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Epstein-Barr virus vaccine development: a lytic and latent protein cocktail

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1. ABSTRACT

Epstein-Barr Virus (EBV) is the causative agent of acute infectious mononucleosis and associates with malignancies such as Burkitt lymphoma, nasopharyngeal carcinoma, and non-Hodgkin's lymphoma. Additionally, EBV is responsible for B-lymphoproliferative disease in the context of HIV-infection, genetic immunodeficiencies and organ/stem-cell transplantation. Here we discuss past and current efforts to design an EBV vaccine. We further describe preliminary studies of a novel cocktail vaccine expressing both lytic and latent EBV proteins. Specifically, a tetrameric vaccinia virus (VV) -based vaccine was formulated to express the EBV lytic proteins gp350 and gp110, and the latent proteins EBNA-2 and EBNA-3C. In a proof-of-concept study, mice were vaccinated with the individual or mixed VV. Each of the passenger genes was expressed *in vivo* at levels sufficient to elicit binding antibody responses. Neutralizing gp350-specific antibodies were also elicited, as were EBV-specific T-cell responses, following inoculation of mice with the single or mixed VV. Results encourage further development of the cocktail vaccine strategy as a potentially powerful weapon against EBV infection and disease in humans.

2. INTRODUCTION

2.1. Epstein-Barr Virus

The Epstein-Barr virus (EBV) was first discovered in 1964 by Tony Epstein and Yvonne Barr (1, 2), a discovery initiated by the successful expansion of Burkitt lymphoma (BL) lines in tissue culture followed by the use of electron microscopy to visualize associated virus particles. EBV is now known to be a gammaherpesvirus comprising a protein core, double stranded DNA, nucleocapsid, tegument proteins and an outer envelope spiked with gp350/220 glycoproteins (3, 4).

EBV is lymphotropic with specificity for the B cell lineage. Once infected, B cells may continue to produce virus (lytic state) or may convert to various forms of latency, each characterized by a unique gene expression pattern. Type III latency, for example, characterizes EBV-immmortalized cells grown in tissue culture and initially within the human host, whereas Type I latency occurs in BL and in latently infected proliferating cells *in vivo*. EBV-immmortalized cells express a variety of EBV nuclear antigens (EBNAs) as well as latent membrane proteins (LMPs). Within latently infected cell populations

(particularly those in culture) there may also be rare cells that are spontaneously permissive for virus replication (1, 2, 5).

EBV is the causative agent of acute infectious mononucleosis and is associated with numerous malignancies such as BL, nasopharyngeal carcinoma, and non-Hodgkin's lymphoma (6). EBV-associated tumors are endemic in Asian and African countries, and are thus responsible for significant morbidity and mortality (1). Additionally, EBV is responsible for B-lymphoproliferative diseases associated with HIV-infection, genetic immunodeficiencies or organ/stem-cell transplantation. Treatments such as drug (7, 8) or cytotoxic T cell immunotherapies (9-16) may be curative in some instances, but yield variable results and are largely unavailable in developing countries. Despite many efforts to formulate a successful vaccine (6, 17-19), none has yet been shown to completely protect humans from EBV.

The chronic infections associated with some viruses often discourage vaccine developers, who argue that classical B- and T-lymphocytes are inherently incapable of recognizing these pathogens. However, mechanisms required for the prevention and clearance of a virus need not be the same. Recent successes with the development of VZV and papilloma virus vaccines demonstrate this concept and encourage further efforts toward the development of a vaccine for EBV.

2.2. Past and current endeavors to develop an EBV vaccine

Most early attempts to develop an EBV vaccine specifically targeted the lytic antigens (18-22). For example, in the 1980s, a number of delivery systems (including recombinant Western Reserve (WR) vaccinia virus (VV)) were used to immunize cotton top tamarins with the gp350 (previously termed gp340) EBV protein. Immune responses were elicited toward this antigen and were able to block the emergence of malignant lymphomas in EBV-infected animals (22-24). Several years later, a VV-gp350 vaccine was reported to elicit partial protection against EBV in a clinical trial, as only three of nine vaccinated infants were infected during a 16-month follow-up period, as compared to ten of ten controls (19, 25). In recent years, a gp350 subunit vaccine has also been tested in phase I/II clinical trials. The vaccine safety profile of this vaccine was good, although there was one serious adverse event considered by investigators to be possibly related to the vaccine. Based on trial results, investigators suggested that the vaccine did not confer sterilizing immunity to study participants, but that it had the potential to inhibit EBV-related disease (26). Future trials are needed to further address this hypothesis.

Another strategy of recent interest has been the development of peptide or poly-peptide vaccines, focused primarily on the elicitation of CD4+ and/or CD8+ T cell responses directed toward latent EBV antigens. Polypeptide vaccines are often designed with an intent to partner conserved EBV peptides with frequently-expressed Human Leukocyte Antigens (HLA, e.g. HLA-A2) for the elicitation of cytotoxic T lymphocyte (CTL) activity specific for EBV-infected/transformed cells (5, 27-32). Animal studies have demonstrated that functional EBV-specific T-cell responses can be generated in response to these peptide-based vaccines (27, 28, 31, 33-35).

Recent research has also involved the adoption of new delivery systems such as modified pox virus vectors (e.g. modified vaccinia virus Ankara, MVA), mammalian DNA plasmids or plant-based plastids (32, 36, 37). In the context of these new formulations, debates continue concerning (i) the most appropriate protein target for an EBV vaccine, (ii) the focus of vaccines on B-cell and/or T-cell responses, and (iii) the relative importance of representing lytic or latent stages of infection in EBV vaccines (21, 28, 33-36, 38-40).

2.3. The cocktail vaccine approach

Here we describe the preliminary testing of an EBV vaccine strategy that is based on the hypothesis that a fully protective product should target antigens of both lytic and latent EBV infections, and should elicit both neutralizing B-cell and T-cell responses. To develop such a vaccine, we have tested cocktails in proof-of-concept studies. Specifically, we created a mixture of four individual recombinant vaccinia viruses (VV), each expressing a single EBV protein (the lytic proteins gp350 and gp110 and the latent proteins EBNA-2 and EBNA-3C), for testing in a small animal model. Our results showed that a tetrameric cocktail elicited responses to each of the four individual proteins, encouraging continued study of the EBV cocktail vaccine approach.

3. METHODS FOR THE STUDY OF A TETRAMERIC VACCINE APPROACH

3.1. Mice

C57BL/6 female mice were purchased from the Jackson Laboratories (Bar Harbor, ME) and were housed in the St. Jude Children's Research Hospital (SJCRH) animal facilities under conditions specified by AAALAC guidelines. Recombinant VV expressing EBV proteins were grown on MC57G cells. Infected cell lysates were administered to mice by intraperitoneal injections. The VV were either used singly or were mixed with equal ratios. The total immunizing dose was 10^7 plaque forming units (pfu) per animal in all experiments (VV titers were measured by plaque formation on fibroblast monolayers). Control mice received a VV (VV-hPIV-HN) expressing the hemagglutinin-neuraminidase (HN) protein from parainfluenza virus-type 1 (hPIV-1) or no injection.

3.2. Vaccinia virus recombinants

Recombinant VV expressing EBV proteins were kindly provided by Drs. M. Kurilla and J. Allan (41-44). These EBV vaccinia virus recombinants were originally produced by recombination between VV and psc11-based vectors (45-47).VV-hPIV-HN was kindly provided by Drs. A. Portner and K. Ryan.

3.3. Vaccinia virus ELISA

The antibody response to VV was measured using ELISA plates coated with a wild-type VV lysate.

