Vaccine development for Epstein-Barr Virus

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Abstract

Epstein-Barr virus (EBV) is the primary cause of infectious mononucleosis and is associated with several malignancies, including nasopharyngeal carcinoma, gastric carcinoma, Hodgkin lymphoma, Burkitt lymphoma, and lymphomas in immunocompromised persons, as well as multiple sclerosis. A vaccine is currently unavailable. While monomeric EBV gp350 was shown in a phase 2 trial to reduce the incidence of infectious mononucleosis, but not the rate of EBV infection, newer formulations of gp350 including multimeric forms, virus-like particles, and nanoparticles may be more effective. Vaccine that also include additional viral glycoproteins, lytic proteins or latency proteins might also improve the effectiveness of an EBV gp350 vaccine. Clinical trials to determine if an EBV vaccine can reduce the rate of infectious mononucleosis or post-transplant lymphoproliferative disease should be performed. The former is important since infectious mononucleosis can be associated with debilitating fatigue as well as other complications, and EBV infectious mononucleosis is associated with increased rates of Hodgkin lymphoma and multiple sclerosis. A vaccine to reduce EBV post-transplant lymphoproliferative disease would be an important proof of principle to prevent an EBV associated malignancy. Trials of an EBV vaccine to reduce the incidence of Hodgkin lymphoma, multiple sclerosis, or Burkitt lymphoma would be difficult, but feasible.

Keywords

Most primary infections with Epstein-Barr virus (EBV) occur in infants or young children and these infections are asymptomatic or result in non-specific symptoms (Cohen 2000). The virus infects epithelial cells in the oropharynx where it replicates and subsequently infects B lymphocytes, or it may infect B cells in the tonsillar crypts directly. These B cells circulate throughout the body and may undergo lytic infection with production of progeny virus or, more often, undergo latent infection with very limited viral gene expression.

The burden of EBV

Epstein-Barr virus is the principal cause of infectious mononucleosis and is a cofactor for several epithelial and lymphoid cell malignancies. The incidence of infectious mononucleosis in the United States is about 500 cases per 100,000 persons each year (Luzuriaga and Sullivan 2010). While infectious mononucleosis is often thought of as a mild disease, about 20% of patients will have persistent fatigue at 2 months and 13% at 6 months

(Rea et al. 2001). About 1% of patients will have severe neurologic, hematologic, or liver complications from the disease. Infectious mononucleosis is the most common cause of lost time for new Army recruits.

EBV is associated with several malignancies; the criteria for association of EBV with cancer includes finding the viral genome in every tumor cell, presence of viral gene expression, and evidence that EBV is clonal (or oligoclonal) in the tumor cells. Each year worldwide there are about 84,000 cases of gastric carcinoma, 78,000 cases of nasopharyngeal carcinoma, 29,000 cases of Hodgkin lymphoma, 7,000 cases of Burkitt lymphoma, and 2,000 cases of lymphoma in transplant recipients associated with EBV (reviewed in Cohen et al. 2011). About 9% of gastric carcinomas are associated with EBV; 90% of gastric lymphoepitheliomas, 7% of moderately to well-differentiated adenocarcinomas, and 6% of poorly differentiated gastric adenocarcinomas are EBV-positive. Virtually all anaplastic nasopharyngeal carcinomas contain EBV genomes. The incidence of nasopharyngeal carcinoma is particularly high in southern China with a rate of 80 per 100,000 in men > 40 years old. About 30% to 40% of Hodgkin lymphomas in developed countries are EBV-positive, while 80% to 90% of these lymphomas are EBV-positive in developing countries. About 85% of Burkitt lymphomas in Africa are EBV-positive, while about 15% of these tumors in the United States are virus-positive. In sub-Sahara Africa, the incidence of Burkitt lymphoma is 20 per 100,000 in children between the ages of 5 and 9 years old. The rate of EBV post-transplant lymphomas varies among the type of transplant ranging from about 1% in renal and hematopoietic stem cell transplant recipients to about 10% in intestinal transplant recipients. Up to 10% of seronegative children receiving a solid organ transplant may develop EBV post-transplant lymphoproliferative disease. Patients with HIV are at increased risk for EBV associated malignancies including Burkitt lymphoma, diffuse large B cell lymphoma, Hodgkin lymphoma, immunoblastic lymphoma, primary central nervous system lymphoma, and smooth muscle tumors. EBV is also associated with other tumors in otherwise healthy persons including angioimmunoblastic T cell lymphoma, extranodal NK/T cell nasal lymphoma, diffuse large B cell lymphoma, and peripheral T cell lymphoma,

