

# Epstein-Barr virus (EBV) reactivation and therapeutic inhibitors

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## ABSTRACT

Epstein-Barr virus (EBV) is a ubiquitous human virus which infects almost all humans during their lifetime and following the acute phase, persists for the remainder of the life of the individual. EBV infects B lymphocytes leading to their immortalisation, with persistence of the EBV genome as an episome. In the latent phase, EBV is prevented from reactivating through efficient cytotoxic cellular immunity. EBV reactivates (lytic phase) under conditions of psychological stress with consequent weakening of cellular immunity, and EBV reactivation has been shown to occur in a subset of individuals with each of a variety of cancers, autoimmune diseases, the autoimmune-like disease, chronic fatigue syndrome/myalgic encephalitis and under other circumstances such as being an inpatient in an intensive care unit. Chronic EBV reactivation is an important mechanism in the pathogenesis of many such diseases, yet is rarely tested for in immunocompetent individuals. This review summarises the pathogenesis of EBV infection, EBV reactivation and its role in disease, and methods which may be used to detect it. Known inhibitors of EBV reactivation and replication are discussed, including drugs licensed for treatment of other herpesviruses, licensed or experimental drugs for various other indications, compounds at an early stage of drug development and nutritional constituents such as vitamins and dietary supplements.

## INTRODUCTION

Epstein-Barr virus (EBV) is a ubiquitous human virus which infects almost all humans during their lifetime and persists for the remainder of the life of the individual. EBV reactivates under psychological stress, and EBV reactivation has been shown to occur in a subset of individuals with each of a variety of autoimmune diseases and cancers. It is recognised that chronic EBV reactivation is an important mechanism in the pathogenesis of these diseases. Yet EBV reactivation is rarely suspected in clinical practice, is rarely tested for in immunocompetent individuals and, even if identified, there are no licensed treatments. In this review, the importance of EBV reactivation will be considered in the pathogenesis of disease in general, along with diagnostic approaches and therapeutic inhibitors, including drugs, vitamins and supplements.

## EBV infection

EBV is a hugely successful virus which maintains a global infection rate in humans of 95% and persists lifelong in individuals following the acute phase of infection.<sup>1</sup> In immunocompetent individuals, this

persistence is not associated with clinical symptoms. Primary EBV infection is usually asymptomatic and for many occurs during childhood, but when it occurs in adolescence or adulthood, 30%–50% cases manifest clinically as infectious mononucleosis (IM).<sup>2</sup> Primary and secondary immunodeficiency facilitates virus reactivation, unchecked proliferation of EBV-infected B lymphocytes and eventual development of EBV+ B lymphoproliferative disease.<sup>1 3–6</sup> EBV infection of T cells/natural killer (NK) cells may result in haemophagocytic lymphohistiocytosis,<sup>7</sup> chronic active EBV infection<sup>8</sup> and T-cell/NK-cell lymphomas,<sup>9</sup> which tend to be aggressive in presentation. EBV also causes Burkitt lymphoma (BL), nasopharyngeal carcinoma (NPC), gastric adenocarcinoma, AIDS-associated lymphoblastic and primary central nervous system lymphoma, post-transplant lymphoproliferative disease (PTLD), nasal T-cell/NK lymphoma, Hodgkin's disease, lymphoepithelioma-like carcinoma and leiomyosarcoma.<sup>2 10 11</sup> Altered immune responses to EBV have been documented in a wide variety of autoimmune diseases, including multiple sclerosis (MS), Sjogren's syndrome (SS), systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), inflammatory bowel disease (IBD) and type 1 diabetes mellitus (T1DM). EBV infection has been associated with a higher risk of developing autoimmune disease, particularly MS, dermatomyositis, SLE, RA and SS.<sup>12</sup>

EBV is a gamma-herpesvirus, containing double-stranded linear DNA of 170–175 kb, which is transmitted mainly by salivary transfer of EBV-infected B cells, but also by aerosol.<sup>2 10</sup> Although it is generally accepted that early subclinical infection is predominant in developing countries, 80% Japanese children have acquired the virus by the age of 3.<sup>1</sup> In the oropharynx, the virus infects B cells via the C3d complement receptor, CD21.<sup>10</sup> It has been shown that the epithelial lining of the oropharynx is discontinuous allowing the virus direct access to the underlying B cells of the tonsils. EBV infection of B lymphocytes in vitro leads to the production of immortalised lymphoblastoid cell lines exhibiting restricted cellular and EBV gene expression.<sup>13</sup> After the initial replicative (lytic) phase of infection, the EBV genome circularises to be maintained as a multicopy plasmid in the B cell nucleus. In almost all EBV-infected B cells, the virus infection exists in a latent state, with the capacity for cellular immortalisation. In vivo, latent EBV+ B cells include immunoblastic B cells, memory B cells and resting non-immunogenic B cells. Immunoblastic B cells are highly immunogenic and are rapidly removed during IM.