The mouse sera were diluted 1:100 and 1:1000 in phosphate buffered saline (PBS) with 1% bovine serum albumin (BSA) and 50 µl were added to each well. After 18 to 24 hr at 4°C, the wells were washed with 10 mM PBS, pH 7.4 containing 0.1% Tween 20. Goat anti-mouse Ig conjugated with alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL) was then added to the plates. After 1.5 hr at room temperature, the wells were washed. The substrate for color development was p-nitrophenyl phosphate (Sigma, St Louis, MO). Reactions were terminated with 3 N NaOH after approximately 30 min. The absorbance was read at 405 nm.

3.4. Immunoprecipitation assay for antibodies to EBNA-2 and EBNA-3C

To detect EBNA-2- and EBNA-3C-specific antibodies, products from an *in vitro* transcription and translation reaction were used for immunoprecipitations. The plasmids coding for the EBNA-2 and EBNA-3C proteins were pSG5-EBNA-2 (48) and pSG5-EBNA-3C (49), respectively. The plasmids were linearized using Bgl II for pSG5-EBNA-2 and Sal I for pSG5-EBNA-3C. The linearized plasmids were transcribed using the mCAP mRNA Capping kit (Stratagene, LaJolla, CA). The mRNA was then translated with an *in vitro* rabbit reticulocyte lysate system (Promega, Madison, WI) using Promix -L-(^{35}S) *in vitro* cell labeling mix as the source of (^{35}S)-labeled methionine.

The immunoprecipitation reaction mixture was 3-5 µl of the translation product with 5 microliters of mouse or human sera in 500 microliters of RIPA buffer (20 mM Tris-HCl, 0.15 M NaCl pH 7.4, 1% Triton X-100, 1% deoxycholic acid, 0.1% BSA and 10 mM EDTA). The mixture was incubated at 4°C with rotation. After 30 min, 7 µl of goat anti-mouse Ig were added to each mixture and incubated for 30 min at 4°C. The immune complexes were precipitated with protein A-sepharose for 30 min at 4°C. The sepharose pellets were washed 5 times in RIPA buffer. The pellets were resuspended with 50 microliters of sample buffer for resolution by SDS-PAGE (see below). A sample of pooled human sera was used as a positive control.

3.5. Immunoprecipitation assay for antibodies to gp350 and gp110

MC57G cells were infected with VV-gp350 or VV-gp110 at a multiplicity of infection of four. After 18 hr, the cells were starved in RPMI deficient in methionine and cysteine (Bio-Whittaker, Walkersville, MD) for 30 min at 37°C. The infected MC57G cells were labeled with 100 microcuries of Promix -L-(^{35}S) *in vitro* cell labeling mix (Amersham, Arlington Heights IL) for 4-5 hr at 37°C, 5% CO_2 and chased with RPMI 1640 (with methionine and cysteine) for 30 min. The labeled cells were pelleted and lysed in 20 mM Tris-HCl, pH 8.0, 0.15 M NaCl, containing 1% Triton X-100 and 1 mM PMSF for 30 min. The lysed supernatants were used for immunoprecipitations.

The labeled lysates were preabsorbed with control sera from mice vaccinated with VV-hPIV-HN. For preabsorption, 500 microliters of the VV-gp350 or gp110 labeled lysate were added to 50 microliters of control mouse serum and 1.0 ml of protein A-sepharose slurry (one part sepharose in four parts RIPA buffer) and the mixture was incubated at 4°C for 1 hr with rotation. After centrifugation and removal of sepharose, 75 microliters of the lysate were mixed with 5 µl of a test mouse serum (or pooled human sera as the positive control) and incubated for 4 hr at 4°C with rotation. The antibody-antigen complexes were precipitated with 100 microliters protein A-sepharose (Pharmacia, Piscataway, NJ) for 1 hr at 4°C with rotation. The protein A-sepharose complexes were washed 3 times with RIPA buffer. The pellets were resuspended in 50 µl of sample buffer for resolution by SDS-PAGE (see below).

3.6. SDS-PAGE and autoradiography

Electrophoresis was performed using a 5% acrylamide-bis resolving gel with a 4% acrylamide-bis stacking gel for resolution of gp350 and gp110 (50). For resolution of EBNA-2 and EBNA-3C, a 7.5% acrylamide-bis gel with a 4% acrylamide-bis stacking gel was used. After electrophoresis the gels were fixed and impregnated with Amplify (Amersham). Autoradiography was with Kodak BioMax-MR X-ray film.

3.7. Immunofluorescence

An indirect immunofluorescence assay was used to measure antibodies to EBV. The EBV-infected B95-8 lymphoid cells (51, 52) were dried on coated-glass slides at 3-5 x 10^4 cells per well, fixed in cold methanol:acetone (1:1) for 3 min and air dried. The mouse sera were diluted 1:20 in PBS and one drop of each sample was added for a 1 hr incubation at room temperature. Slides were washed with PBS and incubated for 1 h r at room temperature in the dark with goat anti-mouse Ig-FITC (Becton Dickinson, Mountain View, CA) diluted 1:100 in PBS. Slides were then washed in PBS, mounted with buffered-glycerol and examined under a fluorescence microscope. A monoclonal antibody specific for gp350 (2L10, kindly provided by Dr. J. Sixbey) was used as a positive control, while PBS or sera from mice lacking EBV-specific antibodies were used as negative controls.

3.8. Neutralization assay

The neutralization assay was based on inhibition of the transformation of human B-lymphocytes. The assay was a modified procedure from that of Moss and Pope (53). The supernatant from B95-8 cells was pre-incubated with serially diluted (1:10, 1:20 and 1:40) sera for 1 hr at 37°C. The serum-virus mixtures were added to human peripheral blood mononuclear cells (PBMC) plated at 2.5 x 10^5 cells per well in cRPMI (4 mM glutamine, 50 µg/ml gentamicin, 100 U/ml penicillin, 100 µg/ml streptomycin, 5 x 10^{-5} M 2-ME, 10 micrograms/ml ciprofloxacin, 20% fetal calf serum) and 1 microgram/ml cyclosporin A in a 96 well plate with 5 wells per serum dilution. The culture medium was replaced every 5-7 days. After 3 weeks the wells were observed for the transformation of B-lymphocytes. The last serum dilution showing a lack of transformation in at least 3 of the 5 wells was considered the endpoint titer. Wells containing mouse sera with no EBV were included as controls.

3.9. T-cell ELISPOT assay

Overlapping peptides were prepared by the Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children's Research Hospital to represent the entire sequence of the EBNA-2 protein. Peptides were generally 15 amino acids in length and initiated at every 5th amino acid in the EBNA-2 sequence. Peptides were assembled into pools with approximately 5 peptides per pool.

Immunized mice were rested for at least 20 days following VV-immunizations, after which spleens were harvested for ELISPOT analyses. Cells were enriched for CD4+ T-cells prior to testing. For this purpose, effector cells were treated with rat anti-mouse monoclonal antibodies specific for MHC class II (TIB 120 cell supernatants) and CD8 (53-6.7.2 cell supernatants), followed by sheep anti-mouse and sheep anti-rat IgG-coated Dynabeads (Dynal, Oslo, Norway). The cell samples were then exposed to a magnet to remove the MHC class II-positive cells, the CD8+ T-cells, and the Ig-positive populations. Antigen presenting cells were prepared from naive mouse spleens by depleting T-cells with an anti-mouse Thy1.2 (AT83) antibody and complement (one part rabbit and five parts guinea pig complement (Cedarlane, Ontario, Canada) in Hanks Balanced Salt Solution plus 0.1% BSA), followed by irradiation with 2500 rad in a cesium irradiator. ELISPOT plates (96-well filtration plates, Millipore, Bedford, MA) were prepared by overnight incubation with 10 micrograms/ml anti-mouse IFN-gamma (clone R4-6A2, BD Biosciences, San Diego, CA) in PBS (100 microliters/well) at 4^{o}C. Plates were washed three times with PBS and blocked for at least 1 h at 37^{o}C with complete tumor medium (CTM (54, 55), a modified Eagle's medium (Invitrogen, Grand Island, NY) supplemented with 5-10% fetal calf serum, dextrose (500 µg/ml), glutamine (2 mM), 2-mercaptoethanol (3 x 10-5 M), essential and non-essential amino acids, sodium pyruvate, sodium bicarbonate, and antibiotics). Then 0.5-1 × 10^6 CD4+ T-cells, 5 × 10^5 antigen presenting cells and peptide pools (at a final total concentration of approximately 10µg/ml) were added to each well. Concanavalin A (4µg/ml; Sigma, St. Louis, MO) served as the positive control. After 24–48 h incubation at 37^{o}C/10% CO_2, wells were washed 5X with PBS and 5X with wash buffer (PBS with 0.0.5% Tween-20). Plates were then incubated for 2 h with 100µl of 5 micrograms/ml biotinylated rat anti-mouse IFN-gamma (clone XMG1.2., BD Biosciences) in PBS with 0.05% Tween-20 and 1% fetal calf serum at 37^{o}C. Wells were then washed 5X with wash buffer before incubation with streptavidin-conjugated-alkaline phosphatase (1:500 in wash buffer) for 1 h. After 5X rinses with wash buffer, spots were developed with BCIP/NBT alkaline phosphatase substrate (Sigma). Plates were rinsed with water, air dried and spots were counted with an ELISPOT reader (Axioplan 2 Imaging; Zeiss, München-Halbergmoos, Germany).

