Development of an Improved Epstein-Barr Virus (EBV) Neutralizing Antibody Assay to Facilitate Development of a Prophylactic gp350-Subunit EBV Vaccine

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Abstract. No licensed vaccine is available for prevention of EBV-associated diseases, and robust, high-throughput bioanalytical assays are needed to evaluate immunogenicity of gp350 subunit-based candidate EBV vaccines. Here we have developed an improved EBV-GFP based neutralization assay for such a vaccine’s pre-clinical and clinical validation to measure EBV specific neutralizing antibodies in human donors. The supplementation of guinea pig complement of our previously published high-throughput EBV-GFP fluorescent focus (FFA)-based neutralization assay allowed the detection of complement-dependent neutralizing antibodies using a panel of heat-inactivated healthy human sera. Anti-gp350 antibody titers, which were evaluated using a previously optimized anti-gp350 IgG ELISA assay, were moderately correlated to the FFA-based neutralization titers. Overall, this improved high-throughput neutralization assay is capable of characterizing the serologic neutralizing antibody response to natural EBV infection, with applications in evaluating EBV antibody status in epidemiologic studies and immunogenicity of candidate gp350-subunit EBV vaccines in clinical studies.

Keywords: Epstein-Barr Virus; Gp350; Vaccine; Anti-gp350 IgG ELISA; Neutralization assay.

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Introduction. Epstein-Barr virus (EBV) is a prevalent gamma herpes virus and is the causative agent of infectious mononucleosis (IM), a clinical syndrome characterized by fever, pharyngitis, and cervical lymphadenopathy primarily afflicting adolescents and young adults.¹² The virus is transmitted mainly via saliva and is able to infect naïve B cells through binding of the major viral surface glycoprotein gp350 to CD21 (also called CR2) on the B-cell surface via gB and gH/gL/gp42, whereas virus infection of epithelial cells in the absence of CR2 can utilize gH/gL without gp42.³ Following primary infection in B-cells, the virus establishes latency in B cells where it persists for life. This chronic B-cell reservoir can undergo recurrent lytic cycle reactivation, asymptomatic shedding of virus into the saliva, and spread to uninfected individuals, thus facilitating near complete permeation of the human population. In addition to IM, EBV has been associated with a variety of malignant diseases including Burkitt and Hodgkin lymphomas, nasopharyngeal carcinoma, gastric adenocarcinoma, and post-transplant lymphoproliferative disorder.⁴⁷
Despite the ubiquitous prevalence of EBV and associated acute IM disease, the immune response to EBV infection is not completely understood. Of particular interest are the immune correlates associated with symptomatic EBV infection, or IM, compared with asymptomatic EBV infection.

At present, no therapeutic or prophylactic options are approved for the prevention or treatment of EBV-associated diseases. Within the prophylactic approach, both adjuvanted gp350 subunit and vaccinia-vectored gp350 approaches have been evaluated in humans based upon the identification that the majority of gp350 approaches have been evaluated in humans both adjuvanted gp350 subunit and vaccinia-vectored associated diseases. Within the prophylactic approach, both adjuvanted gp350 subunit and vaccinia-vectored gp350 approaches have been evaluated in humans based upon the identification that the majority of neutralizing factors present in EBV-positive serum that is directed against the viral surface glycoprotein, gp350. Safety and efficacy trials have been performed using a CHO-derived soluble gp350 subunit antigen mixed with 3-O-desacyl-4’-monophosphoryl A (AS04) adjuvant. These studies successfully demonstrated safety, tolerability, and immunogenicity in young adults, where the vaccine induced strong antibody responses to gp350. The small phase 2 proof-of-concept trial also revealed a high level of efficacy at preventing acute IM, reaching 100% protection following the third dose. Although clinical protection was observed in this study, a limited evaluation of the immune response was performed.

