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## Vaccines and Antiviral Drugs for Diseases Associated with the Epstein-Barr Virus

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### 1. Introduction

The Epstein-Barr virus (EBV) is a globally prevalent  $\gamma$ -herpesvirus that infects over 90% of humans and persists for the lifetime of the person. The EB virion is composed of a linear dsDNA molecule, an icosahedral capsid, an amorphous tegument, and an envelope containing viral glycoprotein spikes on its surface (figure 1A). The EBV primarily infects human B-lymphocytes, establishes a latent phase that persists as an incomplete virus, and then induces the transformation as well as proliferation of the infected cells. Under certain circumstances, latent EBV infection can be reactivated, subsequently giving rise to the production of infectious progeny that reinfects cells of the same type. The reactivated virus can also be transmitted to another individual.<sup>1</sup>

EBV-infected B lymphocytes harbor the latent EBV genome as a multicopy episome. There is compelling evidence that most EBV-associated malignancies have escaped this potent virus-specific cytotoxic T-lymphocytes (CTL) response by restricting viral gene expression. EBV expresses more than 80 lytic antigens, whereas latent EBV does not produce progeny virions. However, latent EBV expresses a limited set of viral gene products that maintain the viral genome as well as promote host-cell survival and proliferation. Latent EBV-infected cells express up to nine proteins (figure 1B) and several non-translated RNAs.<sup>2</sup> Among the nine latent EBV proteins, six are EBV nuclear antigens (EBNA1, 2, 3A, 3B, 3C, and LP) and three are latent membrane proteins (LMP1, 2A, and 2B). Based on the latent viral gene expression pattern, the latency is characterized as three main types, namely, types I, II, and III.<sup>3</sup> A type I infected cell only expresses EBNA1; EBNA1, LMP1, and LMP2 are found in type II infected cells. A type III infected cell expresses the full spectrum of latent EBV proteins.

The role of the immune system in the defense against EBV-associated diseases has recently become a popular topic. One hypothesis suggests that numerous neoplasms express viral antigens that should potentially enable them to be recognised and destroyed by the immune system. The EBV infection of B cells is mainly controlled by CD8<sup>+</sup> T cells, in addition to

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natural killer (NK) cells. CD4<sup>+</sup> T cell response is also probably important as a source of either effector cells or cytokine help for the massive CD8<sup>+</sup> T cell.<sup>4</sup>