EBV has also been associated with multiple sclerosis. A meta-analysis of 14 studies showed a relative risk of 2.3-fold for multiple sclerosis after EBV infectious mononucleosis (Thacker et al. 2006). A case-control study of persons who developed multiple sclerosis showed that 100% of EBV seronegative persons became EBV seropositive before the onset of multiple sclerosis, while only 36% of persons without multiple sclerosis became EBV seropositive during the same time frame (Levin et al. 2010). A prospective study of military personnel showed that the risk of multiple sclerosis increased as serum titers to the anti-EBV nuclear antigen complex increased; the risk was 36-fold higher in persons with titers ≥ 320 compared to those with titers <20 (Munger et al. 2011).

EBV is associated with lymphoproliferative disease in immunodeficient patients (reviewed in Cohen 2015). Boys with X-linked lymphoproliferative disease type 1, who have mutations in *SH2D1A*, can develop fatal infectious mononucleosis with infiltration of multiple organs by lymphocytes and histiocytes. Mutations in other genes including *BIRC4, CD27, CD70, CORO1A, FAAP24, LRBA*, and *MAGT1* predispose patients to severe EBV disease, usually in the absence of increased susceptibility to other pathogens. Mutations in *STXBP2, PRF1*,

or *UNC13D* predispose to severe EBV infections and hemophagocytic lymphohistiocytois. Finally, mutations in other genes including *ATM*, *CARD11*, *CTPS1*, *FCGR3A*, *GATA2*, *MCM4*, *PIK3CD*, *PIK3R1*, and *STK4*, as well as genes associated with severe combined immunodeficiency, increase the risk of severe EBV disease as well as infections due to other pathogens.

EBV glycoproteins as vaccine candidates

Glycoproteins, present on the surface of viruses and virus-infected cells, have typically been primary candidates for development of vaccines to prevent infection and/or disease. EBV infection of B cells requires the function of several glycoproteins (reviewed in Longnecker et al 2013). EBV glycoprotein gp350 is important for attachment of the virus to B cells. EBV gp350 binds to its receptor- CD21 (also known as complement receptor CR2) or CD35 (also termed complement receptor 1). This results in attachment of the virus to the B cell and the virus is then taken up by endocytosis with fusion of the viral envelope to the host cell membrane mediated by EBV gp42 binding to MHC class II. Thereafter, gH/gL are thought to activate gB for fusion of the viral membrane to the plasma membrane of B cells. gH/gL and gB are essential for herpesvirus infection of cells and gp42 is required for EBV entry into B cells.

gp350 is a type I membrane protein and is the most abundant glycoprotein on the surface of virus-infected cells and on virions. gp350 is not strictly essential for virus infection, but is important for efficient infection of B cells in vitro (Janz et al. 2000). The amino acid sequence of gp350 is highly conserved among different isolates especially in the amino terminal region; however, there are differences in the amino terminal region between EBV types 1 and 2 (Lees et al. 1993; Kawaguchi et. al 2009). Recent sequencing of clinical isolates indicates that gp350 is less conserved than other glycoproteins important for infection (Palser et al. 2015; Santpere et al. 2014), likely due to pressure to evolve in response to its role as a target for cytotoxic T cells. There is little change in gp350 amino acid sequence in individuals between the time of acute infectious mononucleosis and convalescence; the few changes that do occur are located outside the CR2 binding domain (Weiss et al. 2016). In general, the CR2 binding site on gp350 is highly conserved.

EBV infection of epithelial cells involves EBV BMRF2 binding to integrins, followed by gH/gL binding to integrins, triggering activation of gB and fusion of the viral envelope to the plasma membrane of the epithelial cell. EBV infection of epithelial cells occurs at the cell surface, not through endocytosis.