Historically, EBV neutralizing titers have been quantified using a B cell transformation assay. This method has low sensitivity and is time consuming as it traditionally requires a 4—6 week incubation period for B cell transformation followed by a calculation of neutralizing antibody titers. More recently, the development of a rapid EBV neutralization assay utilizing Raji cells has been described. This is based on infection of an in vitro human B cell line with an EBV encoding green fluorescent protein (GFP) allowing for detection of neutralizing antibodies by flow cytometry. This flow cytometry-based method enables the calculation of neutralizing titers within 48 hours and correlates highly with the historically utilized transformation assay. Although a significant improvement on the traditional B cell transformation assay, the flow cytometry-based platform exhibited low throughput and reduced sensitivity. An adherent cell line, SVK, expressing the native EBV receptor CD21 (also called CR2; SVK-CR2), was utilized for EBV neutralization assay. To increase sample throughput, a fluorescent focus assay (FFA)-based EBV micro-neutralization assay was also developed with SVK-CR2 cells. The report by Lin et al. facilitated our previously described utilization of IsoCyte™ instrumentation, an automation-friendly benchtop laser scanning cytometer, to allow for higher-throughput sample testing.

Based upon previous studies where guinea pig complement has been shown to expand the linear range of viral neutralization assays, the current study demonstrates that the inclusion of guinea pig complement improves both the flow cytometry-based Raji and FFA-based SVK-CR2 assays by allowing detection of complement-dependent neutralizing antibodies. Together these results facilitate the supplementation of guinea pig complement of the high-throughput EBV-GFP SVK-CR2 FFA-based and EBV-GFP Raji FACS-based micro-neutralization assays to determine EBV neutralizing titers in EBV vaccine research and development.

An anti-gp350 IgG ELISA assay, which was previously optimized through gp350 coating concentration and sera dilutions and described by Wu et al., was utilized to quantify the human anti-gp350 antibody titers across a panel of healthy human donors whose EBV antibody status was pre-determined using commercial ELISAs.

**Materials and Methods.**

**Human sera panel to assess anti-gp350 antibody and neutralization antibody titers.** Healthy human donor serum samples were purchased from AllCells, LLC and Bioreclamation, LLC (n=39). Samples were heated at 56 °C for 30 minutes to inactivate complement, and then the EBV antibody status was determined using commercial kits (Diamedix, Miami Lakes, FL) to measure IgG and IgM antibody responses to EBV viral capsid antigen (VCA). The panel of sera was then examined with the anti-gp350 IgG ELISA, flow cytometry (FACS)-based micro-neutralization assay and FFA-based micro-neutralization assay with or without guinea pig complement supplement.

**Anti-gp350 IgG ELISA.** Immulon 4HBX High-binding 96-well plates (ThermoFisher Scientific) were coated with 0.25 μg/ml recombinant purified gp350 (suspension CHO cell line produced) in PBS overnight at 4 °C (all subsequent steps were performed at room temperature). The plates were washed 4 times with PBST (PBS with 0.05% Tween-20) and blocked with Superblock (ThermoFisher Scientific) for 1 hour. Human serum samples along with positive and negative control serum were serially diluted 1:5 in dilution buffer containing 0.1% Superblock in PBST (1:10 to 1:97,656,250 for sample, 1:10 to 1:781,250 for positive control, and 1:10 to 1:1250 for negative control) at 100 μL/well and incubated for 1 hour. After washing, HRP-conjugated secondary antibody (Rabbit anti-human IgG HRP, Dako® P0214) was diluted to 1: 7,500 in dilution buffer and incubated for 1 hour, followed by 4 washes in PBST. TMB substrate (SIGMA) was added at 100 μL/well for colorimetric reading. After adding 1N HCl at 100μL/well to stop the reaction, the absorption was measured at OD450 on a SpectraMax plate reader. The antisera endpoint titer was quantified as the reciprocal dilution factor using SoftmaxPro to calculate the 4-fold rise above the

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ELISA assay background. The lower limit of detection (LOD) of this assay is 10. Negative samples were assigned an artificial titer of 0.1 for graphing purpose.

**Cells.** Akata-BX1-g, a lymphoma cell line engineered to express GFP in the EBV virus genome, was received from Dr. Lindsey Hutt-Fletcher (Louisiana State University). Akata-BX1-g cells were grown in suspension in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, Penicillin/Streptomycin, L-Glutamine, and 500 µg/mL G418 at a maximum passage density of 2 x 10^6 cells/ml. SVK-CR2, an epithelial cell line that overexpresses CD21, was grown in high glucose DMEM supplemented with 10% FBS, Penicillin/Streptomycin, L-Glutamine, 10 ng/mL Cholera Toxin, and 400 µg/mL G418. Raji cells (ATCC) were grown in suspension in RPMI1640 medium supplemented with 10% FBS, Penicillin/Streptomycin, and L-Glutamine.