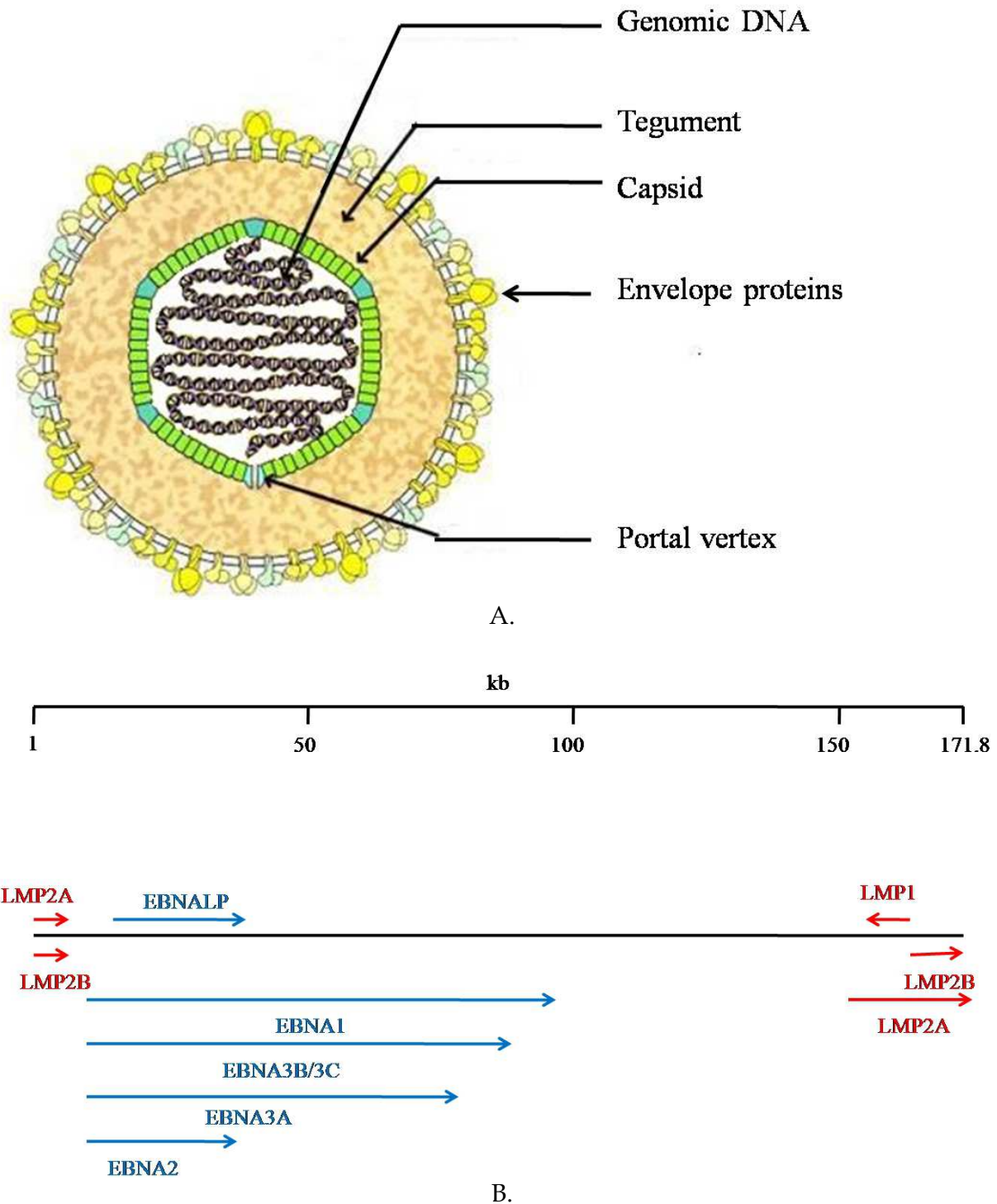


Fig. 1. A) Structure of EB virion. B) Schematic diagram of the EBV genome and location of nine genes expressing latent proteins. The location and polarity of the EBV nuclear antigens (EBNAs) encoding region are shown with blue arrows, latent membrane proteins (LMPs) encoding region are shown with red arrows.

## 2. EBV-associated diseases

EBV is best known as the aetiological agent of infection mononucleosis (IM), which is most common among adolescents and young adults. IM, a self-limiting disease, is characterised by the appearance of heterophile antibodies in the serum and an atypical lymphocytosis.<sup>5</sup> In developing countries, people are exposed to the virus in their early childhood when they are unlikely to produce noticeable symptoms. In developed countries, such as the United States, the age of first exposure may be delayed to late childhood and young adulthood age when symptoms are more likely to manifest.

### 2.1 EBV-associated cancers

EBV is associated with an increasing number of lymphoproliferative processes and epithelial neoplasias not only in immunodepressed or immunodeficient patients, but also in immunocompetent persons. EBV-related tumors are characterised by the active expression patterns of viral gene products (Table 1).

	IM	BL	HL	NPC	PTLD
EBNA1	+	+	+	+	+
EBNA2	+	-	-	-	+
EBNA3A	+	-	-	-	+
EBNA3B	+	-	-	-	+
EBNA3C	+	-	-	-	+
EBNALP	+	-	-	-	+
LMP1	+	-	+	+	+
LMP2A	+	-	+	+	+
LMP2B	+	-	+	+	+
EBERs	+	+	+	+	+
BARTs	+	+	+	+	+
Latency	III	I	II	II	III

Table 1. Expression pattern of latent EBV infected genes in EBV- related diseases. IM: infection mononucleosis; BL: Burkitt's lymphoma; HL: Hodgkin's lymphoma; NPC: Nasopharyngeal carcinoma; PTLD: Posttransplantation lymphoproliferative disorder.

Burkitt's lymphoma (BL), first described by Denis Parsons Burkitt in 1956, are classified into three forms: endemic, sporadic, and AIDS-associated BL.<sup>6</sup> Endemic BL, initially found in

Africa, is the most common childhood lymphoma in western countries and accounts for approximately 5% of all adult lymphomas.<sup>7</sup> About 97% of endemic BL patients are EBV-positive, suggesting the strong association of endemic BL with EBV. On the other hand, EBV DNA can be detected in 20%-40% of sporadic and AIDS-associated BL.<sup>6</sup> EBNA1 is the only EBV antigen expressed in BL.

Hodgkin's lymphoma (HL) has first been described by Thomas Hodgkin in 1832.<sup>8</sup> Approximately 25%-50% of classical HL cases are associated with the presence of EBV in Reed-Sternberg (RS) mononuclear, and multinuclear cells,<sup>9</sup> which are major components of the tumor.<sup>10</sup> RS cells produce cytokines and chemokines, including TGF- $\beta$ , IL-10, and TARC. These cytokines and chemokines possibly enable RS cells to modulate the immune response and escape CTL detection.<sup>11</sup> The human tumor-associated antigen RCAS1 expressed in RS cells induces the apoptosis of activated cytotoxic T cells and natural killer (NK) cells. EBV-positive RS cells expressing RCAS1 may evade the host immune response.<sup>12</sup>

Nasopharyngeal carcinoma (NPC), the most common tumor that develops in the nasopharynx, is extremely common in Southeast Asia and Africa.<sup>13</sup> The EBV genome has been coincidentally found in all NPC specimens,<sup>14</sup> i.e. NPC shows a 100% association with EBV. The latent EBV gene expression pattern in NPC is generally very similar with that detected in most EBV-related HL cases. However, there is no detectable IL-10 and TARC expression in NPC tumor cells, suggesting that the mechanism for escaping immune recognition and destroying NPC tumor cells is different from HL.<sup>11</sup> LMP1 is known to have oncogenic properties during latent infection in NPC, and is thought to be a key modulator in the pathogenesis of NPC.<sup>13</sup> LMP1 triggers the NF- $\kappa$ B, AP-1, and STAT signaling pathways in NPC. Ultimately, all signaling cascades triggered by LMP1 lead to the disruption of the cell cycle, inducing cell transformation.<sup>13</sup>