EBV lytic proteins as vaccine candidates

The symptoms of infectious mononucleosis are thought to be due to the T cell response to the virus (Silins et al. 2001). T cell responses are important for controlling reactivation of the virus and the level of virus in the blood (reviewed in Taylor et al. 2015). Therefore, a vaccine that induced an effective T cell response to EBV might reduce symptomatic disease and/or lower the viral load. The level of virus in the blood has shown to be a risk factor for development of lymphoproliferative disease after hematopoietic cell transplantation (van

Esser et al. 2001; Aalto et al. 2007). Since EBV seronegative recipients of solid organ transplants typically become infected from EBV in the transplanted organ, a vaccine to control proliferation of virus-infected cells in the organ may require T cells in addition to antibody.

Different types of immunogens to induce T cell responses have been suggested for a prophylactic EBV vaccine (Brooks et al 2016). EBV immediate-early proteins Zta (encoded by BZLF1) and Rta (encoded by BRLF1) are the first genes expressed during infection and these are produced before most of the immune evasion genes are expressed which dampen T cell responses (reviewed in Longnecker et al. 2013). Destruction of EBV-infected cells expressing Zta or Rta would reduce the likelihood of the cells producing late proteins and virions. Zta and Rta are important T cell targets in patients with infectious mononucleosis (Callan et al. 1998, Steven et al. 1997; Precopio et al. 2003). Patients with EBV post-transplant lymphoproliferative disease who resolve their disease after a reduction in immunosuppression have an increase in CD8 T cells to Zta (Porcu et al. 2002). Zta and Rta are recognized by CD8 T cells more often than early or late proteins (Pudney et al. 2005). However, a recent study suggests that CD8 T cells do not recognize Zta or Rta within the first day after EBV infection in vitro (Brooks et al. 2016).

Another approach is to induce T cell responses to early or late lytic EBV proteins. While initial studies showed that CD4 T cell responses that lyse virus-infected B cells are directed against structural proteins including EBV gp350 and glycoprotein B (Adhikary et al. 2006, Adhikary et al. 2007), more recent studies show that multiple lytic proteins including BMLF1 (a post-transcriptional regulatory protein), BMRF1 (polymerase-associated processivity factor), BNRF1 (the major tegument protein), BORF1 (DNA packaging protein), BcLF1 (major capsid protein), and BXLF1 (thymidine kinase) are targets of CD4 cells (Long et al. 2011; reviewed in Taylor et al. 2015). BMLF1 and BMRF1 are expressed early in infection, before virus structural proteins are made, and are targets of both CD4 and CD8 cells. EBV structural proteins in the nucleocapsid or envelope are presented directly to newly infected cells and can be processed and recognized by CD4 T cells. These proteins can be detected by CD4 T cells early after infection (Adhikary et al. 2006), and gp350, gH, and gB are recognized by CD4 T cells within the first day after infection in vitro (Brooks et al. 2016).

EBV latent proteins as vaccine candidates

Another approach for a prophylactic EBV vaccine is to induce T cell responses to EBV latency proteins, such those initially expressed during EBV infection of B cells. By 12 hours after infection EBV nuclear antigen 2 (EBNA-2) and EBNA leader protein (EBNA-LP) are detected (Alfieri et al. 1991). A recent in vitro study showed that several epitopes within EBNA2 induce immunodominant CD8 T cell responses, and that EBNA-2 CD8 T cells recognize EBV infected B cells within one day after virus infection of B cells before CD8 T cells that recognize other latent proteins (Brooks et al. 2016). These EBNA-2 specific T cell responses inhibit outgrowth of EBV-transformed B cell lines. EBNA-2 and EBNA-LP are also targets of CD4 T cells (reviewed in Taylor et al. 2015). Thus, a prophylactic vaccine

that induces T cell responses to the first viral proteins expressed after infection in B cells, such as EBNA-2 or EBNA-LP, might destroy any newly infected cells.