4 RESULTS

4.1. Antibody responses toward the VV backbone of vaccines

C57BL/6 mice were immunized either with a single recombinant VV (expressing gp350, gp110, EBNA-2 or EBNA-3C) or with a combination of all four VV-recombinants (VV-mix). In each case, mice received a single intraperitoneal injection containing a total of 10^7 pfu. Because all mice received the same dose of VV, it was expected that each animal would respond similarly to the VV vector. This was indeed the case. An assessment of serum antibodies taken one month post-vaccination with a VV-specific ELISA showed similar activities in all animals, regardless of the passenger gene expressed by the VV vaccine (Figure 1).

4.2. Antibody responses toward the passenger EBV genes of the VV vectors

Additional assays were conducted to ensure that each VV, whether used as a single immunogen or as one component of a mixture, was capable of eliciting an immune response toward its passenger gene (EBNA-2, EBNA-3C, gp110 and gp350). For these assessments, animals were immunized with either individual or mixed VV and analyzed for antibody activity one month later. In the case of constructs expressing EBNA-2 or EBNA-3C, *in vitro* transcribed and translated proteins were used in immunoprecipitation studies for the detection of serum antibody. As shown in Figure 2, the mice that were vaccinated with the VV-EBNA-2 or the VV-mix each produced a specific antibody response against EBNA-2 (Figure 2-top panel). Similarly, all mice that received the VV-EBNA-3C construct singly or within the VV-mix produced anti-EBNA-3C-specific antibodies (Figure 2-bottom panel) at one month post-vaccination.

In the case of glycoproteins gp110 and gp350, immunoprecipitation experiments were with MC57G cells infected with VV-gp110 and VV-gp350, respectively, to assess antibody activity. As shown in Figure 3, sera from mice (tested one month post-vaccination) that received the single VV-gp110 construct were able to immunoprecipitate the gp110 protein. This protein co-migrated with that precipitated by EBV-seropositive human serum (Figure 3, positive control). Similarly, sera from mice that received the VV-mix were able to immunoprecipitate gp110. Sera from the mice vaccinated with VV constructs other than VV-gp110 (e.g. VV-gp350) failed to immunoprecipitate gp110 (data not shown). The results with the immunoprecipitation of VV-gp350 are shown in Figure 4. The gp350-specific antibody responses appeared to be relatively weak in this assay, although the majority of mice that received VV-gp350 or VV-mix scored above background. Sera from unimmunized mice, VV-hPIV-HN immunized mice, and mice primed with individual EBV recombinant VV other than VV-gp350 were negative in this assay. In total, the results of immunoprecipitation experiments with each of the four protein targets demonstrated that immune responses to each of the passenger genes could be elicited when immunizations were with individual or mixed VV vaccines

All experiments described above were with four-week sera, but tests were also performed with sera taken at weeks two and eight. In each case, positive responses were demonstrated among mice that received a single VV or the VV-mix (data not shown).

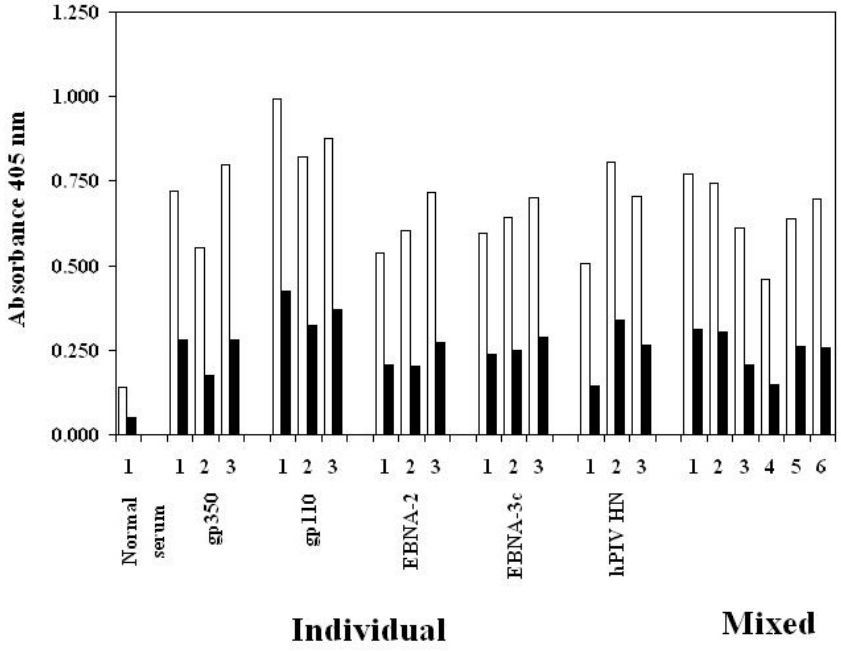

Figure 1. VV-primed mice generate VV-specific antibodies. ELISAs with VV coated plates were used to measure anti-VV specific antibody responses. Serum samples were diluted 1:100 (clear bars) and 1:1000 (solid bars) prior to assay. Numbers along the X-axis represent individual mice. Mice received either individual VV or a combination of VV-gp110, VV-gp350, VV-EBNA-2 and VV-EBNA-3C (mixed).

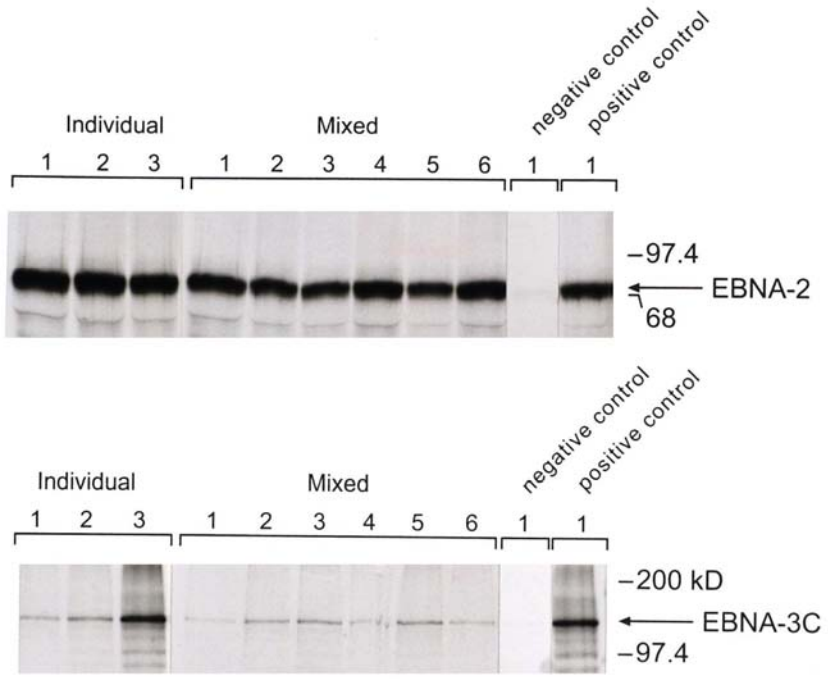

Figure 2. Immunoprecipitation of EBNA-2 and EBNA-3C proteins. *In vitro* translation products were used for immunoprecipitations with mouse sera. Negative controls were sera from mice immunized with VV-hPIV-HN. The positive control was pooled human sera from EBV-sero-positive individuals. Top Panel: Mice (3-6 per group) received VV-EBNA-2 ("Individual") or VV-mix ("Mixed"). Bottom Panel: Mice (3-6 per group) received VV-EBNA-3C ("Individual") or VV-mix ("Mixed").