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Resting non-immunogenic B cells are the latent virus reservoir in the circulation of healthy carriers.<sup>1</sup>

IM is the primary virus infection which occurs in EBV seronegative persons; 80% or more cases of acute IM are due to primary EBV infection. IM symptoms include fever, tender lymphadenopathy, sore throat, hepatosplenomegaly and skin rash. Symptoms characteristic of EBV-associated IM, as opposed to IM due to infection with cytomegalovirus (CMV) or varicella-zoster virus (VZV), are puffy eyelids and tonsillar exudates.<sup>1</sup> Young children more frequently have skin rash and abdominal pain. By the end of the incubation period (2 to 7 weeks post-transmission), EBV has spread to infect approximately 20% B cells in infected adolescents and young adults with acute IM.<sup>2</sup> The proliferation of EBV-infected B lymphocytes is rapidly inhibited during the first 2 weeks by a strong cellular immune response. This was previously believed to be composed of only NK cells, interferon- $\gamma$  (IFN- $\gamma$ )-activated CD8+ T cells and antibody-dependent cellular cytotoxicity.<sup>14</sup> Activated CD8+ T cells (morphologically seen as atypical lymphocytes) may reach 60% peripheral blood mononuclear cells (PBMCs) during the symptomatic phase of acute IM.<sup>15</sup> T lymphocytes specific for EBV lytic phase epitopes have been shown to account for up to 44% of total CD8+ T lymphocytes, in comparison with 1%–2% accounted for by CD8+ T lymphocytes specific for the immunodominant EBV proteins, Epstein-Barr nuclear antigens EBNA3, EBNA4 and EBNA6.<sup>16 17</sup>

More recently, the importance of polyfunctional T cells (PFCs) in control of EBV has been recognised. EBV-specific PFC in long-term carriers produce more cytokines per cell than the single functional T cells and may be functionally superior.<sup>18 19</sup> It has been shown that CD4+ and CD8+ PFC responses occur against immunodominant latent and lytic EBV epitopes during primary EBV infection in children.<sup>20</sup> PFCs have multiple functions, such as simultaneous production of multiple cytokines, for example, interleukin 2, IFN- $\gamma$  and tumour necrosis factor  $\alpha$ , and degranulation of cytotoxic proteins. PFC appear to be associated with more effective control of chronic microbial infections including HIV, hepatitis C virus (HCV) and CMV.<sup>21–25</sup> Frequencies of occurrence of PFC were higher in HIV non-progressors than in progressors.<sup>26</sup> It has recently been shown that polyfunctional and IFN- $\gamma$  monofunctional CD4+ T cells are molecularly distinct and the polyfunctional gene signatures in response to infection with *Plasmodium falciparum* and influenza virus are highly conserved.<sup>27</sup> Therefore, PFC appear to contribute to more robust T-cell immunity in control of virus infections. But how they arise and evolve during primary EBV infection and their role in long-term control of EBV remains unknown.

During the symptomatic phase,  $10^{3-4}$  copies of EBV DNA can be detected in PBMC or serum.<sup>28 29</sup> Occurrence and progression of EBV-specific antibodies are used for the diagnosis of acute EBV infection. The cellular immune response reduces the number of circulating EBV-infected B cells to 1 in  $10^6$  B cells within 4–6 weeks.<sup>1</sup>

## PATHOGENESIS OF EBV INFECTION

### Latent phase of infection

EBV infection of B lymphocytes may exhibit a latent or lytic phase of infection. In the latent phase of infection, the EBV genomic DNA exists as a closed circular plasmid and behaves like host chromosomal DNA. EBV latency in B cells is associated with the expression of at most 12 latent genes. EBV infects naive B lymphocytes and stimulates differentiation to memory B cells, which are the reservoir of EBV. Coordinated

EBV protein expression facilitates this process, especially latent membrane proteins (LMP1, LMP2A, LMP2B) and EBV nuclear antigens (EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C). Three different latency patterns are recognised depending on the pattern of protein expression, each of which is associated with a different stage of B-cell infection and with particular lymphoproliferative disorders. Latency III is the most elaborate viral expression pattern (EBER1, EBER2, EBNA1–6, LMP1, LMP2A, LMP2B) and is associated with EBV+ post-transplant diffuse large B-cell lymphoma (PT-DLBCL). Latency II is more restricted in its protein expression (EBER1, EBER2, EBNA1, LMP1, LMP2A) and is associated with PT-DLBCL and Hodgkin lymphoma. Latency I is the most restrictive latency programme (expression of only EBER1, EBER2, EBNA1) and is associated with post-transplant BL.<sup>30–32</sup>