Adaptive Immunity to EBV

Infection with EBV induces antibodies and T cells specific for viral proteins. Glycoprotein gp350 is the principal target of neutralizing antibody for EBV infection of B cells (North et al. 1980; Thorley-Lawson and Poodry, 1982). Injection of antibody to gp350 prevents lymphoproliferative disease in an immunocompromised mice model (Haque et al. 2006). Antibody to gp42 also neutralizes infection of B cells, while antibody to gH/gL (Li et a. 1995) and BMRF1 (Tugizov et al. 2003) neutralize EBV infection of epithelial cells. EBV neutralizing antibody in human sera correlates better with levels of antibody to gp350 than gp42 (Sashihara et al. 2009). B cells neutralizing antibody reaches peak levels at a median of about 180 days after the onset of infectious mononucleosis (Bu et al 2015). Immunopreciptating antibody to EBV gp350 and gp42 achieves peak levels only at a median of about 900 and 400 days, respectively after onset of symptoms. Thus, antibody maturation can take over a year to occur after primary EBV infection. These findings are consistent with other activities mediated by antibody to EBV proteins which also require time to develop. Antibody-dependent cellular cytotoxicity (ADCC) directed against cells expressing gp350 was not detected in sera from persons at the onset of infectious mononucleosis, but was detected in healthy EBV-seropositive persons (Xu et al. 1998). Similarly, antibody-dependent cell-mediated phagocytosis (ADCP) was rarely detected during the initial phase of infectious mononucleosis, but was frequently present 6 months later (Weiss et al. 2016). At present it is unknown which activities mediated by antibodies are most important for protection against EBV infection or disease.

CD8 T cell responses during infectious mononucleosis are targeted to EBV lytic antigens including gp350, gH, gL, and gB (reviewed in Taylor et al. 2015); over time the number of CD8 T cells recognizing lytic antigens declines and T cells recognizing EBV latency proteins increase (Hislop et al. 2002). CD8 T cells during infectious mononucleosis recognize immediate-early proteins most often and late proteins least often (Pudney et al. 2005); the EBNA-3 proteins are the predominant latency proteins targeted by CD8 T cell targets (Steven et al 1997). In contrast, during infectious mononucleosis CD4 T cells are directly more towards latent antigens, especially EBNA-3 proteins (Woodberry et al 2005). CD4 T cells recognize immediate-early, early, and late proteins without a preference for the kinetic class of gene expression in EBV seropositive persons (Long et al. 2011). Glycoproteins including gp350, gH, gL, gp42, and gB are also recognized by CD4 T cells (reviewed in Taylor et al. 2015). These findings suggest that a vaccine targeting CD8 T cells might focus more on lytic antigens especially immediate-early proteins, while a vaccine targeting CD4 T cells might focus on EBNA-3.

EBV glycoprotein vaccines: immunogenicity in animals

EBV gp350 has been shown to induce EBV neutralizing antibodies, ADCC, or T cell responses in animals using a number of different platforms. EBV neutralizing antibodies were first reported in rabbits (Thorley-Lawson 1979) and cottontop tamarins (Morgan et al.

1984) immunized with gp350 purified from virus-infected cells. Owl monkeys immunized with gp350 purified from EBV-infected cells developed EBV neutralizing and ADCC antibodies (Qualtiere et al. 1982). Subsequently recombinant gp350 purified from mammalian cells was shown to induce neutralizing EBV antibody in rabbits (Emini et al. 1988; Jackman et al. 1999) and cottontop tamarins (Finerty et al. 1992). Mice vaccinated with recombinant gp350 adjuvanted with a TLR4 agonist (glucopyranosyl lipid A) in emulsion developed EBV neutralizing antibodies and gp350-specific CD4 T cell responses (Heeke et al. 2016). Immunization of HLA-A2 transgenic mice with a gp350 peptide induced cytotoxic T cell responses and protected the animals against vaccinia virus expressing gp350 (Khanna et al. 1999). Immunization of mice with a plasmid expressing gp350 induced antibodies that mediated ADCC and gp350-specific cytotoxic T cells (Jung et al. 2001). In another approach, neutralizing antibodies were detected in mice immunized with vaccinia virus expressing gp350 or a combination of four vaccinia viruses expressing gp350, gB, EBNA-2, or EBNA-3C mixed together (Lockey et al. 2008). CD4 T cell responses to EBNA-2 were detected in mice vaccinated with the combination of the four vaccinia viruses.