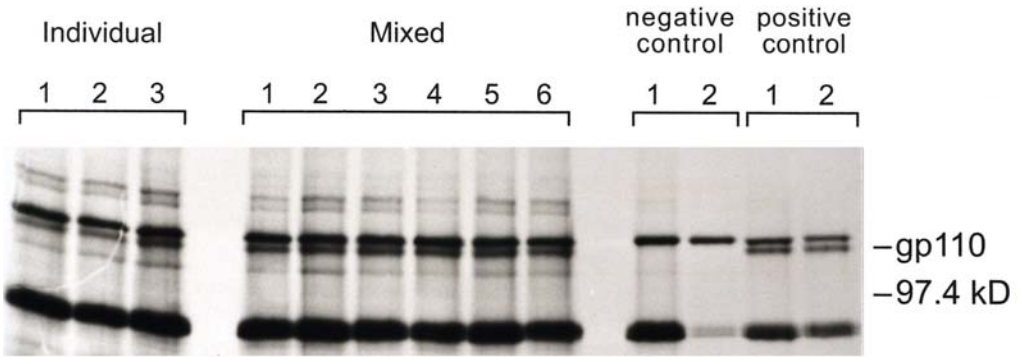

Figure 3. Immunoprecipitation of VV-gp110 lysates. Immunoprecipitation of VV-gp110 cell lysate was with sera from mice vaccinated with VV-gp110 ("Individual") and VV-mix ("Mixed"). MC57G cells infected with VV-gp110 were labeled with (^{35}S)-methionine and cell lysates were used in immunoprecipitation experiments. Samples were pre-treated and precipitated with sera specific for VV. Negative controls were with sera from VV-hPIV-HN immunized mice. Positive controls were with human sera.

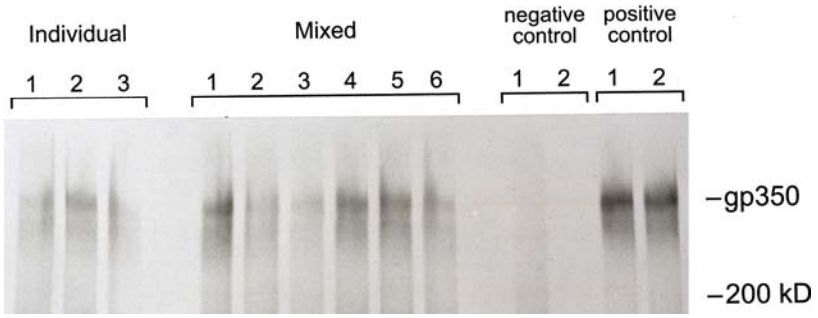

Figure 4. Immunoprecipitation of VV-gp350 lysates**.** Immunoprecipitation of VV-gp350 cell lysate was with sera from mice vaccinated with VV-gp350 ("Individual") and VV-mix ("Mixed"). MC57G cells infected with VV-gp350 were labeled with (^{35}S) methionine and cell lysates were used in immunoprecipitation experiments. Samples were pre-treated and precipitated with sera specific for VV. Negative controls were with sera from VV-hPIV-HN immunized mice. Positive controls were with human sera.

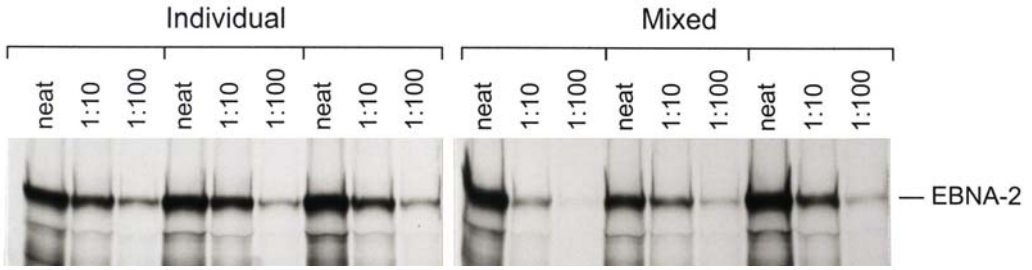

Figure 5. Immunoprecipitation of EBNA-2. The EBNA-2 antibody response was measured by testing serially-diluted sera in immunoprecipitation assays. Mice received either VV-EBNA-2 ("Individual") or VV-mix ("Mixed").

To roughly quantify the immune responses, immunoprecipitations were performed with serially diluted mouse sera from week four and the EBNA-2 protein. Results are shown in Figure 5. Sera from the mice that received VV-EBNA-2 or VV-mix were tested undiluted and at 1:10 and 1:100 dilutions. As shown in the Figure, activities were seen with sera at all dilutions. However, the sera from animals immunized with VV-mix showed the weakest signals at the 1:100 dilution. Possibly, the reduction of VV-EBNA-2 in the VV-mix vaccine (from 1 x 10^7 to 2.5 x 10^6 pfu/dose) yielded a marginal reduction in the associated immune response.

4.3. Gp350-specific binding and neutralizing antibodies generated toward single and mixed vaccines

EBV gp350 is present on the surface of the EBV particle and is therefore an important target of neutralizing antibodies. We therefore focused on the finer definition of gp350-specific antibodies. Immunofluorescence studies were first used to determine if gp350-specific antibodies

Table 1. Neutralization activity of sera from vaccinated mice

Mouse Immunization Group	Mouse #	Neutralization titer[1]
VV-gp350	1	Negative
	2	1:10
	3	1:10
VV-mix	1	1:10
	2	1:10
	3	1:10
	4	1:10
	5	1:10
	6	1:20
Normal Mouse Sera		Negative
Human Sera		1:40

[1]Sera were tested at 1:10, 1:20 and 1:40 dilutions. Neutralization titer was defined as the highest serum dilution to inhibit the transformation of human PBMC in at least three of five test wells, and in at least one of two independent assays.