### Lytic phase of infection

During lytic infection (EBV replication), on the other hand, the EBV genome is amplified up to 1000-fold by the viral replication machinery. The lytic phase is also associated with the expression of nearly 100 EBV genes. The lytic programme arrests cell cycle progression and favours the S-phase which provides the cellular machinery necessary for viral replication.<sup>33</sup> The expression of the immediate early proteins, Zta and Rta (encoded by BZLF1 and BRLF1, respectively), initiate the EBV lytic phase of infection.<sup>34–36</sup> Zta and Rta activate the expression of one another and trigger the expression of a panel of early lytic proteins (BMRF1, BALF1, BHRF1 and others). Thus, viral DNA replication and later, expression of late lytic proteins are initiated by the immediate early and early EBV lytic proteins.<sup>37</sup> In the complete lytic cycle, viral DNA is replicated as large, complete molecules which are later cleaved and packaged into viral progeny which are released to infect neighbouring cells.<sup>33</sup>

EBV reactivation has been shown to occur following impairment of the cellular immune response caused by psychological stress of various types, including student examination stress,<sup>38 39</sup> marital stress,<sup>40</sup> attachment anxiety or fear of abandonment and rejection,<sup>41</sup> and loneliness.<sup>42</sup> EBV reactivation has also been shown to occur with greater than 5 to 7 days spent as a patient in intensive care,<sup>43 44</sup> which is well recognised to be associated with psychological stress. Physical stress by itself does not have this effect.<sup>45</sup> EBV reactivation is paralleled by changes in a large variety of immune markers of cellular immunity which are important in the long-term control and suppression of replication of persistent and asymptomatic EBV in the normal person.<sup>46 47</sup> EBV reactivation has been shown to occur in a variety of chronic autoimmune diseases, including SLE, SS, RA, MS<sup>48</sup> and chronic fatigue syndrome/myalgic encephalomyelitis (CFS/ME).<sup>49 50</sup>

Chronic uncontrolled EBV reactivation has been shown to be an important factor in the pathogenesis of NPC<sup>51</sup> and PTL. It has been shown that an NPC-associated BZLF1 variant is associated with the enhancement of lytic EBV infection.<sup>53</sup> PTL has also been shown to be associated with detectable circulating EBV genome and ZEBRA, the gene product of BZLF1.<sup>54</sup>

Indirect evidence for EBV reactivation is provided by the upregulation of the human EBV-induced 2 (EBI2) gene, which is the most upregulated human gene in EBV-infected BL cells.<sup>55</sup> EBI2 upregulation has been demonstrated in melanoma metastasis, lymphoblastic leukaemia, glioblastoma, bone cancer metastasis, SLE, chronic rhinosinusitis with nasal polyps, T1DM<sup>56</sup> and CFS/ME.<sup>57</sup> EBI2 is a human G-protein coupled receptor (GPCR) which is activated by oxysterols and pertussis toxin-sensitive heterotrimeric G proteins, resulting in decreased cyclic

AMP, mobilisation of calcium and activation of the extracellular signal-related kinase (ERK) pathway.<sup>56–58</sup> The oxysterol, 7 $\alpha$ ,25-dihydroxycholesterol (7 $\alpha$ 25HC) is a high-affinity EB12 agonist.<sup>59–60</sup> Aberrant oxysterol signalling has been demonstrated in MS, experimental allergic encephalomyelitis Alzheimer's disease, Parkinson's disease, motor neuron disease, cerebrotendinous xanthomatosis, hereditary spastic paraplegia type 5, Huntington disease, age-related macular degeneration, atherosclerosis, IBD and osteoporosis.<sup>56</sup>

EBV reactivation may be identified in individuals by detection of IgA antibody to EBV early antigen (EA), neutralising IgG to EBV DNA polymerase and EBV dUTPase,<sup>49–61</sup> IFN- $\gamma$  release from T lymphocytes (ELISpot assay) against EBV latent and lytic viral proteins, circulating EBV genome, and EB12 gene and protein expression.<sup>56</sup>

### Oral hairy leucoplakia (OHL) as a model of EBV reactivation

Although circulating resting memory B lymphocytes are believed to be the reservoir of latent EBV,<sup>62–63</sup> EBV can replicate in human oral epithelial cells<sup>64–66</sup> and EBV may be detected in oral tissue as a latent infection.<sup>67–68</sup> OHL is a benign oral epithelial disease presenting as white patches which is associated with active EBV infection of oral epithelial cells which is frequently seen in HIV infection, IM and with psychological stress.<sup>69</sup> Treatment of this condition with drugs that inhibit EBV replication leads to resolution without eradication of latent infection.<sup>67</sup> Therefore, OHL has been used as a model for EBV reactivation, factors involved and potential therapeutics.

### Therapeutic oncolytic therapy for EBV-associated neoplasia

While EBV reactivation is an important causative mechanism of disease for many patients, it also represents a potentially effective therapeutic intervention for others. Deliberate reactivation of the EBV lytic cycle is a therapeutic strategy that exploits the presence of EBV genome in cancer cells. Induction of EBV lytic cycle can directly induce apoptotic cell death in EBV-infected cell lines.<sup>70–73</sup> And it has been demonstrated that EBV reactivation using tetradecanoyl phorbol acetate results in chromosomal DNA fragmentation in Raji BL cells.<sup>70</sup> Such oncolytic therapy has been used to sensitise EBV+ cancer cells to anti-EBV drugs and is a potential therapeutic strategy.