Posttransplantation lymphoproliferative disorder (PTLD) is an uncontrolled proliferation of B lymphocytes occurring in immunocompromised patients following organ transplant with immunosuppressant medication.<sup>15</sup> The relationship between EBV and PTLD has first been noted by Crawford et al. in 1980.<sup>16</sup> PTLD has a complex clonal diversity ranging from polymorphic B-lymphocyte hyperplasia to malignant monoclonal lymphoma.<sup>5</sup> The B-lymphoma cells of PTLD patients express a full spectrum of latent EBV genes.<sup>17</sup>

In HIV-associated lymphomas, the HIV-induced immunodeficiency may increase the traffic of EBV-infected B-cells, leading to a wide variety of AIDS-related lymphomas.<sup>18</sup> The incidence of non-HL (NHL) in AIDS has increased. Primary cerebral lymphoma (or primary central nervous system lymphoma), a form of NHL, is strongly related to EBV because EBV DNA is present in cerebrospinal fluid.

## 2.2 EBV-associated autoimmune diseases

There is increasing evidence that EBV is a possible triggering factor of many human-autoimmune diseases.<sup>19</sup>

Multiple sclerosis (MS) is a neurological disease characterised by chronic inflammation and demyelination within the central nervous system. A higher frequency of EBV seropositivity and a higher prevalence of high anti-EBV antibody titres exist in patients with MS compared

with controls.<sup>20</sup> About 99% of MS patients are EBV-seropositive.<sup>21</sup> To determine whether antibodies to EBV are elevated before the onset of MS, Levin et al. have conducted a study on the blood samples of more than three million US military personnel. The results demonstrate that the presence of high EBV antibody titres in human increases the risk for developing MS by 34-fold.<sup>22</sup> Although there is no sufficient evidence to conclude that EBV virus causes MS, in some cases, the first attack of MS occurs at the time of primary EBV infection.<sup>23</sup> Apparently, T cells controlling EBV-infected B cells in MS patients are impaired.<sup>24</sup>

Systemic lupus erythematosus (SLE), an autoimmune chronic inflammatory disease that generates a multi-systemic rheumatic disorder, which ultimately causes organ failure.<sup>25</sup> Compared with controls, SLE patients have increased EBV viral load,<sup>26</sup> anti-EBV antibody levels<sup>27</sup>, and numbers of latently infected peripheral B cells. The functional T cell responses of SLE patients are also impaired, and they are positive for the presence of EBV DNA. About 99% of young SLE patients are EBV seropositive.<sup>26</sup> Verdolini et al.<sup>28</sup> have reported a 22-year-old woman who immediately developed SLE after contracting EBV-induced IM. The data obtained from this case suggest that EBV infection can work as a trigger of SLE in some cases, particularly if the patient is genetically susceptible. The T cells controlling the EBV-infected B cells in SLE become defected that cannot control the numbers of EBV-infected B cells.<sup>29</sup>

Rheumatoid arthritis (RA) is a widespread autoimmune disease characterized by the infiltration of CD4+ T cells and NK cells into synovial joints.<sup>30</sup> Compared with controls, RA patients exhibit increased viral load, anti-EBV antibody titres, and frequency of circulating EBV-infected B cells.<sup>31</sup> The high frequency of EBV-infected B cells in RA patients may be explained by the impaired control of infected B cells by EBV-specific T cells.<sup>32</sup>

Sjögren's syndrome is an autoimmune disorder characterised by lymphoid infiltrates in the salivary gland. Patients with this disorder have elevated levels of anti-EBV antibodies<sup>33</sup> and decreased EBV-specific T cell cytotoxicity.<sup>34</sup>

Other autoimmune disorders, such as autoimmune thyroid disease,<sup>35</sup> scleroderma,<sup>36</sup> autoimmune liver disease (primary biliary cirrhosis and autoimmune hepatitis),<sup>37</sup> inflammatory bowel disease (ulcerative colitis and Crohn's disease),<sup>38</sup> as well as cryptogenic fibrosing alveolitis<sup>39</sup> are also associated with EBV. Patients with any of these disorders all have increased EBV DNA loads and increased serum levels of anti-EBV antibodies.

### 3. Vaccines

EBV is known to be associated with a large number of human malignancies in immunocompetent and immunosuppressed individuals. Prophylactic vaccines against some pathogenic viruses are excellent public health interventions in terms of safety and effectiveness. Accordingly, there is a great demand for effective vaccines against EBV. Interest in formulating an effective vaccine against EBV is increasing, but only a few clinical trials have been conducted. No candidate vaccine has yet been proven sufficiently effective as to warrant commercialisation.

Vaccines should be able to either block primary EBV infection or significantly reduce the EBV load during primary EBV infection. Almost all, if not all, EBV-associated malignancies

develop years after the primary EBV infection. Given that immunisation with whole viral proteins does not elicit an efficient CTL response, focus has been directed towards developing peptide vaccines based on defined epitope sequences. Two broad approaches being considered to design effective vaccines for controlling EBV-associated diseases are discussed in the following subsections.