New approaches have recently been developed to express gp350 in a multimeric configuration. First, a tetrameric gp350 construct was expressed in Chinese hamster ovary cells that induced 19-fold higher levels of neutralizing antibodies in mice than soluble gp350; however, the neutralization assay did not use EBV, but instead the ability to block binding of gp350 to a cell line expressing CD21 (Cui et al. 2013). In a follow-up paper from the same group, mice immunized with the tetrameric gp350 showed 4-fold higher titers compared with animals immunized with monomeric gp350 using a virus neutralizing assay (Cui et al. 2016). Second, the ectodomain of gp350 was fused to the F protein of Newcastle disease virus and the chimeric protein was incorporated into the membrane of virus-like particles (VLPs) composed of the Newcastle disease virus matrix and nucleoprotein. Mice immunized with these gp350 VLPs produced higher levels of EBV neutralizing antibodies than those immunized with soluble gp350, although the differences were not statistically significant (Ogembo et al. 2015). Third, a portion of the ectodomain of gp350 (containing the CR2 binding domain of gp350) was fused to ferritin or encapsulin and nanoparticles were produced that contain 24 or 60 copies of gp350, respectively (Kanekiyo et al. 2015). Immunization of mice with the nanoparticles induced neutralizing titers that were about 1,000-fold higher than that obtained with soluble gp350; immunization of LCV-seropositive cynomolgus monkeys with the nanoparticles induced 3- to 10-fold higher neutralizing titers than that obtained with soluble gp350. Vaccination of mice with ferritin-gp350 nanoparticles protected the animals from challenge with vaccinia virus expressing gp350; vaccination with encapsulin-gp350 nanoparticles did not protect the mice.

Other EBV glycoproteins have also been used to induce neutralizing antibody to EBV or T cell responses in mice and rabbits. Vaccination of rabbits with trimeric gB, monomeric gH/gL, or trimeric gH/gL induced 18-fold, 20-fold, or >100-fold higher levels of EBV neutralizing antibody than monomeric gp350 (Cui et al 2016). In another approach, two Newcastle disease virus VLPs were constructed; one containing the ectodomain of EBV gH fused to the Newcastle disease virus F protein, the EBV gL ectodomain fused to the Newcastle disease virus HN protein, and the carboxyl half of EBV EBNA1 fused to the

Newcastle disease virus NP protein (Perez et al. 2016). The second VLP contained the ectodomain EBV gB fused to the Newcastle disease virus F protein and EBV LMP2 fused to the Newcastle disease virus NP protein. Mice immunized with either of the two EBV Newcastle disease virus VLPs produced high levels of EBV neutralizing antibodies and EBV-specific T cell responses in mice. Immunization of HLA-A2 transgenic mice with gH peptides induced cytotoxic T cell responses and protected the animals again vaccinia virus expressing gH (Khanna et al. 1999).

Another approach to an EBV vaccine used EBV VLPs. A producer cell line containing an EBV genome deleted for EBNA2, LMP1, EBNA3A, and EBNA3C, but still containing viral proteins needed for assembly and release of virions was used to produce EBV VLPs (Hettich et al. 2006). Mouse immunized with these EBV VLPs produced neutralizing antibody and T cell responses to viral proteins (Ruiss et al. 2011).

EBV lytic proteins: immunogenicity in animals

EBV BZLF1 encodes the immediate-early protein (Zta). SCID mice injected with human peripheral blood mononuclear cells from an EBV-seropositive donor were vaccinated with dendritic cells transduced either by an adenovirus expressing Zta or adenovirus with an empty vector (Hartlage et al. 2015). Mice receiving dendritic cells expressing Zta developed Zta-specific T cell responses and had delayed development of EBV lymphoproliferative disease compared with animals receiving dendritic cells not expressing Zta.