Although no drug has been licensed for the treatment of EBV infection, there are a variety of antiviral and other drugs, as well as vitamins and plant extracts which effectively inhibit EBV replication (table 1 and figure 1). In each case, the anti-EBV activity is directed toward the prevention of replication, and none has any effect on the latent phase of infection.

### Anti-herpesvirus drugs

Aciclovir is a nucleoside analogue approved for the treatment of herpes simplex virus (HSV) and VZV infections. The antiviral effect of aciclovir is mediated by the interaction of aciclovir triphosphate with herpesvirus DNA polymerase with much higher affinity than for cellular polymerases. Aciclovir triphosphate is incorporated into the viral DNA where it irreversibly stops chain elongation. Anti-EBV activity of acyclovir is significantly less than its activity against HSV and VZV. Aciclovir is not clinically useful in EBV-associated IM, but it has been shown to reduce EBV shedding.<sup>74</sup>

Ganciclovir (dihydroxypropoxymethyl guanine) exhibits greater anti-EBV activity than aciclovir, but it is much more toxic which makes its use in otherwise normal persons more difficult to justify.<sup>74</sup> Valganciclovir is the L-valyl ester of ganciclovir which

is metabolised to ganciclovir by the intestine and liver following oral administration. Valganciclovir is licensed for the treatment of CMV disease and has been shown to significantly reduce the level and duration of EBV shedding in IM in a randomised, double-blind, placebo-controlled study.<sup>75</sup>

Omaciclovir (H2G) is a carbocyclic analogue of aciclovir which is active against several herpesviruses and has good anti-EBV activity.<sup>76</sup> Valomaciclovir is an L-valine ester of H2G, with higher oral bioavailability. Valomaciclovir was the subject of a clinical trial in IM and was shown to mediate faster clinical improvement than placebo recipients, although the effect was not statistically significant. Valomaciclovir significantly decreased EBV load in the mouth, compared with placebo (ClinicalTrials.gov trial: NCT00575185).

Maribavir (MBV) is an investigational oral benzimidazole L-ribose with significant activity against CMV and EBV, mediated by an effect on the protein kinases. MBV inhibits EBV through a unique dual effect of inhibition of viral DNA replication and viral transcription.<sup>77</sup>

Cidofovir ((S)-1-(3-hydroxy-2-phosphonylmethoxypropyl) cytosine) is a nucleoside analogue which has been used for the treatment of human papillomavirus-associated lesions.<sup>78</sup> Cidofovir has been shown to have an antiproliferative effect although the mechanism of this is not understood. Cidofovir has also been shown to inhibit the growth of EBV+ NPC xenografts in nude mice.<sup>79–80</sup>

### Other drugs with anti-EBV activity

Cimetidine was the first H<sub>2</sub> antagonist and has multiple anti-cancer activities including antiproliferative activity on cancer cells, immunomodulatory effects, effects on cell adhesion and antiangiogenic activity. Histamine is associated with an immunosuppressive tumour microenvironment through an increase in CD4+CD25+ regulatory T cell activity, reduced antigen-presenting activity of dendritic cells (DCs), reduced NK cell activity and increased myeloid-derived suppressor cell activity.<sup>81–82</sup> Cimetidine therapy has been reported anecdotally to benefit patients with chronic EBV reactivation,<sup>83–84</sup> although this has not been studied formally in clinical trials. Possible mechanisms include inhibition of the T helper suppressor cell, resulting in potentiation of cytotoxicity of CD8+ T cells, and antiproliferative activity.<sup>81–82–85</sup>

Antiretroviral drugs have been reported, in several reports, to induce prolonged remission in MS.<sup>86–88</sup> These patients were taking zidovudine and lamivudine,<sup>86</sup> efavirenz/emtricitabine/tenofovir-disoproxil fumarate (ATRIPLA)<sup>87</sup> and emtricitabine/tenofovir and nelfinavir.<sup>88</sup> 3'-Azido-3'-deoxythymidine (Zidovudine) was shown to inhibit EBV replication in vitro in P3HR-1 cells.<sup>89</sup> L(-)FMAU (Clevudine), L(-)I-OddC and Br(-)Br-OddU have also been shown to exhibit anti-EBV replication activity.<sup>90</sup>

Valpromide is an amide derivative of valproic acid, which unlike valproic acid, inhibits the expression of BRLF1 and BZLF1 and is not a histone deacetylase (HDAC) inhibitor and so does not induce the expression of cellular genes.<sup>91</sup> Valpromide did not activate, and even decreased, the expression of cellular immediate-early genes, FOS and EGR1, which during EBV infection occurs upstream of the EBV lytic cycle. Valpromide does not alter the expression of several other cellular immediate-early genes which are induced by HDAC inhibitors in cells refractory to EBV lytic induction. Therefore, valpromide inhibits both viral and cellular genes involved in EBV lytic infection.<sup>91</sup>

Bromodomain and extraterminal family members block two different steps in the sequential cascade of the lytic EBV cycle.