### 3.1 EBV structural antigens as target antigens

The EBV is enveloped by a membrane composed of four major virus-specific proteins, namely, gp350, gp220, gp85, and p140.<sup>40</sup> The EBV mainly binds to B cells, via the interaction of the gp220 present in the envelope of the virus with cell receptor CD21. This interaction fosters infection. Later, the EBV produces a latent infection mainly in B cells.<sup>41</sup> Most strategies for developing EBV vaccines have focused on the virus membrane antigen, which consists of at least three glycoproteins.

Prophylactic vaccines are known to function primarily via the induction of virus-neutralising antibodies. Gp350 contains the main neutralisation epitope and is the primary target of the virus-neutralising antibody response. These features suggest that gp350 is a primary potential vaccine candidate. In the past several decades, there have been several efforts of developing vaccines mainly focused on the use of a subunit preparation of gp350 (recombinant and affinity purified). Abundant recombinant formulations of gp350 presenting as a subunit antigen or expressing from recombinant viral vectors, generated to induce high load neutralising antibodies, have shown significant protection against EBV-induced B-cell lymphomas in cotton-top tamarins. The recombinant gp350 vaccine is able to elicit neutralising antibodies in a phase I/II trial,<sup>42</sup> has a good safety profile, and is well tolerated.<sup>43</sup> The vaccine is proven effective in preventing the development of EBV-induced IM, but has no efficacy in preventing asymptomatic EBV infection.<sup>44</sup> Indeed, highly purified gp350 induces high levels of neutralising antibodies and inhibits tumor formation in cotton-top tamarins when administered subcutaneously administered with adjuvants such as muramyl dipeptide or immune-stimulation complexes.<sup>45</sup> A number of recombinant vectors, including vaccinia-gp350 and adenovirus 5-gp350, have also been successfully used in these animals to block tumor outgrowth.<sup>46</sup> Nevertheless, the development of neutralising antibody titres in vaccinated animals does not always correlate with protection.<sup>47</sup> Yao et al. have demonstrated that very low levels of neutralising anti-gp350 antibodies are present in the saliva of healthy EBV-immune donors. This finding suggests that such antibodies are unlikely to be the basis of long-term immunity in healthy seropositive individuals.<sup>48</sup> Apparently, a vaccine solely based on gp350 does not completely prevent the infection of every single B lymphocyte or epithelial cell.

Wolf et al.<sup>49</sup> have expressed poly-antigens containing several antigenic determinants of gp220 and gp350. These proteins are useful in the prophylaxis and therapy of EBV-related diseases because they are able to modulate the immune responses of patients suffering from diseases such as NPC, IM, or EBV-related BL. Mond et al.<sup>50</sup> have enhanced B-cell activation and immunoglobulin secretion by co-stimulation of the receptor for antigen gp350/220.

gp85 is also a potential target for vaccine design.<sup>51</sup> Burrows et al.<sup>52</sup> have successfully identified CTL epitopes within the EBV structural antigen gp85. Using *ex vivo* primary

effectors, strong reactivity to gp85 peptides is observed. An animal model system further reveals that gp85 epitopes are capable of generating structural antigen-specific CTL responses and reducing infections with the virus expressing gp85. Queensland Institute of Medical Research has developed a vaccine including several CTL epitopes that provides protection to more than 90% of the Caucasian population.<sup>53</sup> In 1995, the recombinant vaccinia virus expressing the major virus membrane antigen has first been used in humans.<sup>54</sup>

### 3.2 Latent antigens as potential vaccine candidates

EBV structural antigens are not expressed in latently infected B-lymphocytes. Hence, therapeutic EBV vaccine efforts have been focused on latency antigens expressed in EBV-associated diseases. EBNA1 has been identified as a vaccine antigen. In a specific embodiment, a purified protein corresponding to EBNA1 elicits a strong CD4<sup>+</sup> T cell response.<sup>55</sup> Another vaccine for EBNA2 with the aim of treating and/or preventing PTDL has been developed.<sup>56</sup> LMP1 and LMP2 are the only target antigens available for expanding CTL responses in patients with HD and NPC. Duraiswamy et al.<sup>57</sup> have generated a recombinant poxvirus vaccine that encodes a polyepitope protein derived from LMP1. Human cells infected with the vaccine are efficiently recognised by LMP1-specific CTLs from HLA A2 healthy individuals. The outgrowth of LMP1-expressing tumors in HLA A2/K<sup>b</sup> mice is also reversed by the vaccine.

EBNA1 is a protein expressed during both the latent and lytic phases of the EBV. EBNA1 is the only viral protein expressed in all EBV-positive proliferating cells in healthy EBV carriers and in all EBV-associated malignancies.<sup>58</sup> Therefore, a possible vaccine would include EBNA1 added to another latent or lytic gene. A group has developed a vaccine comprising a synthetic polypeptide with a plurality of different segments of parent EBV polypeptides, including EBNA1, LMP1, and LMP2.<sup>59</sup> The vaccine is mainly aimed at treating NPC, HL, and PTLN. Taylor et al.<sup>60</sup> have generated a modified vaccinia virus Ankara recombinant, MVA-EL, which expresses the CD4<sup>+</sup> epitope-rich C-terminal domain of EBNA1 fused to full-length LMP2. LMP2 is the source of subdominant CD8<sup>+</sup> T cell epitopes. MVA-EL has immunogenicity to both CD4<sup>+</sup> and CD8<sup>+</sup> T cells.