Animal studies using EBV challenge models

Most prophylactic EBV vaccines have used gp350. Initial experiments focused on cottontop tamarins, which develop EBV-positive mono- or oligoclonal B cell large-cell lymphomas after parental inoculation with high titers of virus (Cleary et al. 1985). The first proof of principle for an EBV vaccine was reported by Epstein and colleagues (1985). Tamarins vaccinated intraperitoneally with purified cell membranes containing gp350, isolated from virus infected (B95–8) cells, developed neutralizing antibody to EBV and were protected from EBV-tumors after challenge with virus. In additional experiments, animals vaccinated with gp350 incorporated into liposomes developed neutralizing antibody and were also protected against challenge with EBV. Subsequent experiments performed in cottontop tamarins showed that purified gp350 in immunostimulating complexes (ISCOMs) or muramyl dipeptide in squalene, recombinant gp350 in alum or muramyl dipeptide in squalene, or adenovirus or vaccinia virus expressing gp350 protected animals from lymphoma after challenge with EBV (reviewed in Cohen 2015). Infection of common marmosets with EBV results in lymphocytosis and development of heterophile antibodies similar to those seen with infectious mononucleosis (Wedderburn N et al. 1984). Vaccination of common marmosets with vaccinia or adenovirus expressing gp350 reduced EBV replication after challenge with the virus (reviewed in Cohen 2015).

Analysis of these studies showed that neutralizing antibody did not always correlate with protection from disease. In two studies, cottontop tamarins immunized with adenovirus or vaccinia virus expressing gp350 did not develop detectable levels of EBV neutralizing

antibody, but were protected from challenge with EBV (Morgan et al. 1988; Ragot et al. 1993). In another study, not all cottontop tamarins that developed high neutralizing titers to EBV after vaccine were protected against challenge with the virus (Epstein et al. 1986). The reasons for these findings is not clear at present and may due to insufficient time for maturation of neutralizing antibody responses or to other activities of antibodies including ADCC, ADCP, or complement-dependent cytotoxicity. Alternatively these vaccines could induce protective CD4 or CD8 T cell immunity.

Another model that has been used to test gp350 vaccines is the rhesus lymphocryptovirus (LCV). Virtually all adult rhesus macaques are naturally infected with rhesus LCV. Oral inoculation of seronegative animals results in atypical lymphocytes in the blood, lymphadenopathy, latent infection in circulating B cells, virus shedding from the saliva, and antibody responses to lytic and latent EBV antigens (Moghaddam et al. 1997). Rhesus LCV was used as a model to test different types of vaccines (Sashihara 2011). Rhesus macaques were vaccinated with rhesus LCV gp350, virus-like replicon particles expressing rhesus LCV gp350, a mixture of replicon particles expressing rhesus LCV gp350, EBNA-3A, and EBNA-3B, or saline. The highest levels of antibodies to gp350 were observed in animals vaccinated with soluble gp350. Rhesus LCV-specific CD4 and CD8 T cell responses were observed in animals vaccinated with virus-like replicon particles expressing EBNA-3A and EBNA-3B, but not with particles expressing gp350. Rhesus macaques vaccinated with rhesus LCV gp350 had the best level of protection after challenge; animals that still became infected after challenge had the lowest level of rhesus LCV DNA in the blood nearly three years after infection. These results emphasize the important role of immune responses to gp350 for protecting animals from infection and for reducing the level of EBV in the blood in animals that still become infected after challenge.

EBV vaccine trials in humans

The first EBV vaccine trial in humans used live recombinant vaccinia virus expressing gp350 (Gu et al. 1995). Vaccination of adults that were seropositive for both EBV and vaccinia virus did not induce increased titers to EBV. Vaccination of children that were both EBV-seropositive and vaccinia virus-seronegative boosted EBV neutralizing antibody titers. Vaccination of infants that were seronegative for both EBV and vaccinia virus induced neutralizing antibodies in all 9 infants; one-third became infected with EBV within 16 months after vaccination, while 10 of 10 unvaccinated control infants became infected. The numbers were too small to prove efficacy. Lve vaccinia virus is no longer a practical platform for a vaccine.

A recombinant gp350 vaccine produced in Chinese hamster ovary cells was tested in two double-blind randomized controlled studies (Moutschen et al. 2007). In a phase 1 study that included EBV seropositive and seronegative adults, all seronegative vaccine recipients produced ELISA antibody to gp350; more subjects who received the vaccine in alum/monophosphoryl lipid A (MPL) adjuvant developed neutralizing antibodies than those receiving the vaccine in alum alone. In a phase 1/2 study, EBV seronegative adults received gp350 in alum, alum/MPL or no adjuvant. ELISA antibody titers to gp350 were induced in all the subjects in this study; neutralizing titers developed in 50–60% of persons, and more

persons receiving the vaccine in alum adjuvant developed neutralizing titers than those receiving vaccine in alum/MPL or no adjuvant. One serious adverse event occurred that was suspected to be related to the vaccine; a subject who received gp350 in alum/MPL developed headache, meningismus, and oligoarthritis which resolved after 2 months.