**Table 1** Therapeutic inhibitors of EBV reactivation and replication

Inhibitor of EBV reactivation	Mechanism of action	References
<b>Anti-herpesvirus drugs</b>		
Aciclovir	Aciclovir triphosphate is a specific inhibitor of herpesvirus DNA polymerase leading to obligate chain termination.	74
Ganciclovir/valganciclovir	Preferential inhibitor of herpesvirus DNA polymerase and competitive inhibitor of dGTP incorporation into DNA.	75
Omaciclovir (H2G)/valomaciclovir	H2G triphosphate is a specific inhibitor of herpesvirus DNA polymerase leading to limited chain elongation.	76
Maribavir	Oral benzimidazole L-riboside which inhibits HCMV and EBV protein kinases.	77
Cidofovir	Cidofovir diphosphate selectively inhibits viral DNA polymerase.	78–80
<b>Other drugs</b>		
Cimetidine	H <sub>2</sub> antagonist, inhibition of T suppressor cells, cellular proliferation, adhesion and angiogenesis.	81–85
Zidovudine	Thymidine analogue which selectively inhibits HIV reverse transcriptase. Anti-EBV effect not proven nor elucidated.	86–88 90
Clevudine	Clevudine triphosphate inhibits multiple steps in the intracellular life cycle of hepatitis B virus. Anti-EBV effect not proven nor elucidated.	86–89
Valpromide	Prevention of expression of immediate-early EBV genes, BZLF1 and BRLF1.	91
JQ1, and bromodomain and extraterminal	JQ1 inhibits the growth of EBV+ nasopharyngeal cancer cells; proapoptotic, antiproliferative and enhancement of radiological sensitivity.	92 93
Artesunate	Inhibition of immediate-early EBV protein synthesis.	94 95
H31 sequence-specific inhibitor	Inhibition of EBNA1-dependent OriP sequence-specific DNA-binding activity.	96 97
EBI2 inhibitor, GSK682753A	Inhibition of oxysterol-induced EBI2 activation, $\beta$ -arrestin recruitment and chemotaxis in B lymphocytes.	98 99
EBI2 inhibitor, NIBR189	EBI2 inhibition; blocks migration in U937 monocytes.	100
<b>Vitamins</b>		
Vitamin C	Inhibition of EBV activation in human lymphoblastoid cells. Killing of EBV+ Burkitt lymphoma cells and EBV-transformed cells in vitro.	101–103
Vitamin D	Direct inhibition of enveloped viruses. Upregulation of antimicrobial peptides LL-37 and human $\beta$ -defensin. LL-37 may disrupt viral envelope.	104–109
Retinoic acid	Negative regulator of EBV BZLF1 and thus inhibits EBV lytic cycle. Irreversible inhibition of EBV-transformed B lymphocytes.	110–113
<b>Dietary constituents and supplements</b>		
Resveratrol	Inhibition of EBV lytic cycle through effects on multiple molecular targets.	114–116
Luteolin	Inhibition of promoter activity of EBV immediate-early genes, BRLF1 and BZLF1. Reduces genomic instability and suppresses tumourigenicity of EBV.	117–124
Apigenin	Inhibition of EBV BRLF1 and BZLF1 activity.	125
Astragalus extract	Inhibition of expression of BZLF1, BRLF1 and EA-D during EBV lytic cycle.	126
Epigallocatechin-3-gallate	Downregulation of LMP1. Inhibition of EBV-induced B-lymphocyte transformation via suppression of RelA acetylation.	127–131
Delta-9-tetrahydrocannabinol (THC)	THC inhibits replication of $\gamma$ -herpesviruses. Mechanism not understood.	132
L-arginine	Suppression of EBV replication through enhancement of iNOS and nitric oxide.	133
Sulforaphane	Inhibition of transactivation of Rta, but not Zta.	134
Curcumin	Enhanced apoptosis-mediated inhibition of proliferation of EBV-transformed lymphoblastoid cell line. Inhibition of BZLF1 transcription.	135–137
Baicalein	Inhibition of EBV+ NPC through repression of activity of EBNA1 Q-promoter.	138
(+)-Rutamarin	Cellular topoisomerase II catalytic inhibitor.	139 140

EAD, early antigen D; EBI2, EBV-induced 2 gene; EBNA1, EBV nuclear antigen 1; EBV, Epstein-Barr virus; HCMV, human cytomegalovirus; NPC, nasopharyngeal carcinoma; iNOS, inducible nitric oxide synthase.