**EBV-GFP virus induction, harvest, and titration.** The EBV virus was obtained by induction of virus production from Akata-BX1-g cells. EBV-GFP virus was purified from 4 x 10^9 Akata-BX1-g cells that had been pelleted down and re-suspended at a concentration of 4 x 10^6 per mL in 1 liter of virus growth medium (RPMI 1640 medium supplemented with 1% heat-inactivated FBS, Penicillin/Streptomycin, and L-Glutamine) and induced with 60 µg goat anti human-IgG (MP Biomedicals #55049) for a final concentration of 60 µg/mL for 5 days. On day 2, an additional 1 liter of virus growth medium was added to achieve a final anti-human-IgG concentration of 30 µg/mL. On day 5, supernatants were harvested by centrifuging at 4000g for 15 minutes to remove cells, filtering on a 0.8 µm Nalgene MF75 filter unit, and then centrifuging at 16,000g for 90 minutes to pellet the virus. Viral pellets were re-suspended in 20 mL RPMI containing 100 µg/mL bacitracin (Sigma), aliquoted and stored at -80 °C.

EBV-GFP virus was titrated as previously described. SVK-CR2 cells were seeded in 96 well plates one day prior to assay. EBV-GFP with a titer of 500 FFU per well was pre-incubated with the serially-diluted monoclonal control antibody (72A1, Rockland Inc.) or heat-inactivated serum samples in triplicates for 30 minutes, and then SVK-CR2 cells were infected with these serum/virus mixtures as described in above section. The assay was performed either with or without 1% guinea pig complement in RPMI complete medium. 25 µL of the diluted virus was added to each well of 96-well U bottom plates before the addition of an equal volume of 1:2 serially diluted heat-inactivated serum in triplicates beginning at 1:20 dilution. Viral and serum dilutions were co-incubated for 2 hours at 37°C before the addition of 10^5 Raji cells in 200 µL volume, followed by one-day incubation at 37°C. Cells were pelleted by centrifugation at 1,200 rpm for 5 min and fixed with 200 µL/well of 4% paraformaldehyde for 5 minutes at room temperature. Fixed cells were washed, re-suspended in DPBS buffer, and analyzed by flow cytometry on a Guava instrument (Millipore) to determine the percentage of GFP positive Raji cells. Results were analyzed with non-linear regression fit using Graphpad Prism software. The serum dilution at 50% inhibition of viral infection (IC50) was quantified as titer, and Log2-transformed data were compared.

**EBV-GFP FFA-based micro-neutralization assay in SVK-CR2 cells.** SVK-CR2 cells were seeded in 96 well plates one day prior to assay. EBV-GFP with a titer of 500 FFU per well was pre-incubated with the serially-diluted monoclonal control antibody (72A1, Rockland Inc.) or heat-inactivated serum samples in triplicates for 30 minutes, and then SVK-CR2 cells were infected with these serum/virus mixtures as described in above section. The assay was performed either with or without 1% guinea pig complement in SVK-CR2 culture medium. Neutralization titers were calculated as IC50 value in Log2 (serum dilution factor) of non-linear regression fit in GraphPad Prism. The maximum infection is normalized to virus only as 100%, and the minimum infection is normalized to cell only as 0%. IC50 corresponds to antibody dilution at 50% of (Max – Min infection).

Statistics. Correlation between FFA-based micro-neutralization assay, FCS-based micro-neutralization assay, and anti-gp350 IgG ELISA were evaluated by Pearson test using GraphPad Prism software for statistical analysis.

**Results.**

**Quantitation of human anti-gp350 antibody titers with anti-gp350 IgG ELISA.** A total of 39 serum samples were tested for the presence of anti-gp350 antibody titers using the optimized anti-gp350 IgG ELISA assay. Quantitative analysis, obtained by determination of gp350 endpoint dilution titers from this sample set (n=39), ranged from negative (LOD is 10) to a titer of...
25830.5, demonstrating a wide range of antibody titers across this population. Of the 39 samples, six sera tested negative for VCA IgM and IgG. Three of these six were also below the limit of detection for gp350 and were classified as anti-gp350 negative. The remaining three VCA negative sera had positive gp350 titers of 220.6, 445.4, and 612.8. Of the 33 EBV VCA seropositive sera, two sera were VCA IgM positive and VCA IgG negative, and 31 sera were VCA IgG positive and VCA IgM negative or “equivocal.” No sample tested positive for both VCA IgG and VCA IgM. The two VCA IgM positive sera were gp350 IgG negative, which makes sense in that the VCA IgM response occurs within 7—10 days after onset of symptoms, whereas the gp350 IgG antibody response takes months to develop. Evidently, these two individuals were in the early stages of a primary EBV infection. All 31 VCA IgG positive sera were also gp350 IgG positive. The gp350 titers ranged from 26.2 to 25830.5, with the median titer at 991.04.