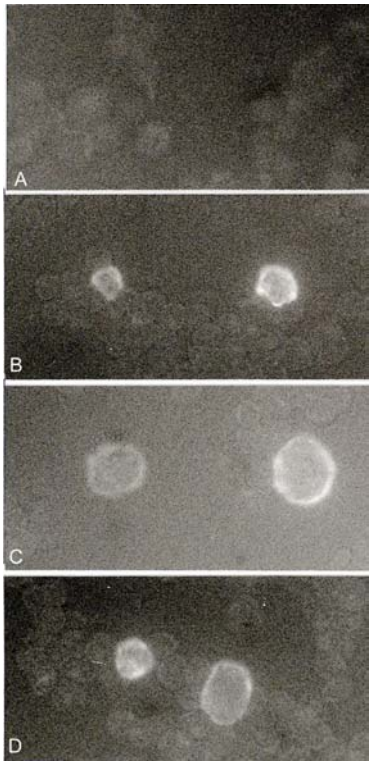

Figure 6. Immunofluorescence assay for the detection of EBV-specific antibodies. Serum samples and controls were mixed with B95-8 cells that were subsequently stained with a secondary, fluorescein-labeled goat anti-mouse Ig antibody and analyzed by fluorescence microscopy. Shown are results with (A) the negative control, PBS, (B) the positive control, monoclonal antibody to gp350 (2L10), (C) mouse serum from a VV-gp350 vaccinated mouse, and (D) mouse serum from a VV-mix vaccinated mouse. Sera from VV-hPIV-HN primed mice scored negatively in this assay (not shown).

were able to recognize their target antigen in the context of EBV-infected cells. For this study, we utilized B95-8 cultures, as 5-10% of these cells produce mature virions and thus stain with monoclonal anti-gp350 antibodies. Using a double-staining procedure with a fluorescein-labeled goat anti-mouse Ig as a secondary reagent, we compared sera from primed mice with a control monoclonal anti-gp350 antibody for B95-8 cell staining. As shown in Figure 6, brightly positive staining results were demonstrated with sera from mice that received the VV-gp350 or the VV-mix vaccines. In contrast, sera from mice primed with VV-gp110 or no VV showed weak or negative staining of cells (data not shown, gp110 is only weakly expressed in the un-induced B95-8 line (56)).

To determine whether gp350-primed mice generated neutralizing activity, mouse sera were used to inhibit EBV transformation of human PBMC. In preliminary experiments, a low level of neutralization activity was detected against EBV among sera from mice that received the VV-gp350 or VV-mix vaccines. As shown in Table 1, two of three mice that received the VV-gp350 vaccine and all of the mice that received the VV-mix had some detectable neutralizing activity. No significant difference between mice that received VV-gp350 and VV-mix could be identified. It should be noted that sera were not toxic to cells, as transformation occurred in all experiments with negative control sera.

4.4. CD4+ T-cell responses generated toward tetrameric EBV vaccine

Experiments were also conducted to determine if CD4+ T cell immune responses were detectable toward an EBV antigen presented in the context of a VV vaccine. We were particularly interested in T-cells responsive to latent antigens as these may be key to the control of EBV-transformed cells (57). For this analysis, we therefore examined responses to EBNA-2, a representative of the latent EBV antigens. To test for T-cell activities, we first prepared overlapping synthetic peptides to represent the entire sequence of the EBNA-2 protein. Peptides were generally 15 amino acids in size, initiated at every fifth amino acid. Peptide pools were then assembled with approximately five peptides per pool (see Figure 7 for locations of peptide pools). Mice were grouped for immunizations with the VV-EBNA- 2 construct or with the VV-mix. Negative control mice received no injection. Mice were rested for at least 20 days, after which spleens from each mouse group were removed and combined for ELISPOT analyses with the individual peptide pools. In Figure 8 are shown experimental results demonstrating that CD4+ T-cells from the mice primed with VV-EBNA-2 were particularly responsive to pools 17 and 18 (panel A). These pools encompassed peptides located near the C-terminus of the EBNA-2 protein. Responses toward other peptide pools were negative (representative negative responses toward pools 1 and 19 are shown in this Figure). Mice that were immunized with the VV-mix also recognized pools 17 and 18 (Figure 8B). T-cells from naïve mice were non-responsive in these assays (Figure 8C). Results provided a preliminary demonstration that T-

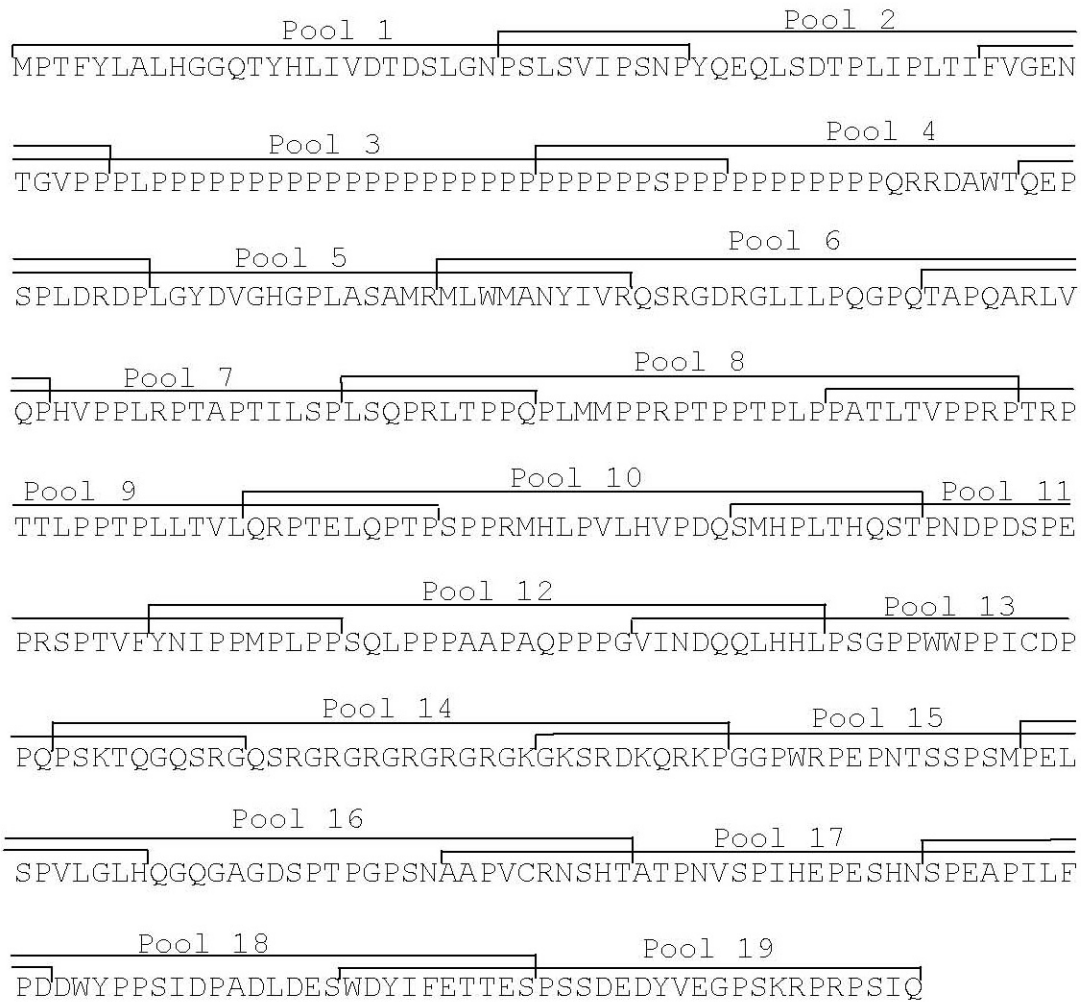

Figure 7. EBNA-2 sequence and peptide pools. Overlapping peptides were prepared to span the EBNA-2 sequence. Peptide pools were prepared with approximately five peptides per pool for ELISPOT analyses. Peptide pool positions are shown within the EBNA-2 sequence.

cell activity was generated toward a latent antigen following mouse immunizations with the VV-EBNA-2 or the VV-mix vaccine.

5. DISCUSSION

5.1. Proof-of-concept support for a cocktail EBV vaccine

A number of vaccine strategies have been, and are being pursued as a means to combat EBV disease in humans, but no vaccine has yet been taken to advanced stages of clinical trial. Most of the vaccine strategies considered to date have targeted either lytic or latent stages of EBV infection/transformation. Our strategy differs from those described previously in that a cocktail vaccine has been formulated to include both lytic (gp110 and gp350) and latent (EBNA-2 and EBNA-3C) EBV proteins, with an intent to elicit immune responses toward more than one stage of EBV infection.