First, they prevent the expression of the EBV immediate-early gene, BZLF1. JQ1 reduces transcription of BZLF1 through an effect on genes controlled by host protein BACH1, and BACH1 knockdown reduces BZLF1 expression. JQ1 also localises to the lytic origin of replication preventing late gene expression. JQ1 reduces BRD4 recruitment during reactivation to prevent replication initiation.<sup>92</sup> JQ1 preferentially represses EBV+ NPC cells partially through repressing c-Myc and is therefore a promising therapeutic candidate for advanced NPC.<sup>93</sup>

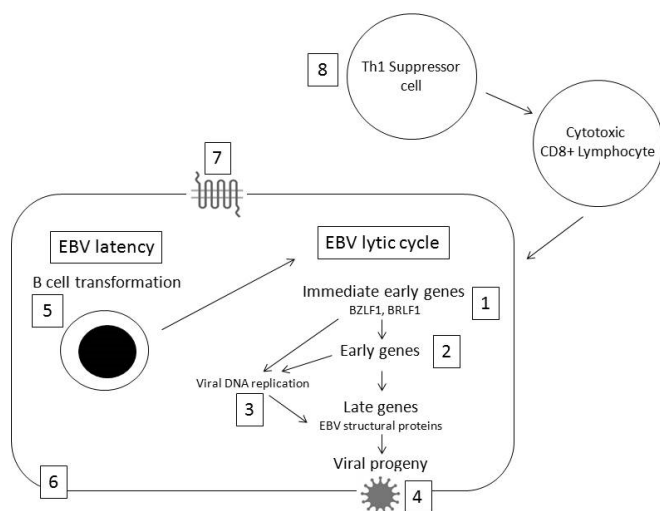
Artesunate, best known as an antimalarial drug, is known to inhibit herpesvirus replication.<sup>94</sup> Artesunate has been shown to inhibit EBV replication in both epithelial cells and lymphocytes. The mode of action is through a block on viral immediate early protein synthesis.<sup>95</sup>

H31 is a novel small molecule inhibitor of sequence-specific DNA binding of EBNA1 to the EBV OriP element.<sup>96</sup> The EBV OriP is a 1.7 kb region of the EBV chromosome that supports the

replication and stable maintenance of plasmids in human cells.<sup>97</sup> Replication and persistence of extrachromosomal EBV episome in latently infected cells is dependent on the binding of EBV-encoded nuclear antigen 1 (EBNA1) to the EBV oriP element. H31 repressed the EBNA1-dependent transcription, replication and persistence of an EBV oriP plasmid. H31 induced progressive loss of EBV episome. In addition, H31 selectively retarded the growth of EBV-infected lymphoblastoid cell lines or BL cells. H31 inhibition of EBNA1-dependent DNA binding decreases transcription from and persistence of EBV episome in EBV-infected cells.<sup>96</sup>

The human GPCR, *EBI2*, is a key receptor in B cells, T cells and DCs, modulating the T and B cell response to bloodborne antigens.<sup>98</sup> *EBI2* is highly upregulated in response to EBV infection,<sup>55</sup> T1DM, RA, SLE, MS, CFS/ME and several cancers.<sup>56 57</sup> There are two *EBI2* modulators in development. GSK682753A is a small molecule, potent *EBI2* antagonist which blocks 7 $\alpha$ 25HC





**Figure 1** Schematic diagram showing Epstein-Barr virus (EBV) latency with B-cell transformation, and the steps comprising the EBV lytic cycle (immediate-early proteins, early proteins, viral genome replication, late (structural) proteins and virion production). The role of cytotoxic CD8+ lymphocytes is also shown responding to the infection, under the influence of Th1 suppressor cells. Numbers 1–8 have been added to the diagram to indicate different mechanisms of action of anti-EBV drugs, vitamins and nutritional supplements. Number 1, inhibition of transcription or function of the immediate-early genes, BZLF1 and BRLF1, which represent the first step in the EBV lytic cycle (valpromide, artesunate, vitamin C, retinoic acid, luteolin, apigenin, astragalus extract, sulforaphane, curcumin, resveratrol). Number 2, inhibition of the function of Epstein-Barr nuclear antigen 1 (H31, baicalein). Number 3, inhibition of viral DNA replication through either inhibition of cellular topoisomerases I and II which are required for EBV genome replication ((+)-rutamarin) or inhibition of viral DNA polymerase (aciclovir, ganciclovir, omaciclovir, cidofovir). Number 4, direct inhibition of the EBV protein kinases (maribavir) or envelope (vitamin D). Number 5, inhibition of B-cell transformation (epigallocatechin-3-gallate). Number 6, a cellular antiproliferative effect (retinoic acid, JQ1, cimetidine). Number 7, inhibition of the function of the human G-protein coupled receptor, EBV-induced gene 2 (GSK682753A, NIBR189). Number 8, inhibition of Th1 suppressor cells, resulting in enhanced CD8+ cell cytotoxicity (cimetidine).