## 4. Antiviral drugs

### 4.1 Targeting lytic DNA replication/EBV-encoded DNA polymerase

Lytic phase EBV causes a cell-to-cell infection in the same host or transmits the virus to another individual. Until now, the most successful therapeutic interventions used against EBV infection and its associated diseases target the lytic replication of EBV.

DNA polymerase performs a key step in DNA replication. The polymerase 'reads' an intact DNA strand as a template and uses it to synthesise the new strand. During the lytic phase of the EBV life cycle, EBV DNA polymerase mediates viral DNA replication. Compounds that target EBV DNA polymerase are used to treat diseases associated with lytic EBV infection, and are widely used in various clinical settings. Drugs that may be possible candidates for targeting viral DNA polymerase are categorised into two groups, namely, nucleoside analogues and non-nucleoside DNA polymerase inhibitors.



#### 4.1.1 Nucleoside analogues

Nucleosidic antivirals have been used in the clinical treatment of EBV-associated diseases since the late 1970s. Acyclovir (ACV; 9-(2-hydroxyethoxymethyl) guanine), a synthetic acyclic nucleoside compound, has been initially shown to have a potent inhibitory activity against herpes simplex virus (HSV) infected cells.<sup>61</sup> Subsequently, ACV has been proven as an effective inhibitor of viral DNA replication in lytic EBV-infected cells, but without the same function in latently infected ones.<sup>62</sup> Given that ACV is only effective in the lytic phase by selectively inhibiting EBV DNA polymerase, efficacious compounds urgently need to be developed. Nucleoside analogues are prodrugs that require phosphorylation by viral thymidine kinase to become active. Inspired by ACV, nucleoside analogues such as ganciclovir (GCV; 9-(1,3-dihydroxy-2-propoxymethyl) guanine)<sup>63</sup> and penciclovir (PCV; 9-(4-hydroxy-3-hydroxymethylbut-1-yl) guanine),<sup>64</sup> as well as nucleotide analogues including cidofovir (CDF; (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl) cytosine)<sup>65</sup> and adefovir (9-(2-phosphonylmethoxyethyl) adenine, PMEAs)<sup>66</sup> have been developed. GCV reduces the risk of EBV-associated PTLD in renal transplant recipients, and may be more efficacious than ACV.<sup>67</sup> The inhibitive activity of PCV to EBV has also been evaluated in assays, wherein infectious virus production, viral antigen expression, and viral DNA synthesis are measured. The obtained data suggest that PCV is a selective inhibitor of EBV in cell cultures.<sup>68</sup> CDF, an acyclic nucleoside phosphonate analogue, decreases EBV oncoproteins and enhances radiosensitivity in EBV-associated diseases.<sup>69</sup> In vitro, adefovir is a potent inhibitor against a few viruses including EBV.<sup>70</sup> Nevertheless, efficiencies of these compounds as inhibitors of EBV are limited. To improve bioavailability, the orally available prodrugs valaciclovir (VACV),<sup>71</sup> valganciclovir (VGC; the valine ester of GCV)<sup>72</sup> and famciclovir (FCV)<sup>73</sup> have been introduced in the mid-1990s. VACV, the L-valyl ester of ACV, is rapidly and almost completely converted to ACV in vivo, as well as provided three to five times increase in ACV bioavailability.<sup>74</sup> The pharmacokinetics of the orally administered VGC, the valine ester of GCV, has been studied compared with the pharmacokinetics of oral and intravenous GCV. VGC results in the improved oral absorption of GCV in liver transplant recipients.<sup>75</sup> FCV, the oral form of PCV, is converted to PCV in vivo.<sup>76</sup> Despite the impressive efficiency of these nucleoside analogues in the treatment of herpes simplex infection, all these compounds suffer from the same drawbacks, including toxic side effects, poor oral bioavailability, and potential mutagenesis. Nearly all clinically effective nucleoside analogues also target the same active sites on viral DNA polymerase molecules, such that mutant viruses resistant to one drug are commonly resistant to others.<sup>77</sup>

#### 4.1.2 Non-nucleoside inhibitors

Given the success of ACV and its analogues, additional inhibitors of DNA polymerases have been expectedly identified. For example, foscarnet (the trisodium salt of phosphonoformic acid),<sup>78</sup> apparently inhibits EBV replication within the range of 2 $\mu$ M to 3 $\mu$ M, which is nontoxic to normal cellular growth. The inhibitory effects of foscarnet are exerted at the pyrophosphate binding site of DNA polymerase. Given that foscarnet is not activated by viral kinases, it is often used as an alternative treatment for EBV, and for ACV- or GCV-resistant patients. However, foscarnet is more toxic than ACV, has profound metabolic side effects, and must be intravenously administered.<sup>77</sup> A novel class of non-nucleoside inhibitors against DNA polymerases, 4-oxo-dihydroquinolines (represented as PHA-529311 and PHA-570886), has great inhibitory activity against multiple herpesviruses. These

inhibitors also show activity against ACV-resistant HSV and varicella-zoster virus isolates, as well as GCV- or foscarnet-resistant cytomegalovirus isolates.<sup>79</sup>

## 4.2 Targeting latent infections/EBV-encoded latent proteins

Most EBV-associated tumors harbor the latent viral genome as a multicopy episome in the nucleus of the transformed cells. During latent infection, the EBV does not produce progeny virions, but expresses a limited set of viral gene products that promote host-cell survival and proliferation. The EBV-encoded proteins involved in latency that have received the most attention are EBNA1, EBNA2, EBNA3A, EBNA3C, LMP1, and LMP2A. These latent proteins can induce the immortalisation and proliferation of infected cells, and are involved in immune response evasion, which are essential for neoplasias.

