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Viral pathophysiology of multiple sclerosis: A role for Epstein-Barr virus infection?

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Abstract

Multiple sclerosis (MS) is the most common inflammatory demyelinating and degenerative disease of the CNS. The cause of MS is unknown but environmental risk factors are implicated in MS. Several viruses have been proposed as a trigger for MS, and lately Epstein-Barr virus (EBV) has become the leading candidate. An infectious aetiology fits with a number of epidemiological observations in addition to the immunopathological features of the disease. In this review we will summarize the emerging evidence, which demonstrates a strong association between EBV infection and MS. The conundrum remains as to whether EBV is directly involved in the pathophysiology of MS, or alternatively if the immunopathology of MS somehow affects the regulation of EBV infection.

Keywords: Multiple sclerosis; Epstein-Barr virus; Pathophysiology; B cell; Infectious mononucleosis; Lymphoma; CD8+ T cells

1. Introduction

Among the environmental risk factors proposed for MS, an infectious aetiology fits with a number of epidemiological observations as well as immunopathological features of the disease [1]. Several viruses are proposed as a trigger for MS, and of these Epstein-Barr virus (EBV) has become the leading candidate in recent years [1]. The emerging evidence demonstrates a strong association between EBV infection and several autoimmune diseases, including MS.

EBV is a human γ-herpesvirus that has primary tropism for resting B cells. EBV is mainly transmitted via saliva; however, sexual transmission of the virus is also reported [2]. More than 90% of the adult population worldwide has serological evidence of prior infection with EBV. EBV is capable of causing latent infection within B cells, although other cell types can also be infected (T cells or epithelial cells) [3]. Primary infection with EBV follows a bimodal distribution, with peaks in young childhood and adolescence/early adulthood [4]. In early childhood acute infection is usually asymptomatic, whereas acute infection occurs in adolescence or adulthood, subjects may develop infectious mononucleosis (IM). Following infection with EBV, life-long latent viral presence is established within B cells, with periodic reactivation resulting in virus shedding in the saliva [5], during which times carriers may infect others.

EBV has been implicated in the pathogenesis of several diseases, mainly cancers. It is difficult to demonstrate the causative role of EBV in autoimmune disease, e.g. systemic lupus erythematosus [6], as it is yet to be elucidated why viral infection results in disease in only a few individuals despite high levels of EBV seropositivity within the general population; and as the virus resides in memory B cells, which traffic into inflamed tissues, the presence of EBV could be

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a bystander phenomenon leading to misinterpretation. There is evidence for an association of EBV with a high number of cases of B cell malignancies (Burkitt's lymphoma, Hodgkin's lymphoma and B cell lymphoma in patients with immunosuppressive disease), T cell malignancies (nasal NK-T cell lymphoma, hemophagocytic syndrome T cell lymphoma), and epithelial cell malignancies (nasopharyngeal carcinoma, lymphoepithelioma-like carcinoma) [5,7].

Several features support a pathogenic role of EBV in MS. Therefore in this review we will be focusing on EBV as a contributory factor for MS and will discuss several lines of evidence which strengthen this association.

2. Epidemiological evidence

Since the early 1980s remarkable similarities between the epidemiology of MS and IM have been noted [8]. Several epidemiological features of MS, such as the association with higher socio-economic status, occurrence of clusters and epidemics, changes in the prevalence with latitude and changes in the risk of the disease with migration, could possibly be explained by a role for EBV in the pathophysiology of MS [9].

In addition, several studies have shown that individuals with a history of infectious mononucleosis are at increased risk of developing MS. A meta-analysis of 14 case–control and cohort studies of IM and MS calculated the combined relative risk of MS after IM was 2.3 (95% CI, 1.7–3.0; $p < 10^{-8}$) [10]. Apart from indicating a potential role for EBV in the pathogenesis of MS, these findings suggest that the timing of primary EBV infection is an important factor in developing MS. Those who are infected in adolescence or young adulthood have a greater risk of developing MS than those infected during childhood [10]. It has been shown that in high prevalence regions for MS, seroconversion for EBV occurs during or after puberty in a large proportion of the population [11]. A Danish study of more than 25,000 patients with IM observed more than two-fold increased risk of MS in the IM cohort, further confirming the association between IM and MS [12]. They also found that the risk of developing MS was increased for more than 30 years after IM [12].