stimulation of the *EBI2* receptor in a recombinant system.<sup>99</sup> NIBR189 is a potent selective antagonist of *EBI2*, being developed in particular for cardiovascular disease.<sup>100</sup>

### Vitamins with anti-EBV activity

Higher plasma levels of vitamin C have been correlated with lower levels of EBV viral capsid antigen IgM and EA IgG in patients with acute and prolonged symptomatic EBV infection.<sup>101</sup> Vitamin C has been shown to kill EBV+ BL cells and EBV-transformed B cells in vitro.<sup>102</sup> Vitamin C was found to abrogate EBV activation in human lymphoblastoid cells.<sup>103</sup>

Vitamin D deficiency occurs with apparent increased frequency in acute IM.<sup>104</sup> An inverse correlation was reported between vitamin D status and EBV load but not EBNA1 antibody level in relapsing, remitting MS (RR-MS).<sup>105</sup> High-dose oral vitamin D<sub>3</sub> supplementation was shown to lower anti-EBNA1 antibody level in patients with RR-MS.<sup>106</sup> Vitamin D levels which reach a nadir during late winter and early spring are correlated with increased disease activity, clinical severity as well as relapse rates in several autoimmune diseases including MS, non-cutaneous

flares of SLE, psoriasis and RA.<sup>107</sup> The seasonality of infectious disease in general is markedly influenced by sunlight and vitamin D level, which explain the winter peaks of incidence of symptomatic infection with various respiratory viruses.<sup>108 109</sup>

Retinoic acid is a metabolite of vitamin A<sub>1</sub> (all-*trans*-retinol) which mediates the functions of vitamin A<sub>1</sub> that are required for growth and development and is required in all higher animals. All-*trans*-retinoic acid binds the retinoic acid receptor (RAR) which modifies transcription of different sets of genes depending on cell type. Retinoic acid is a negative regulator of EBV BZLF1 and thus inhibits EBV reactivation.<sup>110</sup> Retinoids have also been shown to irreversibly inhibit in vitro growth of EBV-transformed B lymphocytes,<sup>111</sup> through upregulation of the cyclin-dependent kinase inhibitor, p27Kip1.<sup>112</sup> This antiproliferative effect has also been demonstrated in EBV-transformed B cells with an activated c-Myc oncogene.<sup>113</sup>

### Nutritional supplements with anti-EBV activity

Resveratrol (3,5,4'-trihydroxy-trans-stilbene) is a type of natural phenol called a stilbenoid, and a phytoalexin produced by plants as a response to injury or infection. Sources of resveratrol in food include the skin of grapes, blueberries, raspberries, mulberries and peanuts. Resveratrol prevents EBV transformation and inhibits the outgrowth of EBV-transformed B lymphocytes<sup>114</sup> and EBV-infected BL cells.<sup>115</sup> Resveratrol also inhibits EBV lytic cycle in BL cells through effects on multiple molecular targets.<sup>116</sup>

Luteolin is a flavone with a flavonoid 2-phenylchromen-4-one ring structure and has a yellow crystalline appearance. Luteolin has also been shown to significantly inhibit EBV reactivation by suppressing promoter activities of two immediate early genes, BRLF1 and BZLF1.<sup>117</sup> It also reduces genomic instability and suppresses tumourigenic features induced by repeated EBV reactivation, suggesting that inhibition of EBV reactivation is a novel target to prevent NPC relapse.<sup>118</sup> Luteolin is an effective free radical scavenger and inducer of tumour apoptosis<sup>119</sup> and has been shown to have valuable anticancer effects.<sup>120</sup> It is antiangiogenic, antimetastatic, anti-inflammatory and antioestrogenic, and regulates many signalling pathways.<sup>121 122</sup> Luteolin has been shown to have profound antiviral properties.<sup>123 124</sup> Natural sources include celery, broccoli, green pepper, parsley, thyme, dandelion, perilla, chamomile, carrots, olive oil, peppermint, rosemary, navel oranges and oregano.

Apigenin (4',5,7-trihydroxyflavone) is a natural product of many plants. It has a yellow crystalline appearance and has been used to dye wool. Apigenin inhibits EBV reactivation through suppression of the activities of two immediate early EBV genes, BRLF1 and BZLF1.<sup>125</sup> It is also a potent inhibitor of the enzyme CYP2C9, which metabolises many drugs in the body. It also activates monoamine transporters, is a weak anxiolytic and sedative, is a non-selective antagonist of all three opioid receptors, and may have an important pharmacological effect on the endocannabinoid system.<sup>125</sup> Potential health benefits are stimulation of generation of neuronal cells, prevention of amyloid- $\beta$  deposition and anticancer activity, related to its ability to suppress EBV reactivation.