### 4.2.1 LMP1 as a target protein

LMP1 is an integral membrane protein containing a short N-terminal cytoplasmic tail of 17 amino acids, 6 hydrophobic transmembrane-spanning domains, and a large cytoplasmic C-terminal domain of 200 amino acids.<sup>80</sup> LMP1, is the main transforming protein of EBV, is identified as the principal oncoprotein because it can transform rodent fibroblasts and is essential for the immortalisation of B cells.<sup>81</sup> LMP1 is a functional homologue of the TNF receptor CD40, which that can deliver a signal to rescue cells from apoptosis and drive proliferation.<sup>82</sup> LMP1 mimics CD40 in activating multiple downstream signaling pathways, such as the NF- $\kappa$ B and JNK pathways. Subsequently, LMP1 up-regulates the expression of cellular genes involved in cell proliferation, cytokine secretion, angiogenesis, and tumor metastasis.<sup>83</sup> The expression of LMP1 induces EBV-associated lymphomas in transgenic mice.<sup>84</sup> Based on these characteristics, LMP1 is a potential target for EBV-associated diseases.

Antisense oligonucleotides (AODs) are effective in inhibiting gene expression in a sequence-specific manner.<sup>85</sup> A number of research groups have used antisense molecules for silencing LMP1. This process is performed with the notion of modulating the course of EBV-associated lymphoproliferative disorders because the modulation is vital for B-cells transformation. As expected, silencing the expression of LMP1 rendered the EBV-positive lymphoblastoid cell lines susceptible to chemotherapeutic agents by abrogating Bcl-2 upregulation and consequently enhancing apoptosis.<sup>86</sup> Galletti et al.<sup>87</sup> have examined the efficacy of liposomes, dendrimers or transferrin-polylysine-conjugated oligonucleotides (ONs) for antisense molecules. The data have indicated that only the delivery system exploiting the transferrin receptor pathway internalised active molecules for silence LMP1 expression. Intracellular single-chain antibodies (sFvs), the smallest domain region of an antibody that retains the binding specificity of the parental antibody, could selectively knockout viral or cellular oncoproteins. Piche et al.<sup>88</sup> have reported that an anti-LMP1 sFv increases the sensitivity of EBV-transformed B lymphocytes to drug-induced cell death. The authors suggest that an anti-LM1 sFv used in combination with conventional chemotherapy may be useful for the therapy of EBV-related lymphomas in immunocompromised patients.

### 4.2.2 LMP2A as a target protein

LMP2A can promote the survival of latently infected cells and prevent EBV reactivation from the latent phase to the lytic phase.<sup>89</sup> LMP2A signaling does not cause B cells to grow,

but delivers a critical signal that is essential for the survival of all B cells.<sup>82</sup> In *in vitro* infected B cells, the LMP2A N-terminal cytoplasmic domain blocks B cell antigen receptor (BCR) signal transduction, preventing the change from the latent to the lytic cycle, thereby maintaining latency.<sup>90</sup> This domain interacts with Syk and Lyn protein tyrosine kinases via multiple phosphotyrosines arranged in ITAM- and SH2-protein binding motifs. Hence, Syk and Lyn are prevented from binding to the cytoplasmic B cells. Syk and Lyn binding to cytoplasmic B cells are able to induce the lytic cycle.

Monroe et al.<sup>91</sup> have used peptide homologues (synthetic ITAM analogues) to inhibit the interaction of proteins and the ITAM-protein binding motif of viral proteins. They have shown that the blocking association of LMP2A ITAM with cellular molecules and the blocking of LMP2A ITAM-mediated signaling are effective strategies for the treatment and prevention of metastases of EBV-induced malignancies.

#### 4.2.3 EBNA1 as a target protein

EBNA1 is an extremely attractive target for preventing EBV infection and treating EBV-related malignancies. EBNA1 is the only viral protein expressed in all EBV-positive proliferating cells in healthy EBV carriers and in all EBV-related malignancies.<sup>92</sup> EBNA1 is essential for the persistence of the EBV episome, and is anti-apoptotic in contributing to infected-cell survival.<sup>93</sup> EBNA1 also has well-defined biochemical and structural properties. It consists of several functional domains, including a well-defined carboxyl-terminal DNA binding domain.<sup>94</sup> This domain is essential for interacting with the viral oriP. OriP consisting of a series of 30 bp repeats acts in *cis* to permit linked DNAs to replicate as plasmids in cells containing EBV DNA. EBNA1 regulates the function of oriP to which EBNA1 binds an 18 bp palindromic-sequence as a homodimer.<sup>95</sup> The DNA binding and dimerisation interface have been solved by high resolution X-ray crystallography in the apo- and DNA-bound forms.<sup>96</sup>