3. EBV serology

Several studies have compared the seroprevalence for common EBV antibodies between MS patients and healthy individuals. A systematic review of eight case–control studies demonstrated that nearly all MS patients are infected with EBV, compared with only about 90% of controls [13]. This study reported an odds ratio of developing MS is 13.5 (95% CI, 6.3–31.4) in EBV-seropositive individuals. A second systematic review including thirteen case–control studies confirmed the relation of EBV seropositivity and MS, reporting an odds ratio of developing MS for seronegative individuals of 0.06 (95% CI 0.03–0.13) [14]. In addition a more recent study confirmed that the risk of MS in EBV-negative individuals is very low, however, it increases dramatically in the same individuals after seroconversion [15].

The seropositivity for EBV is more prominent in pediatric MS. It has been reported that there is an EBV seropositivity rate of nearly 99% in pediatric MS patients compared to only 72% in age-matched controls [16]. A more recent study, showed that 86% of the pediatric MS patients were seropositive for EBV compared to 64% of matched controls ($p = 0.025$) [17]. However, the comparison of clinical manifestation in EBV-positive and EBV-negative pediatric MS patients showed no difference other than earlier or younger age, suggesting that EBV may not fundamentally influence the clinical manifestation of MS in children [18].

Further serological evidence comes from longitudinal prospective studies, in which blood samples were collected prior to the onset of MS. Analysis of these sera showed that the risk of acquiring MS is strongly associated with increased levels of EBV antibody titers prior to disease onset, and the strongest association was found for IgG antibodies binding to a EBV latent nuclear antigen (EBNA1) [19–22]. In addition a recent longitudinal study confirmed that the risk of MS in EBV negative individuals is very low, however, it increases dramatically in the same individuals after seroconversion [15].

It is believed that the protective response of the immune system against EBV is modulated via the secretion of neutralizing antibodies (NAbs) (these NAbs block the spread of free viruses to uninfected cells). Only one study has measured the EBV NAb titres in MS patients and healthy controls and no significant difference between the two groups was found [23]. In this study there was no significant correlation between the IgG NAb, EBNA1, and early antigen (EA) responses in either group [23].

There is evidence that the EBV serological findings might be associated with clinical and radiological disease activity in MS. EBV reactivation appears to be linked to disease activity in early MS. It has been found that MS patients in clinical relapse, compared to patients in clinical remission, had evidence of peripheral EBV reactivation as evidenced by increased IgM and IgA responses to EBV EA, and detectable EBV DNA in serum [24]. It has also been shown that patients with anti-EBV early antigen (EA) antibodies have more radiological disease activity in terms of gadolinium-enhancing lesions on MRI [25]. Recently MRI activity, as measured by Gd-enhancing lesions, was shown to also be correlated with anti-EBNA1 IgG and EBNA1/virus capsid antigen (VCA) IgG ratio [26]. In addition, anti-EBNA1 IgG titres but not anti-VCA IgG levels were correlated with T2-lesion volume changes over 5 years [26]. Anti-EBNA1 IgG titres were also a predictor of change in Expanded Disability Status Scale (EDSS) during the study follow-up period [26]. The latter could imply an association between anti-EBNA1 antibodies and disease progression in MS. Other antibodies against