A phase 2 trial double-bind placebo-controlled trial was then performed in EBV seronegative adults using 50 ug of gp350 in alum/MPL (Sokal et al. 2007). 88 of 90 persons in the vaccine group and 90 of 91 in the placebo completed the study. Subjects received 3 doses of vaccine or placebo at 0, 1 and 5 months and were followed for symptoms of infectious mononucleosis for 18 months after the second dose of vaccine. In the according to protocol analysis there were fewer cases of infectious mononucleosis in the vaccine group, but the difference did not reach statistical significance (p=0.06); in the intention to treat analysis the difference was significant (p=0.03) with a vaccine efficacy to prevent infectious mononucleosis of 78%. The incidence of asymptomatic EBV infection was similar in both groups. One month after the third dose of vaccine, 99% of subjects had gp350 antibodies and these antibodies persisted for 18 months; 70% of vaccinated subjects developed competition ELISA antibodies (a surrogate for neutralizing antibody); EBV DNA levels in the blood were not measured. No serious adverse events were reported.

A phase 1 trial using 12.5 ug or 25 ug of EBV gp350 vaccine in alum adjuvant was performed in EBV seronegative children with chronic renal insufficiency while waiting for kidney transplants (Rees et al. 2009). All 13 children who could be evaluated developed antibody to EBV, but only 4 developed EBV neutralizing antibody. Antibody levels fell rapidly and 2 of 13 children became infected with EBV. The authors concluded that additional doses of vaccine and/or a more potent adjuvant would be needed for such a vaccine to reduce EBV disease.

A randomized single-blind, placebo-controlled phase 1 study of two doses of an EBV peptide vaccine was tested in HLA B*801 EBV seronegative young adults (Elliott et al. 2008). Subjects received 5 ug or 50 ug of an EBNA-3A peptide with tetanus toxoid in a water in oil emulsion (Montanide ISA 720) or placebo. None of the 10 subjects who received the vaccine developed infectious mononucleosis, while 1 of the 4 placebo recipients developed the disease; 4 of the 10 vaccine recipients developed asymptomatic EBV infection, while 1 of the 4 placebo recipients had an asymptomatic infection. EBV-specific T cell responses were detected in 8 of 9 subjects who received the vaccine. No serious adverse events were noted. While the numbers were small, the results resembled the phase 2 gp350 trial with a possible reduction in infectious mononucleosis with the vaccine without a reduction in asymptomatic EBV infection.

Future vaccine trials

The phase 2 gp350 trial showed that gp350 in alum/MPL reduced the rate of infectious mononucleosis by 78% (Sokal et al. 2007). A phase 3 trial of a vaccine that includes gp350 should be tested in young seronegative adults to determine if the vaccine definitely can reduce the rate of infectious mononucleosis. Infectious mononucleosis is associated with increased rates of Hodgkin lymphoma and multiple sclerosis. About 1:800 persons in

Sweden and Denmark with EBV-positive infectious mononucleosis developed Hodgkin lymphoma (Hjalgrim et al. 2003). The risk increased within one year after onset of mononucleosis and declined back to the rate seen in controls at about 12 years. The median time from infectious mononucleosis to EBV-positive Hodgkin's lymphoma was 4 years and the relative risk of EBV-positive Hodgkin's lymphoma was 4.0 after EBV infectious mononucleosis. Therefore, a vaccine that reduces infectious mononucleosis might reduce Hodgkin lymphoma, although the relatively low rate of lymphoma would require a very large study.

The prevalence of multiple sclerosis in the United States is about 1 to 1.5 per 1000. A meta-analysis of 18 studies showed that the relative risk of multiple sclerosis after EBV infectious mononucleosis was 2.2 (Handel et al 2010). In a nested, case-controlled study of persons who developed multiple sclerosis who had serial serum samples stored, the mean interval between primary EBV infection and onset of multiple sclerosis was estimated to be 5.6 years (with a range of 2.3 to 9.4 years) (Levin et al. 2010). These data suggest that a vaccine that prevents infectious mononucleosis might reduce the rate of multiple sclerosis. Importantly, such a vaccine might definitively demonstrate (or refute) a causative role of EBV in multiple sclerosis.