The approach of using an AOD to target a single selected viral gene product is promising for the treatment of EBV infections.<sup>97</sup> The treatment of EBV-transformed B cells with EBNA1 antisense ONs inhibits the proliferation of EBV-immortalised cells by at least 50% compared with scrambled antisense sequences.<sup>98</sup> In contrast to primary B cells, EBV-transformed B lymphoblastoid cell lines express alpha-v integrins, the adenovirus internalisation receptor, and are also susceptible to adenovirus-mediated gene delivery. The adenovirus delivery of a specific EBNA1 ribozyme to lymphoblastoid cell lines as well as suppressed EBNA1 mRNA, and protein expression, significantly reduce the number of EBV genomes.<sup>99</sup> Recently, Sun et al.<sup>100</sup> have demonstrated that Hsp90 inhibitors can be used to inhibit EBNA1 expression and translation. This effect requires the EBNA1 Gly-Ala repeat domain. Hsp90 inhibitors induce the death of established, EBV-transformed lymphoblastoid cell lines at doses that are nontoxic to normal cells. Hsp90 inhibitors prevent the EBV transformation of primary B cells and strongly inhibit the growth of EBV-induced lymphoproliferative disease in severe combined immunodeficiency (SCID) mice. The authors suggest that Hsp90 inhibitors may be particularly effective for treating EBV-induced diseases requiring the continued presence of the viral genome. Li et al.<sup>101</sup> have identified a new class of small molecule compound inhibitors of EBV latent infection based on their ability to inhibit the DNA binding function of EBNA1. The molecules have been discovered via high throughput *in silico* virtual screening and further validated by biochemical as well as cell-based assays. Four

compounds are identified to have biochemical activity, and two of which have activity in cell-based assays.

#### 4.2.4 EBNA2 and EBNA3 as target proteins

EBNA2 is related to the differentiation and transformation of B cells. EBNA2 acts as a transactivator molecule that binds to cellular sequence-specific DNA-binding proteins, such as the Jkappa recombination signal binding protein (CBF1/RBP Jkappa). Consequently, the cellular genes CD23 and CD21, as well as the viral genes LMP1 and LMP2A are transactivated.<sup>102</sup> However, EBNA3A and 3C can inhibit EBNA2 activation of transcription by interacting with RBP Jkappa.<sup>103</sup> EBNA3A and 3C, other than EBNA3B, are critical to this B-lymphocyte growth transformation.<sup>104</sup> Farrell et al.<sup>105</sup> have synthesised a 10-aa peptide from the CBF1 interaction domain of EBNA2 as a fusion with the protein transduction domain of HIV-1 TAT (transcriptional transactivator). Treatment of an EBV-immortalised lymphoid cell lines (LCLs) with the EBNA2-TAT peptide stops cell growth and reduces cell viability. EBNA2-TAT peptide treatment also down-regulates the viral LMP1 and LMP2 genes as well as cellular CD23 expression while up-regulating the expression of the cyclin-dependent kinase inhibitor p21. As another form of treatment, Kempkes<sup>106</sup> has provided a mutant RBP-J DNA binding protein capable of binding the Notch protein but unable to bind to EBNA2. The RBP-J DNA binding protein presents an amino acid sequence with at least one mutation in the EBNA2 binding domain, thereby preventing immortalisation. EBNA3C regulates cell cycles by targeting critical cellular complexes such as cyclin A/cdk2, SCFSkp2, and Rb. Knight et al.<sup>107</sup> have used a 20-aa EBNA3C-derived peptide fused to an HIV TAT-tag to disrupt the EBNA3C-mediated cell cycle. The peptide has inhibited a hyperproliferation of EBV-infected B cell lines and reduced in vitro immortalization of primary B lymphocytes by EBV. The peptide also inhibited lymphoblastoid outgrowth from the blood of an EBV-positive transplant patient in vitro. These experiments suggest that inhibitors targeted against EBNA2 and EBNA3C may have potential novel anti-EBV therapeutics.

## 5. Therapies

Chemotherapies based on chemical products play important roles in the treatment of EBV-associated diseases. Immunotherapies using antibodies, such as the anti-CD30 and anti-CD20 antibodies (rituximab) are used to treat EBV-related malignancies. Rituximab has been combined with standard chemotherapy for EBV-associated diseases, with promising results.<sup>9, 108</sup> Other therapies such as adoptive immunotherapy, gene therapy and small interfering RNA (siRNA) therapy have also been developed.

### 5.1 Adoptive immunotherapy

The adoptive transfer of antigen-specific cytotoxic T lymphocytes offers a safe and effective therapy for certain viral infections and could prove useful in the eradication of tumor cells. Helen et al.<sup>109</sup> have reported the long-term detection of gene-marked EBV-specific CTLs in immunocompromised patients at risk for the development of EBV lymphoproliferative disease. Infusions of T cell lines have not only restored cellular immune responses against EBV, but have also established populations of CTL precursors that could respond to in vivo or ex vivo challenge with the virus for as long as 18 months. The adoptive transfer of EBV-