EBV antigens have been correlated with MRI parameters [27,28]. Anti-VCA IgG levels have been positively correlated with T2 and T1 lesion volume [27,28], and negatively correlated with atrophy parameters (grey matter fraction-GMF-and brain parenchymal fraction-BPF) [28]. Increased anti-VCA IgG levels were associated with greater decrease in BPF after three years [27,28], denoting an association between EBV antibodies and loss of brain volume [28]. In this study, anti-EBNA1 IgG was only negatively associated with GMF, and there was no association of anti-EBV-EA IgG antibodies with any of the MRI parameters measured [28]. Although it has been demonstrated that EBV viral load is not associated with overall MS risk [29,30], EBV viral load has been associated with increased disease activity [29]. It has been shown that in patients with clinically isolated syndrome (CIS) the immune response IgG against EBNA1 but not other EBV antigens correlates with baseline number of T2 lesions, number of lesions meeting the Barkhof criteria at baseline, number of T2 lesions during follow-up, presence of new T2 lesions, and EDSS during a mean follow-up of 7 years. In addition, they showed that increased anti-EBNA1 IgG responses predict conversion to MS based on McDonald criteria [31]. No correlation was found between any EBV IgG responses and the number of gadolinium-enhancing lesions, although this finding was based on a small proportion of their studied patients.

Elevated EBV-VCA antibodies were also positively associated with other predisposing factors for MS in healthy individuals including female gender, HLA-DR2, current smoking status and the total number of pack-years smoked [32]. When both DR15 and high EBV titers are present, the conferred risk of MS is considerably increased [33–35]. In one study, the relative risk of MS was increased nine-fold for female MS patients with DR15 and elevated titers of anti-EBNA1 antibodies and implied an interaction between these two risk factors when compared to that of DR15-negative woman with low anti-EBNA1 titers [33].

Taken together, these studies strongly indicate that EBV seropositivity is a possible predisposing factor for the development of MS; however, they do not establish a direct causative relation to MS, since the observed results might be part of a more general immune dysregulation as a consequence of MS. In addition, this is not exclusive to MS, as EBV seropositivity is also associated with other autoimmune diseases like systemic lupus erythematosus [6], which might indicate that EBV plays a role in autoimmunity in general.

4. EBV genotypes

Only two studies have investigated whether the strains differ in MS patients and healthy individuals infected with EBV. Both studies analysed the genotype of only a single viral gene. One study showed similar EBNA-6 genotypes in eight MS patients from a small community in Denmark and concluded that they had been infected with the same strain [36]. Another study, however, found different sequences of latent membrane protein-1 (LMP-1) in eleven MS patients and eleven controls [37]. It is unlikely that the same strain of EBV will be found in most MS patients. Future studies should focus on changes in sequence of genes under selection pressures, e.g. affecting immune viral evasion strategies, which may have a role in the pathogenesis of diseases associated with EBV.

5. Humoral and cellular immunity to EBV

Altered cellular immunity to EBV in MS patients may reflect either an altered immune response to infection, or it may be a direct response to EBV infection facilitating an aberrant autoimmune process in an individual. EBV has developed elegant strategies that enable immune-evasion allowing it to persist within the host as a latent viral infection.

5.1. B cells

The main viral envelope glycoprotein gp350/220 binds CD21 (complement receptor 2) on resting mature B cells and initiates EBV infection [38]. EBV entry into other cell-types, such as epithelial cells, depends on gB/gH/gL/gp42 combinations [39]. Nevertheless, the presence of gp42 appears to be essential for the binding and fusion of EBV with MHC class II expressing cells. EBV expresses two proteins, LMP1 and LMP2A that mimic the activated CD40 receptor and antigen-activated B cell receptor (BCR), respectively, on B cells allowing the activation, proliferation and maturation of those B cells into persistent EBV memory B cells independently of T cells [5].

Dendritic cells (DCs) are pivotal in eliciting primary antiviral T cell responses. DCs are responsible for presenting EBV antigens from necrotic or apoptotic B cells to T cells [40]. However, EBV has developed mechanisms to interfere with antigen presentation, e.g. virions avoid entrance into monocytes or EBV may interfere with the development of monocytes into DCs [41,42].