EBV-positive post-transplant lymphoproliferative disease usually occurs within one year of hematopoietic stem cell transplantation and within three years after solid organ transplantation. The rate of EBV post-transplant lymphoproliferative disease is 24–33-fold higher in persons with primary infection after transplant (Preiksaitis and Cockfield, 1998). About 6% of seronegative persons who receive solid organ transplants develop post-transplant lymphoproliferative disease (Sarabu and Hricik, 2016). The EBV level in the blood is predictive of post-transplant lymphoproliferative disease (van Esser et al.2001; Aalto et al. 2007) and rituximab (monoclonal anti-CD20 antibody) given when the viral load is increasing in the blood, usually reduces the viral load to undetectable levels and may reduce post-transplant lymphoproliferative disease (van Esser et al. 2002). Therefore, a vaccine that prevents EBV infection or reduces the viral load during primary infection might reduce the rate of EBV post-transplant lymphoproliferative disease. Similarly, an elevated EBV load in the blood of patients with HIV was associated with a 2.5-fold increased risk of developing systemic B cell lymphoma a median of 10 months after blood was drawn (Leruez-Villet et al. 2012). A prior study in which rhesus macaques were vaccinated with a rhesus LCV gp350 vaccine and challenged with wild-type rhesus LCV, showed that animals that became infected after challenge had a lower viral load nearly 3 years after challenge compared with control animals (Sashihara et al. 2011). Unfortunately, viral loads were not measured in the phase 1, 1/2, and 2 trials of the recombinant gp350 vaccine in humans (Moutschen et al. 2007, Sokal et al. 2007), but sensitive assays can reliably measure EBV DNA levels in health seropositive persons (Hoshino et al. 2009). This may be important since there is a significant correlation between the level of EBV DNA in the blood and the severity of symptoms with infectious mononucleosis (Balfour et al. 2013). Thus, an EBV vaccine that does not prevent infection might still reduce the viral load in the blood after infection and decrease the risk of severe infectious mononucleosis and EBV associated malignancies.

An EBV vaccine might also be used to prevent X-linked lymphoproliferative disease type 1 in EBV-seronegative boys with mutations in *SH2D1A* or in other patients with genetic disorders that predispose to EBV malignancies (reviewed in Cohen 2015) who have not yet become infected. A potential concern is that patients with who have X-linked lymphoproliferative disease type 1 might not have a normal response to the EBV vaccine. Mice with mutations in *SH2D1A* have acute IgG antibody responses, but a near complete absence of antigen-specific long-lived plasma cells and memory B cells (Crotty et al. 2003).

Burkitt lymphoma is a common malignancy in children in sub-Sahara Africa. About 50% of these children are infected with EBV before one year of age, so a vaccine would have to be given at a very young age and recipients would need to be followed for 5 to 10 years to determine if the vaccine reduces the rate of disease.

A vaccine trial to reduce rates of nasopharyngeal carcinoma or EBV-positive gastric carcinoma would difficult to perform due to long latency period between primary infection and development of these carcinomas. Even if such a vaccine does not prevent infection it still might reduce the rate of these malignancies. Higher EBV gp350 and B cell neutralizing antibody levels correlated with a reduced risk of nasopharyngeal carcinoma in one study (Coghill et al 2016). This suggests that a vaccine that induces persistently elevated levels of EBV B neutralizing antibodies might reduce the rate of nasopharyngeal carcinoma.

At a meeting held at the National Institutes of Health in 2011 (Cohen et al. 2011)a, there was a strong consensus that clinical trials be performed with vaccine candidates with a goal to prevent infectious mononucleosis and EBV-associated cancers. In addition, priorities for future research that were identified included determining disease-predictive surrogate markers of EBV malignancies to use as endpoints for EBV vaccine trials, identifying immune correlates of protection from EBV infection and disease, establishing epidemiologic studies to determine the benefit of an EBV vaccine, development of a plan to determine vaccine efficacy for preventing malignancies, and establishing a strategy to facilitate collaborations between academic, industry, and government organizations to accelerate EBV vaccine development.

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