This report describes the preliminary testing of an EBV cocktail vaccine strategy. Results showed that among animals immunized with either individual or mixed vaccines, antibody responses were generated against each of four independent EBV proteins. Neutralizing antibodies were also detected (in VV-gp350-primed animals), as were EBV-specific T-cells. It should be noted that EBV-specific T-cell responses have been characterized in numerous previous reports (9, 32, 33, 42, 43, 57-60). These can be isolated directly from EBV-infected human PBMC and are known to mediate cell death by a number of distinct mechanisms. For example, CD4+ T cell clones specific for EBNA-1 and EBNA-2 were found to inhibit outgrowth of EBV transformed B cells (61-63) and were capable of direct lysis of targets such as BL. Additional analyses of both CD4+ and CD8+ T cells directed toward the lytic and latent EBV antigens of individual and mixed VV vaccines are warranted as a topic of future study.

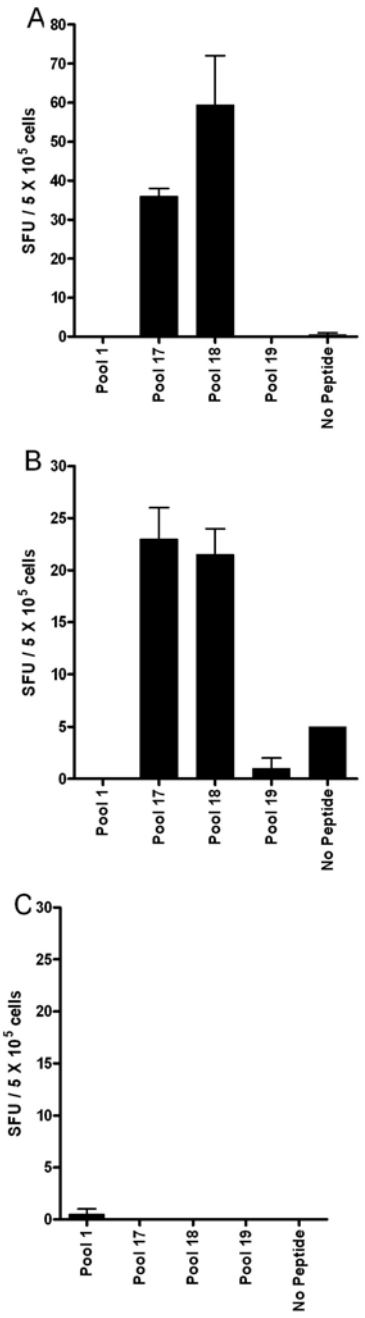

Figure 8. CD4+ T-cell responses toward EBNA-2 elicited by single-component and cocktail vaccines. Mice were immunized with VV-EBNA-2 or VV-mix and rested for at least 20 days, after which spleens were removed. Naïve mice were used as controls. CD4+ T-cells were purified for ELISPOT testing across the array of peptide pools. Panel A: Responses from mice following immunizations with VV-EBNA-2. Panel B: Responses from mice following immunizations with the VV-mix. Panel C: naïve mouse controls. Results show the averages and standard errors from analyses with replicate wells. Results were negative for EBNA-2 peptide pools that are not shown.

We utilized VV as a delivery vehicle in this study, because of its proven ability to harness both humoral and cellular immunity. VV was also an attractive delivery vehicle, because it has been the only vaccine thus far able to eradicate a human disease (smallpox). VV therefore provided a useful reagent for this proof-of-concept study, demonstrating that a combination of EBV antigens could be delivered with a single inoculation to elicit immune activities toward both lytic and latent stages of EBV infection. Perhaps this first demonstration will encourage further studies with EBV cocktail vaccines either using VV or other vehicles for vaccine delivery. Additional lytic and latent antigens (e.g. LMP1, LMP2, EBNA-1) might also be tested as cocktail vaccine targets.

5.2. Conclusion

In conclusion, there have been a number of past efforts to develop an EBV vaccine, targeting either lytic or latent proteins, but no complete successes have yet been attained. Our current working hypothesis is that the partial protection previously demonstrated in a clinical trial with VV-gp350 (19) might be much improved by combining vaccine vectors that express both lytic and latent EBV proteins. If our hypothesis is correct, a cocktail vaccine may eventually combat the acute diseases and multiple malignancies associated with human infections with EBV.

6. PERSPECTIVE

The development of an EBV vaccine may take orphan status, because the most serious diseases associated with this virus infection are relatively rare in Western nations (64). However, EBV infections cause significant morbidity and mortality throughout the world, affecting both immunocompetent and immunosuppressed patient populations. A clinical test of an EBV vaccine in the mid-1990s demonstrated a trend toward protection, encouraging further product development. Perhaps minimal efforts to formulate a combination lytic/latent EBV vaccine may ultimately be sufficient to eliminate the human diseases caused by EBV.

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8. REFERENCES

1. A. B. Rickinson and E. Kieff, *Epstein-Barr virus.* In *Fields Virology*. (Ed. D. M. Knipe, P. M. Howley, D. E. Griffin, M. A. Martin, R. A. Lamb, B. Roizman and S. E. Straus) pp. 2575-2627, Lippincott Williams and Wilkins, Philadelphia (2001)