While in the latent phase, EBV maintains an episomal form, expressing a reduced number of genes and down-regulating the surface expression of MHC class II on B cells. EBV expression of gp42 is able to both mediate and interfere with MHC class II dependent T cell activation. These features allow the virus to evade immune recognition and hence prevent viral clearance. Latent EBV infection of B cells is associated with expression of approximately 11 genes during cell proliferation. In order to facilitate onward transmission of EBV to other cells, the virus is able to transiently switch to the lytic phase, during which approximately 80 genes are expressed. Memory EBV-specific T cells are produced following primary infection with EBV and maintain immunosurveillance of infected B cells, but they are unable to eliminate all cells latently infected with EBV. Cytotoxic

CD8+ T cells are pivotal at eliminating B cells lytically infected with EBV [43,44].

The percentage of EBV+ B cells in peripheral blood of healthy individuals is between 1 and 10 latently infected B cells within 10^6 peripheral blood mononuclear cells (PBMCs) [45]. It is not yet known if these frequencies are similar in MS patients, which would give us an indication as to whether EBV-specific T cell responses are altered in MS patients.

5.2. EBV-specific CD8+ T cells

Viral infections are controlled by circulating cytotoxic CD8+ T cells. Viral proteins undergo proteosomal degradation within infected cells, and the resulting peptides are uploaded onto MHC class I molecules in the endosomal reticulum and are presented on the cell surface to CD8+ cytotoxic T cells. CD8+ T cell responses can be measured by frequencies, IFNγ production and cytotoxic assays. Several studies have investigated the CD8+ T cell responses against EBV in MS patients, with sometimes different results.

An increased frequency of CD8+ T cells reactive to two out of six different EBV peptides was found in MS patients, when response to five HLA-A2 and one HLA-B7 EBV restricted peptides was analyzed in a group of 33 MS patients and 33 healthy controls (HC) [46]. In a study employing seven HLA-B7 restricted EBV peptides no such difference was seen between 73 MS patients and 32 HC [47]. When 18 HLA class I restricted EBV peptides were used, increased CD8+ T cell responses were detected in 35 people with clinically isolated syndrome (CIS) but similar frequencies were found in 73 MS patients and 21 HC [48]. The most recent study described an increased frequency of EBNA1 specific IFN-γ-producing T cells in 28 patients with CIS compared with 30 HC, although the frequency of T cells specific for other EBV-derived immunodominant CD8+ T-cell epitopes did not differ between CIS patients and controls [31].

On the other hand, there is another interesting study using EBV infected B cell lymphoblastoid cell lines (LCL), which express both latent and lytic phase proteins and may represent a more physiological representation of the *in vivo* situation. They report a decreased CD8+ T cell immunity to EBV in 34 MS patients compared to 34 HC [49] with lower mean frequency of PBMCs producing IFN-γ in response to autologous LCL in 34 MS patients when compared to 34 EBV-positive healthy people as measured by ELISPOT. However, similar values for both populations were detected for control antigens including concavalin A and tetanus toxoid. The authors hypothesize that there may be impaired priming by DCs or a genetically determined deficiency in the generation of EBV CD8+ cells that lead to higher numbers of EBV infected B cells in MS patients [49].

EBNA1 is considered an immunogenic protein, but it has developed strategies to avoid immune recognition. There is a long glycine–alanine repeat sequence that hinders proteosomal degradation, limiting its presentation to CD8+ T cells. When this repeat sequence is deleted, immune recognition and elimination of infected B cells proceed normally [50]. Furthermore, the level of EBNA1 expression within infected B cells is enough to maintain the viral episome, but is kept at low levels, thus impairing vigorous immune responses.

5.3. EBV-specific CD4+ T cells

CD4+ T helper cells recognise specific peptides presented by MHC class II molecules on antigen presenting cells. They have multiple roles including the maintenance of CD8+ T cell function and crosstalk with B cells allowing them to be induced to transform into immunoglobulin producing cells.