Quantitation of EBV-GFP neutralization titers by high-throughput fluorescent focus assay (FFA) or flow cytometry (FACS)-based neutralization assay. Two different assay formats were used to measure GFP encoding EBV neutralizing antibody titers in 39 human sera samples: i) the FACS-based neutralization assay in Raji cells and ii) the FABA-based neutralization assay in SVK-CR2 cells. In each assay platform, the human sera were tested in two conditions, supplemented with or without 1% Guinea Pig Complement.

For the FACS-based assay, the mean neutralization titer of the 39 samples produced without guinea pig complement, which was expressed as log2 (IC50), was 3.414 [95% confidence interval (CI), 3.293-3.535]. For the FACS-based assay supplemented with 1% guinea pig complement, the mean titer was significantly (P<0.0001) increased to 4.890 (95% CI, 4.433-5.347) (Figure 1A). For the FFA-based assay, the mean neutralization titer of the 39 samples produced without guinea pig complement was 6.599 (95% CI, 5.863-7.335). For the FFA-based assay supplemented with 1% guinea pig complement, the mean titer was significantly (P<0.0001) increased to 9.314 (95% CI, 8.466-10.16) (Figure 1A). We also compared the two assay formats supplemented with guinea pig complement, the mean titer produced by SVK-CR2 format (9.314) was also significantly (P<0.0001) higher than that produced by Raji format (4.890) (Figure 1A).

For the four conditions, FACS without complement, FACS with complement, FFA without complement, FFA with complement, the detection rate of neutralizing antibodies above the limit of detection (3.32 Log2 Neut. titer) was 12.8%, 69.2%, 87.2%, and 89.7% respectively; the detection rate two folds above the limit of detection (4.32 Log2 Neut. titer) was 2.6%, 61.5%, 71.8%, and 89.7% respectively. The correlation between neutralization titers from FABA-based assay with complement and FACS-based assay with complement were established (Pearson r value=0.5931, P value <0.0001, data not shown). The correlation between neutralization titers from FABA-based assay with complement and human anti-gp350

![Figure 1. Quantitation of EBV neutralization titers in human sera. A. Comparison of neutralization titer tested by Fluorescent Focus Assay (FFA)-based or Flow cytometry (FACS)-based neutralization assay with or without guinea pig complement. Healthy human donor sera samples obtained from AllCells, LLC and Bioreclamation, LLC (n=39) were tested in two different assay formats: FABA-based neutralization in SVK-CR2, the CR2-expressing transfected human epithelial cell line, or FACS-based neutralization assay in Raji, the human B lymphoblastoid cell line. In each assay format, the human sera were also tested under two conditions, supplemented with or without 1% guinea pig complement. The limit of the detection of both assay formats is 3.32 Log2 neutralization titer. The limit of the detection of both assay formats is 3.32 Log2 neutralization titer. B. Correlation of EBV anti-gp350 IgG ELISA and the functional FABA-based neutralization assay supplemented with 1% guinea pig complement. Assay correlation was established with 32 human sera which tested positive in both assays (Pearson r value=0.5844, P value =0.0004).](image-url)
IgG ELISA titers were established as well (for all 39 samples: Pearson r value =0.5573, P value =0.0002, data not shown; for 32 sera samples tested positive in both assay: Pearson r value =0.5844, P value =0.0004, Figure 1B). There was no correlation observed between neutralization titers from FACS-based assay and human anti-gp350 IgG ELISA titer (data not shown).