2. E. Kieff and A. B. Rickinson, *Epstein-Barr virus and its replication.* In *Fields Virology*. (Ed. D. M. Knipe, P. M. Howley, D. E. Griffin, M. A. Martin, R. A. Lamb, B. Roizman and S. E. Straus) pp. 2511-2573, Lippincott Williams and Wilkins, Philadelphia (2001)
3. J. Tanner, J. Weis, D. Fearon, Y. Whang and E. Kieff: Epstein-Barr virus gp350/220 binding to the B lymphocyte C3d receptor mediates adsorption, capping, and endocytosis. *Cell* 50*,* 203-213 (1987)
4. M. Hummel, D. Thorley-Lawson and E. Kieff: An Epstein-Barr virus DNA fragment encodes messages for the two major envelope glycoproteins (gp350/300 and gp220/200) *J Virol* 49*,* 413-417 (1984)
5. E. Landais, X. Saulquin, M. Bonneville and E. Houssaint: Long-term MHC class II presentation of the EBV lytic protein BHRF1 by EBV latently infected b cells following capture of BHRF1 antigen. *J Immunol* 175*,* 7939-7946 (2005)
6. J. Andersson: An Overview of Epstein-Barr Virus: from Discovery to Future Directions for Treatment and Prevention. *Herpes* 7*,* 76-82 (2000)
7. A. B. Jain, A. Marcos, R. Pokharna, R. Shapiro, P. A. Fontes, W. Marsh, R. Mohanka and J. J. Fung: Rituximab (chimeric anti-CD20 antibody) for posttransplant lymphoproliferative disorder after solid organ transplantation in adults: long-term experience from a single center. *Transplantation* 80*,* 1692-1698 (2005)
8. A. Humar, D. Hebert, H. D. Davies, A. Humar, D. Stephens, B. O'Doherty and U. Allen: A randomized trial of ganciclovir versus ganciclovir plus immune globulin for prophylaxis against Epstein-Barr virus related posttransplant lymphoproliferative disorder. *Transplantation* 81*,* 856-861 (2006)
9. S. D. Rencher, K. S. Slobod, F. S. Smith and J. L. Hurwitz: Activity of transplanted CD8+ versus CD4+ cytotoxic T-cells against Epstein-Barr virus immortalized B-cell tumors in SCID mice. *Transplantation* 58*,* 629-633 (1994)
10. K. S. Slobod and J. L. Hurwitz: Adoptive Immunotherapy. *Pediatr Infect Dis J* 18*,* 467-468 (1999)
11. K. S. Slobod, E. Benaim, L. Woodruff, S. Nooner, J. Houston, M. Holladay, T. D. Lockey and J. L. Hurwitz: T cell immunotherapeutic populations control viral infections in bone marrow transplant recipients. *Immunologic Res* 24*,* 289-301 (2001)
12. S. D. Rencher, J. A. Houston, T. D. Lockey and J. L. Hurwitz: Eliminating graft-versus-host potential from T cell immunotherapeutic populations. *Bone Marrow Trans* 18*,* 415-420 (1996)
13. C. M. Rooney, C. A. Smith, C. Y. Ng, S. K. Loftin, J. W. Sixbey, Y. Gan, D.-K. Srivastava, L. C. Bowman, R. A. Krance, M. K. Brenner and H. E. Heslop: Infusion of cytotoxic T cells for the prevention and treatment of Epstein-Barr virus-induced lymphoma in allogeneic transplant recipients. *Blood* 92*,* 1549-1555 (1998)
14. C. A. Smith, C. Y. C. Ng, S. K. Loftin, C. Li, H. E. Heslop, M. K. Brenner and C. M. Rooney: Adoptive immunotherapy for Epstein-Barr virus-related lymphoma. *Leuk Lymph* (1996)
15. H. E. Heslop, C. Y. Ng, C. Li, C. A. Smith, S. K. Loftin, R. A. Krance, M. B. Brenner and C. M. Rooney: Long-term restoration of immunity against Epstein-Barr virus infection by adoptive transfer of gene-modified virus-specific T lymphocytes. *Nat Med* 2*,* 551-555 (1996)
16. H. E. Heslop, M. K. Brenner and C. M. Rooney: Donor T cells to treat EBV-associated lymphoma. *N Eng J Med* 331*,* 679-680 (1994)
17. D. J. Moss, S. R. Burrows, R. Khanna, I. S. Misko and T. B. Sculley: Immune surveillance against Epstein-Barr virus. *Semin Immunol* 4*,* 97-104 (1992)
18. A. J. Morgan: Epstein-Barr virus vaccines. *Vaccine* 10*,* 563-571 (1992)
19. S. Y. Gu, T. M. Huang, L. Ruan, Y. H. Miao, H. Lu, C. M. Chu, M. Motz and H. Wolf: First EBV vaccine trial in humans using recombinant vaccinia virus expressing the major membrane antigen. *Dev Biol Stand* 84*,* 171-177 (1995)
20. M. Mackett and J. R. Arrand: Recombinant vaccinia virus induces neutralising antibodies in rabbits against Epstein-Barr virus membrane antigen gp340. *EMBO J* 4*,* 3229-3234 (1985)
21. L. E. Wallace, J. Wright, D. O. Ulaeto, A. J. Morgan and A. B. Rickinson: Identification of two T-cell epitopes on the candidate Epstein-Barr virus vaccine glycoprotein gp340 recognized by CD4+ T-cell clones. *J Virol* 65*,* 3821-3828 (1991)
22. A. J. Morgan, S. Finerty, K. Lovgren, F. T. Scullion and B. Morein: Prevention of Epstein-Barr (EB) virus-induced lymphoma in cottontop tamarins by vaccination with the EB virus envelope glycoprotein gp340 incorporated into immune-stimulating complexes. *J Gen Virol* 69 (Pt 8)*,* 2093-2096 (1988)
23. M. A. Epstein, A. J. Morgan, S. Finerty, B. J. Randle and J. K. Kirkwood: Protection of cottontop tamarins against Epstein-Barr virus-induced malignant lymphoma by a prototype subunit vaccine. *Nature* 318*,* 287-289 (1985)
24. A. J. Morgan, M. Mackett, S. Finerty, J. R. Arrand, F. T. Scullion and M. A. Epstein: Recombinant vaccinia virus expressing Epstein-Barr virus glycoprotein gp340 protects cottontop tamarins against EB virus-induced malignant lymphomas. *J Med Virol* 25*,* 189-195 (1988)
25. S. Gu, T. Huang, Y. Miao, L. Ruan, Y. Zhao, C. Han, Y. Xiao, J. Zhu and H. Wolf: A preliminary study on the immunogenicity in rabbits and in human volunteers of a recombinant vaccinia virus expressing Epstein-Barr virus membrane antigen. *Chin Med Sci J* 6*,* 241-243 (1991)
26. M. Moutschen, P. Leonard, E. M. Sokal, F. Smets, M. Haumont, P. Mazzu, A. Bollen, F. Denamur, P. Peeters, G. Dubin and M. Denis: Phase I/II studies to evaluate safety and immunogenicity of a recombinant gp350 Epstein-Barr virus vaccine in healthy adults. *Vaccine* 25*,* 4697-4705 (2007)
27. S. A. Thomson, R. Khanna, J. Gardner, S. R. Burrows, B. Coupar, D. J. Moss and A. Suhrbier: Minimal epitopes expressed in a recombinant polyepitope protein are processed and presented to CD8+ cytotoxic T cells: implications for vaccine design. *Proc Natl Acad Sci U S A* 92*,* 5845-5849 (1995)
28. J. Duraiswamy, M. Sherritt, S. Thomson, J. Tellam, L. Cooper, G. Connolly, M. Bharadwaj and R. Khanna: Therapeutic LMP1 polyepitope vaccine for EBV-associated Hodgkin disease and nasopharyngeal carcinoma. *Blood* 101*,* 3150-3156 (2003)

