In our hands, abundant EBV in multiple sclerosis brains as described by Aloisi’s group was not detected. Our data also question the importance and significance of ectopic B cell follicles as a pathogenic feature of multiple sclerosis. Nevertheless the debate on the role that EBV plays in the pathogenesis of multiple sclerosis should continue since the epidemiological data are compelling (Ascherio and Munger, 2007; Zacharsa et al., 2008); however, we may wish to focus on mechanisms explaining how EBV contributes to the early stages of multiple sclerosis, as it is unlikely that ongoing CNS infection with EBV contributes to the later stage multiple sclerosis pathology.

Acknowledgements

The authors thank the Netherlands Brain Bank and the UK Multiple Sclerosis tissue bank for tissues used in this study. All studies were carried out after ethical approval by the local committees.

Funding

This research was funded by grants from Dutch MS Research Foundation Netherlands (608-3 MS to LANP; 608-4 MS to LNRL; 05-538c MS to PvdV; 03-525 MS to IH and JM; 05-490b MS to RH); Stichting Research Fonds Pathologie (PvdV, FL, RMB-B and SA).

Supplementary material

Supplementary material is available at Brain online.

References