A study of 21 MS patients and 20 healthy controls showed that MS patients had increased CD4+ T cells responses to an extensive EBNA1 repertoire of peptides, covering the entire C-terminal region [51]. The great majority of these EBV peptide specific CD4+ T cells had a memory phenotype (CD45RA-RO+). More recently, a cohort of CIS patients was shown to have 1.83-fold higher frequency of CD4+ T cells responses (as elicited by a overlapping library of C-terminus EBNA1 peptides) than healthy EBV carriers [31]. Unlike studies where LCLs were used to elicit responses, the frequency of EBV peptide specific CD4+ T cells was much higher than CD8+ T cells. Interestingly, 3–4% of EBNA1 specific CD4+ T cells in both MS patients and controls also reacted with myelin peptides.

BGLF5, a viral DNase and lytic phase protein, is able to not only reduce the surface expression of MHC class I, but also has a similar effect on MHC class II, reducing the presentation and activation of CD4+ T cells. BZLF1, the EBV immediate-early protein, acts through inhibiting IFN-γ signalling, causing reduced expression of MHC class II molecules through downstream interference with IRF7 and p65 of the NF-κB complex [52]. The lysosomal protease system typically degrades exogenous proteins for presentation by MHC class II molecules to CD4+ T cells. Surprisingly, intracellular EBV proteins expressed in B cells can by-pass the exogenous pathway into the MHC class II pathway [53].

Research into EBV-specific T cells responses in MS patients has led to conflicting findings, some of which may have originated as a result of the fact that some studies employ EBV peptides and others EBV infected B cell lines. There is no consensus yet as to whether EBV-specific T cell responses are decreased or increased in MS patients. Opposing results appear to stem mainly from differences in experimental set up. In summary, EBV-specific CD8+ T cell responses are more prominent when EBV infected LCL are used but increased CD4+ T cell responses are observed in experiments using EBV peptides.

6. Neuropathological studies

Neuropathological studies, along with newer, more sensitive molecular techniques, have been essential both to

generate and to confirm or refute the idea of an infection as a cause of MS, although both technical and practical limitations have sometimes led to confusing results. It must be remembered that brain samples are limited, biased towards more advanced or aggressive forms of the disease, and do not allow for longitudinal analyses. Nevertheless, histopathology remains the gold standard for better understanding the pathophysiology of the disease.

Early pathological studies with electron microscopy found tubular paramyxovirus-like intranuclear inclusions in active MS lesions [54], which were later described as parainfluenza virus [55]. Spheroidal particles interpreted as papovavirus were also described, but both types of inclusions were soon found in other conditions [56], and were thus interpreted as cell organelles or protein artifacts [57]. The development of *in situ* hybridisation studies and PCR allowed further investigation of these viruses, failing to demonstrate the consistent presence of measles, other Paramyxovirus or coronavirus [58–60]. More recently, various members of the herpesvirus family were considered as possible candidates involved in MS pathogenesis. Herpes simplex virus, varicella-zoster virus, Epstein-Barr virus, cytomegalovirus, and human herpesvirus-6 have been found in the brains of both MS patients and control patients using both PCR and Southern blot hybridisation [61,62]. The demonstration of different viral infections in MS brains and the intrathecal synthesis of antibodies directed against several viruses led to the hypothesis that they may not be a single specific underlying viral trigger to MS, but that several common viral infections may play a role in the pathological cascade [63,64]. Studying anti-pathogen responses in detail may aid understanding of host–virus interactions in the CNS and help shed light on immunopathological mechanisms.

Recent stimulating and sometimes controversial pathological studies have fuelled the debate about the role of EBV in MS. Early studies failed to show consistent presence of EBV within MS brains: Sanders et al. found EBV in 27% of MS patients and 38% of controls, and in 5% of active plaques and 10% of inactive plaques using PCR [62], while Hilton et al. did not find EBV in a cohort of 10 MS patients including 4 acute, 11 chronic active, 2 chronic inactive and 4 shadow plaques using *in situ* hybridisation for EBER (EBV encoded RNA) [65]. Suboptimal tissue preservation prevented Opsahl and Kennedy from drawing any conclusions about the presence of EBV in MS brains [66].