29. R. Khanna, S. R. Burrows, A. Neisig, J. Neefjes, D. J. Moss and S. L. Silins: Hierarchy of Epstein-Barr virus-specific cytotoxic T-cell responses in individuals carrying different subtypes of an HLA allele: implications for epitope-based antiviral vaccines. *J Virol* 71*,* 7429-7435 (1997)
30. E. Landais, X. Saulquin, E. Scotet, L. Trautmann, M. A. Peyrat, J. L. Yates, W. W. Kwok, M. Bonneville and E. Houssaint: Direct killing of Epstein-Barr virus (EBV)-infected B cells by CD4 T cells directed against the EBV lytic protein BHRF1. *Blood* 103*,* 1408-1416 (2004)
31. S. A. Thomson, S. R. Burrows, I. S. Misko, D. J. Moss, B. E. Coupar and R. Khanna: Targeting a polyepitope protein incorporating multiple class II-restricted viral epitopes to the secretory/endocytic pathway facilitates immune recognition by CD4+ cytotoxic T lymphocytes: a novel approach to vaccine design. *J Virol* 72*,* 2246-2252 (1998)
32. G. S. Taylor, T. A. Haigh, N. H. Gudgeon, R. J. Phelps, S. P. Lee, N. M. Steven and A. B. Rickinson: Dual stimulation of Epstein-Barr Virus (EBV)-specific CD4+- and CD8+-T-cell responses by a chimeric antigen construct: potential therapeutic vaccine for EBV-positive nasopharyngeal carcinoma. *J Virol* 78*,* 768-778 (2004)
33. R. Khanna, M. Sherritt and S. R. Burrows: EBV structural antigens, gp350 and gp85, as targets for *ex vivo* virus-specific CTL during acute infectious mononucleosis: potential use of gp350/gp85 CTL epitopes for vaccine design. *J Immunol* 162*,* 3063-3069 (1999)
34. R. Khanna, D. J. Moss and S. R. Burrows: Vaccine strategies against Epstein-Barr virus-associated diseases: lessons from studies on cytotoxic T-cell-mediated immune regulation. *Immunol Rev* 170*,* 49-64 (1999)
35. V. Turcanova and P. Hollsberg: Sustained CD8+ T-cell immune response to a novel immunodominant HLA-B*0702-associated epitope derived from an Epstein-Barr virus helicase-primase-associated protein. *J Med Virol* 72*,* 635-645 (2004)
36. S. Jung, Y. K. Chung, S. H. Chang, J. Kim, H. R. Kim, H. S. Jang, J. C. Lee, G. H. Chung and Y. S. Jang: DNA-mediated immunization of glycoprotein 350 of Epstein-Barr virus induces the effective humoral and cellular immune responses against the antigen. *Mol Cells* 12*,* 41-49 (2001)
37. M. Y. Lee, Y. Zhou, R. W. Lung, M. L. Chye, W. K. Yip, S. Y. Zee and E. Lam: Expression of viral capsid protein antigen against Epstein-Barr virus in plastids of Nicotiana tabacum cv. SR1. *Biotechnol Bioeng* (2006)
38. K. Bickham and C. Munz: Contrasting roles of dendritic cells and B cells in the immune control of Epstein-Barr virus. *Curr Top Microbiol Immunol* 276*,* 55-76 (2003)
39. M. Gong, T. Ooka, T. Matsuo and E. Kieff: Epstein-Barr virus glycoprotein homologous to herpes simplex virus gB. *J Virol* 61*,* 499-508 (1987)
40. R. J. Pither, L. Nolan, J. Tarlton, J. Walford and A. J. Morgan: Distribution of epitopes within the amino acid sequence of the Epstein-Barr virus major envelope glycoprotein, gp340, recognized by hyperimmune rabbit sera. *J Gen Virol* 73 (Pt 6)*,* 1409-1415 (1992)
41. R. Gavioli, M. G. Kurilla, P. O. De Campos-Lima, L. E. Wallace, R. Dolcetti, R. J. Murray, A. B. Rickinson and M. G. Masucci: Multiple HLA A11-restricted cytotoxic T-lymphocyte epitopes of different immunogenicities in the Epstein-Barr virus- encoded nuclear antigen 4. *J Virol* 67*,* 1572-1578 (1993)
42. R. Gavioli, P. O. De Campos-Lima, M. G. Kurilla, E. Kieff, G. Klein and M. G. Masucci: Recognition of the Epstein-Barr virus-encoded nuclear antigens EBNA-4 and EBNA-6 by HLA-A11-restricted cytotoxic T lymphocytes: Implications for down-regulation of HLA-A11 in Burkitt lymphoma. *Proc Natl Acad Sci USA* 89*,* 5862-5866 (1992)
43. R. J. Murray, M. G. Kurilla, H. M. Griffin, J. M. Brooks, M. Mackett, J. R. Arrand, M. Rowe, S. R. Burrows, D. J. Moss, E. Kieff and A. B. Rickinson: Human cytotoxic T-cell responses against Epstein-Barr virus nuclear antigens demonstrated by using recombinant vaccinia viruses. *Proc Natl Acad Sci (USA)* 87*,* 2906-2910 (1990)
44. L. Petti, J. Sample, F. Wang and E. Kieff: A fifth Epstein-Barr virus nuclear protein (EBNA3C) is expressed in latently infected growth-transformed lymphocytes. *J Virol* 62*,* 1330-1338 (1988)
45. B. Moss, *Relication of poxviruses.* In *Virology*. (Ed. B. N. Fields) pp. 685-703, Raven Press, New York, N.Y. 1985.
46. G. L. Smith and B. Moss: Vaccinia virus expression vectors: construction, properties and applications. *BioTechniques* Nov/Dec*,* 306-312 (1984)
47. K. W. Ryan, R. J. Owens and J. L. Hurwitz, *Preparation and use of vaccinia virus vectors for HIV protein expression and immunization.* In *Immunology methods manual*. (Ed. I. Lefkovits) pp. 1995-2015, Academic Press, London, England 1997.
48. S. F. Tsang, F. Wang, K. M. Izumi and E. Kieff: Delineation of the cis-acting element mediating EBNA-2 transactivation of latent infection membrane protein expression. *J Virol* 65*,* 6765-6771 (1991)
49. D. Marshall and C. Sample: Epstein-Barr virus nuclear antigen 3C is a transcriptional regulator. *J Virol* 69*,* 3624-3630 (1995)
50. U. K. Laemmli: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227*,* 680-685 (1970)
51. G. Miller, T. Shope, H. Lisco, D. Stitt and M. Lipman: Epstein-Barr virus: Transformation, cytopathic changes, and viral antigens in squirrel monkey and marmoset leukocytes. *Proc Natl Acad Sci (USA)* 69*,* 383-387 (1972)
52. R. Baer, A. T. Bankier, M. D. Biggin, P. L. Deininger, P. J. Farrell, T. J. Gibson, G. Hatfull, G. S. Hudson, S. C. Satchwell, C. Seguin, P. S. Tuffnell and B. G. Barrell: DNA sequence and expression of the B95-8 Epstein-Barr virus genome. *Nature* 310*,* 207-211 (1984)
53. D. J. Moss and J. H. Pope: Assay of the infectivity of Epstein-Barr virus by transformation of human leucocytes *in vitro*. *J Gen Virol* 17*,* 233-236 (1972)
54. J. W. Kappler, B. Skidmore, J. White and P. Marrack: Antigen-inducible, H-2-restricted, interleukin-2-producing T cell hybridomas. Lack of independent antigen and H-2 recognition. *J Exp Med* 153*,* 1198-1214 (1981)

55. D. L. Woodland, M. P. Happ, J. Bill and E. Palmer: Requirement for cotolerogenic gene products in the clonal deletion of I-E reactive cells. *Science* 247*,* 964-967 (1990)
56. B. Neuhierl, R. Feederle, W. Hammerschmidt and H. J. Delecluse: Glycoprotein gp110 of Epstein-Barr virus determines viral tropism and efficiency of infection. *Proc Natl Acad Sci U S A* 99*,* 15036-15041 (2002)
57. R. J. Murray, M. G. Kurilla, J. M. Brooks, W. A. Thomas, M. Rowe, E. Kieff and A. B. Rickinson: Identification of target antigens for the human cytotoxic T cell response to Epstein-Barr Virus (EBV) :Implications for the immune control of EBV-positive malignancies. *J Exp Med* 176*,* 157-168 (1992)
58. K. N. Heller, C. Gurer and C. Munz: Virus-specific CD4+ T cells: ready for direct attack. *J Exp Med* 203*,* 805-808 (2006)
59. K. S. Slobod, A. S. Freiberg, J. E. Allan, S. D. Rencher and J. L. Hurwitz: T-cell receptor heterogeneity among Epstein-Barr Virus-stimulated T-cell populations. *Virology* 196*,* 179-189 (1993)
60. R. Khanna, S. R. Burrows, M. G. Kurilla, C. A. Jacob, I. S. Misko, T. B. Sculley, E. Kieff and D. J. Moss: Localization of Epstein-Barr Virus cytotoxic T cell epitopes using recombinant Vaccinia: Implications for vaccine development. *J Exp Med* 176*,* 169-176 (1992)
61. S. Nikiforow, K. Bottomly, G. Miller and C. Munz: Cytolytic CD4 (+)-T-cell clones reactive to EBNA1 inhibit Epstein-Barr virus-induced B-cell proliferation. *J Virol* 77*,* 12088-12104 (2003)
62. K. Bickham, K. Goodman, C. Paludan, S. Nikiforow, M. L. Tsang, R. M. Steinman and C. Munz: Dendritic cells initiate immune control of epstein-barr virus transformation of B lymphocytes *in vitro*. *J Exp Med* 198*,* 1653-1663 (2003)
63. R. Omiya, C. Buteau, H. Kobayashi, C. V. Paya and E. Celis: Inhibition of EBV-induced lymphoproliferation by CD4 (+) T cells specific for an MHC class II promiscuous epitope. *J Immunol* 169*,* 2172-2179 (2002)
64. D. J. Moss, R. Khanna and M. Bharadwaj: Will a vaccine to nasopharyngeal carcinoma retain orphan status? *Dev Biol (Basel)* 110*,* 67-71 (2002)

Abbreviations: EBV: Epstein-Barr Virus, BL: Burkitt lymphoma, PBMC: Peripheral blood mononuclear cells, VV: Vaccinia virus, CTL: cytotoxic T lymphocyte, HN-hemagglutinin-neuraminidase, hPIV-human parainfluenza virus, CTM: complete tumor medium

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