CTLs has been successfully applied in the treatment of PTLD. In 2010, Helen et al.<sup>110</sup> have tried to address the long-term efficacy, safety, and practicality of EBV-specific CTL immunotherapy. They have studied 114 patients who received infusions of EBV-specific CTLs to prevent or treat PTLD. None of the 101 patients who received CTL prophylaxis has developed EBV-positive PTLD, whereas 11 of the 13 patients treated with CTLs for biopsy-proven or probable PTLD have achieved sustained complete remissions. A gene-marking component is used to demonstrate the persistence of functional CTLs for up to 9 years. The conclusion is that CTL lines provide a safe and effective prophylaxis or treatment for PTLD. However, Subklewe et al.<sup>111</sup> compared dendritic cells (DCs) with LCLs for T cell stimulation against dominant and subdominant EBV antigens. DCs expand tenfold more EBNA3A and LMP2 specific T cells than LCLs, and expand EBV-specific T cell responses more efficiently than LCLs. In a specific embodiment, a vaccine using DCs charged with EBNA1 elicits a strong T cell response.<sup>112</sup> Kuzushima et al.<sup>113</sup> have introduced EBNA1 and LMP1 mRNAs into APCs. These modified cells can induce EBV-specific CTLs, inhibit the outgrowth of EBV-infected B lymphocytes, and then lyse EBV-infected NK lymphomas and NK cells.

## 5.2 Gene therapy

Gene therapy strategies for introducing novel compounds or cytotoxic gene products (e.g., HSV1-TK gene into EBV-infected tumor cells followed by GCV therapy) are being actively developed. Such strategies involve the inhibition of EBV oncoproteins or cellular genes that are critical for virus-associated oncogenesis.

Liu et al.<sup>114</sup> have administered a nucleic acid molecule that can limit tumor cell growth and/or cause tumor cell death. The molecule comprises an EBNA1 responsive promoter region operatively linked to a gene necessary for viral replication. This method can be used to treat and prevent EBV-associated tumors. Franken et al.<sup>115</sup> have introduced a suicide gene regulated by the expression of EBNA2 into latent EBV-infected cells. Cells expressing EBNA2 are demonstrated to be more selectively sensitive to GCV. There is also a complete macroscopic regression of established B-cell lymphomas in SCID mice. However, gene therapy suffers from the common problem of accurate delivery to the appropriate disease sites.

## 5.3 SiRNA therapy

Therapies using drugs targeted at latent proteins mainly expressing in tumors such as LMP1, LMP2A, or EBNA1 are promising. These proteins are critical to the immortalisation and proliferation of cells and for evading immune responses. The efficacy of siRNA is manifested. Mei et al.<sup>116</sup> have constructed a plasmid stably encoding a 21-nt siRNA specifically and efficiently interfering with LMP1. The siRNA can induce apoptosis in EBV-positive lymphoma cells.

## 6. Conclusions

Asymptomatic EBV infection causes a few EBV-associated malignancies and autoimmune diseases. The prevention and treatment of these disorders are long-term and arduous. Chemotherapy based on chemical agents such as ACV and GCV can effectively inhibit the viral DNA polymerase used in the treatment of EBV infection and EBV-associated diseases. However, these agents are only effective in lytically infected cells, but not in latently infected

ones. Standard chemotherapy combined with chemical compounds that transform latent phase cells into lytic phase cells has apparently increased therapeutic efficiency. Employing immunotherapy after chemotherapy also has a prominent effect on prevention and treatment.

Gene and siRNA therapies effectively prevent or inhibit critical genes involved in EBV infection. However, they suffer from the same drawback of accurate delivery. Adaptive immunotherapy is a promising approach against EBV-associated neoplasias. Based on the reactivation and expansion of epitope-specific CTL clones in vitro, the epitope activates and increases the immune response against EBV-associated disorders. An effective vaccine that prevents primary EBV infection and produce long-lasting protective immunity may significantly lessen the occurrence of diseases caused by EBV. Abundant vaccines based on membrane glycoproteins or latent proteins against EBV have been developed, and have promising results. However, an effective vaccine should at least contain promising CD4<sup>+</sup> T cell and CD8<sup>+</sup> cell antigens for both the prevention of symptomatic EBV infection and immunotherapy against EBV-associated diseases.

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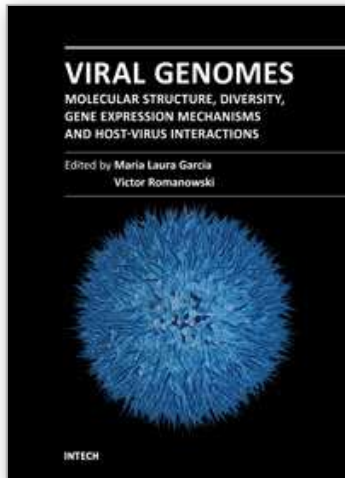
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**Viral Genomes - Molecular Structure, Diversity, Gene Expression  
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Viruses are small infectious agents that can replicate only inside the living cells of susceptible organisms. The understanding of the molecular events underlying the infectious process has been of central interest to improve strategies aimed at combating viral diseases of medical, veterinary and agricultural importance. Some of the viruses cause dreadful diseases, while others are also of interest as tools for gene transduction and expression and in non-polluting insect pest management strategies. The contributions in this book provide the reader with a perspective on the wide spectrum of virus-host systems. They are organized in sections based on the major topics covered: viral genomes organization, regulation of replication and gene expression, genome diversity and evolution, virus-host interactions, including clinically relevant features. The chapters also cover a wide range of technical approaches, including high throughput methods to assess genome variation or stability. This book should appeal to all those interested in fundamental and applied aspects of virology.

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