More recently, Serafini et al. reported the presence of EBV infected B cells and plasma cells in the brains of in 21 of 22 MS cases, and these were not present in other inflammatory neurological diseases (primary cerebral vasculitis, viral encephalitis, mycotic meningitis and encephalopathy of unknown origin) [67]. Eight of the MS cases were known to have rich B cell/plasma cell infiltration and ectopic B cell follicles, while 12 cases were less infiltrated. *In situ* hybridisation for EBER and immunohistochemical techniques for EBV proteins were used. 40–90% of B cells and 50–80% of plasma cells were positive for EBER, with the highest percentage for B cells in ectopic follicles. Of note, the cases with more prominent EBER+ cells accumulation were cases rich in B cells/plasma cell infiltrates and ectopic B cell follicles, while less infiltrated cases had fewer and often isolated EBER+ cells. Viral reactivation, demonstrated by EBNA2 and BFRF1 expression, was restricted to active lesions and ectopic follicles. Cytotoxic CD8+ T cells, key players in the elimination of virally infected cells, were found to accumulate mainly in sites with EBV+ cells.

Three consecutive studies have tried unsuccessfully to reproduce the results [68–70]. Willis et al. examined 63 formalin-fixed, paraffin-embedded tissue specimens from 12 MS patients and from these selected 23 specimens with CD20+ B cell infiltrates [68]. They used a wide range of techniques, including *in situ* hybridisation for EBER, immunohistochemistry for LMP1 and EBNA2 and quantitative real-time PCR to detect genomic EBV and EBER1 RNA in MS lesions [68]. In addition, 12 specimens were examined for B cell infiltration or aggregates within the meninges. B cell meningeal infiltration was found to be either low or absent, but 3 of these cases did have B cell aggregates within the brain parenchyma [68]. EBV was undetectable in these samples by *in situ* hybridisation. Real-time PCR detected low-level EBV infection in only 2 of the cases [68]. Peferoen et al., made similar observations, they undertook a study screening 632 specimens from 94 MS patients, including 11 patients who died before the age of 50, in addition to studying 12 blocks used in the Serafini study [69,67]. Sixteen of the patients had rich B cell infiltrates, although follicle-like structures were not seen in any specimen. EBER was detected only in one tissue specimen by *in situ* hybridisation, which also showed lytic cycle markers. Real-time PCR for EBV genome and encoded RNA were negative in all tissue examined [69]. A more recent study, screened perivascular areas of 12 MS lesions and one case of acute disseminated encephalomyelitis (ADEM) [71]. Active lesions with over-expression of IFN-α had been pre-selected. *In situ* hybridisation showed EBER+ cells in the active MS lesions and also in the case of ADEM [71]. Finally, the most recent study using nested and non-nested real-time PCR to detect cell specific and EBV-specific transcripts in 15 fresh-frozen and 5 formalin-fixed paraffin-embedded MS plaques and in single CSF B-lymphocytes and plasma cells did not reveal any evidence of active EBV infection [70].

Careful analysis of the above studies underlines the difficulties associated with neuropathological work. Further research is urgently needed as we are still trying to optimise molecular techniques to identify persistent viral infections in brain tissue, the detection of which is often hampered by post-mortem delay and tissue fixation. A working consensus on lesion staging may further help interpretation of pathological findings. It would appear that EBV+ cells can be detected in MS brains, mainly within active lesions, but far less frequently than has been reported at times, and that the presence of these EBV+ cells may not be specific to the MS brain.

7. Conclusion

In summary, MS is a complex disease, and while there have been huge advances in the tools used to study this potentially devastating neurological condition, many questions remain. The role of EBV in MS pathogenesis remains unknown at the present time. Future studies will hopefully help us understand the overall clinical impact of infection in affected populations. Since EBV has yet again emerged as a candidate of interest, it may take all our effort to see whether it is deserving of its' place in the limelight.

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