Polysaccharide extract of the Chinese herb, *Astragalus membranaceus*, was shown to inhibit EBV reactivation in EBV-infected Raji cells in vitro. Astragalus polysaccharide extract in a non-cytotoxic concentration of 30  $\mu$ g/mL significantly suppressed the expression of BZLF1, BRLF1 and EA-D during the EBV lytic cycle and is potentially useful as an anti-EBV drug.<sup>126</sup>

Epigallocatechin-3-gallate (EGCG) is one of the green tea polyphenols and has been shown to inhibit EBV replication<sup>127</sup> through ERK1/2 and PI3K/Akt signalling in EBV+ cells,<sup>128</sup> also

involving downregulation of LMP1.<sup>129</sup> EGCG also inhibits EBV LMP1-induced activation of nuclear factor- $\kappa$ B signal transduction pathways.<sup>130</sup> EGCG has been shown to be a histone acetyltransferase inhibitor and inhibits EBV-induced B-cell transformation via suppression of RelA acetylation.<sup>131</sup>

Delta-9-tetrahydrocannabinol ( $\Delta$ -9-THC) was shown to specifically target viral and/or cellular mechanisms required for replication of CMV and EBV, suggesting that the endocannabinoid system is possibly involved in regulating gamma herpesvirus latency and lytic replication. The immediate early gene ORF50 promoter activity was specifically inhibited by THC.<sup>132</sup>

L-arginine supplementation inhibited EBV replication in EBV+ cells through enhanced inducible nitric oxide synthase and increased nitric oxide generation. The expression of EBV EA, immediate-early BZLF1 mRNA and ZEBRA protein, and production of infectious virus were reduced by L-arginine supplementation in a dose-dependent manner.<sup>133</sup>

Sulforaphane is an isothiocyanate compound found in cruciferous vegetables (broccoli, brussel sprouts and cabbage). It is produced when the enzyme myrosinase transforms glucoraphanin, a glucosinolate, into sulforaphane on damage to the plant (such as from chewing), which allows the two compounds to mix and react. Sulforaphane has been shown to inhibit EBV reactivation in NPC cells, through inhibition of transactivation activity of the immediate-early EBV gene, BRLF1, but not BZLF1.<sup>134</sup>

Curcumin, a phenolic extract of the spice turmeric, has been used as a food additive for centuries and has potent anti-inflammatory, antiviral and antitumour properties. Curcumin has been shown to block EBV-induced B-cell immortalisation in a dose-dependent fashion with nearly complete inhibition at 20  $\mu$ M.<sup>135</sup> The mechanism of action is enhancement of apoptosis.<sup>136</sup> Curcumin has also been shown to be an inhibitor of EBV BZLF1 in Raji DR-LUC cells.<sup>137</sup>

Baicalein is a bioactive flavonoid compound purified from the root of *Scutellaria baicalensis* (the flowering plant, Baikal skullcap), *Scutellaria lateriflora* (the flowering plant, blue skullcap), *Oroxylum indicum* (Indian trumpet flower) and thyme. It exhibits anti-inflammatory, immunosuppressive and antitumour properties. Baicalein inhibits the growth of EBV+ NPC cells by repressing the activity of the EBNA1 Q-promoter.<sup>138</sup>

(+)-Rutamarin is a topoisomerase II catalytic inhibitor which has been shown to inhibit the replication of EBV. It is obtained from the *Ruta graveolens* (Rue) plant. Herpesviruses require several cellular proteins for their lytic DNA replication including topoisomerase II. (+)-Rutamarin is effective in inhibiting EBV DNA replication and virion production with little adverse effect on cell proliferation, and therefore has potential to become a safe and effective drug for the treatment of human diseases associated with EBV infection.<sup>139</sup> A variety of rutamarin derivatives also exhibit similar inhibition of EBV replication.<sup>140</sup>

## CONCLUSIONS

EBV is a hugely successful pathogen which infects almost all people globally, and which persists lifelong following the acute phase. EBV reactivation is induced by chronic psychological stress with consequent weakening of the cellular immune response and is an important mechanism in the pathogenesis of various autoimmune diseases, cancers and CFS/ME. This article documents the anti-EBV activity of anti-herpesvirus drugs, other drugs and various dietary constituents and supplements. There are no drugs currently licensed for the treatment of EBV reactivation, although there are a number of anti-EBV drugs in development. It is interesting to note that there are numerous dietary constituents and supplements

with effective anti-EBV activity, demonstrating the importance of a nutritious diet and a healthy lifestyle in the prevention of EBV reactivation.

## Take home messages

- Epstein-Barr virus (EBV) infection infects almost everyone in all populations studied.
- EBV persists life-long following acute infection and is reactivated with prolonged psychological stress which weakens cellular immunity.
- EBV reactivation has been associated with various autoimmune diseases, chronic fatigue syndrome / myalgic encephalomyelitis (CFS/ME) and various malignancies.
- Various drugs, vitamins and nutritional supplements inhibit EBV reactivation and several other critical points in the EBV life cycle.
- A nutritious diet containing sufficient vitamins A, C and D is important in the control of EBV infection and prevention of disease.

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