Discussion. In this study, an improved guinea pig complement-supplemented high-throughput EBV-GFP SVK-CR2 FFA-based neutralization assay was developed for use in clinical investigations of disease outcome following primary EBV infection. This assay also has utility in staging the phase of EBV infection, in epidemiologic studies, and in the clinical development of a prophylactic EBV vaccine or therapeutic agent. In the determination and assessment of assay parameters suitable for use in these studies, sera from healthy human donors were used to confirm the key humoral immune responses against natural EBV infection, characterized by anti-gp350 IgG titer and EBV neutralizing titer.

Determination of clinical efficacy for any prophylactic EBV study is predicated upon the accurate identification and grouping of clinical study participants into those who have been previously infected by the virus and thus are EBV seropositive, and those who have not been infected and are EBV naïve participants. Since we had only collected at a later timepoint from VCA seronegative subjects may have value in confirming their true EBV seronegative status. Based on our results, an extra set of sera might suggest that gp350 specific immune responses might represent a reflection of the difference in timing of EBV VCA vs. gp350 specific response of acute EBV infection. Therefore, the interpretation of a VCA “seronegative” results may not exclude EBV infection, and it may be possible to observe gp350 positive results in these samples as we demonstrate in this study. Based on our results, an extra set of sera collected at a later timepoint from VCA seronegative subjects may have value in confirming their true EBV VCA and anti-gp350 IgG serostatus. Since we had only a small number of VCA seronegative samples (n=6) in this study, it may be worth testing more samples in the future to determine the percentage of anti-gp350 IgG positive samples among the VCA seronegative samples. If they are found with some frequency and are true positives based on a panel of other EBV-specific assays, individuals with this antibody profile would not be considered appropriate for vaccine trials of EBV-naïve participants.

Lastly, understanding the mechanism of action of any prophylactic EBV vaccine is imperative for identifying the correlates of protective immunity to prevent IM in pre-adolescents and young adults. Human antibodies to EBV gp350 and EBV gp42 have been shown to block infection of B cells by EBV. However, anti-EBV gp350 antibodies are reported to neutralize infectivity more effectively than antibody titers to EBV gp42.13 It is also reported that elevated titers of EBV neutralizing antibody and anti-gp350 antibody were low-risk biomarkers for nasopharyngeal carcinoma, an EBV-related epithelial tumor.15 In this study, a moderate correlation22 between anti-gp350 IgG ELISA titer and EBV-GFP SVK-CR2 neutralization antibody titer in healthy human donor sera was established. Thus, the induction of high titer EBV gp350 neutralizing antibodies may represent an essential correlate of protection and mechanism of
action to be monitored.

In this study, two different assay formats, the FFA-based neutralization assay in SVK-CR2 and the FACS-based neutralization assay in Raji were compared to measure EBV neutralizing antibody titers in the same set of 39 healthy human donor sera samples. The development of a higher throughput FFA-based neutralization assay was previously published by MedImmune/AstraZeneca. In our study, we further improved the assay for human sample application by supplementing an equal amount of the guinea pig complement to the heat-inactivated human sera. The heat-inactivation step during the sample processing was to ensure the removal of natural complement, which might exist in various amounts in human sera and contribute to the variation of neutralization titer. Currently, there are two types of neutralizing antibodies that were reported, complement-independent neutralizing antibodies versus complement-dependent neutralizing antibodies. Analysis of heat-inactivated sera would get the result for complement-independent neutralizing antibodies, whereas the addition of guinea pig complement to heat-inactivated sera would allow the assay to detect complement-dependent neutralizing antibodies. For the FFA-based assay, the detection of EBV neutralizing antibodies among the 39 samples increased moderately from 71.8% to 89.7% when the cutoff value was set as two-fold above limit of detection with the inclusion of guinea pig complement. For the FACS-based assay, the detection of EBV neutralizing antibodies increased dramatically from 2.6% to 61.5% with the inclusion of guinea pig complement. Both assay formats are of value, as EBV neutralizing analysis using SVK-CR2 cells detects the titers of neutralizing antibodies against EBV infection of epithelial cells, whereas neutralizing analysis using Raji cells detects the titers of neutralizing antibodies against EBV infection of B cells. Together the results support supplementation of the guinea pig complement of both high-throughput EBV-GFP SVK-CR2 FFA-based neutralization assay and EBV-GFP Raji FACS-based assay for determination of EBV neutralizing titers in human EBV vaccine program. The throughput of the FFA-based assay also supports its use in large scale, multicenter studies.

In summary, an improved guinea pig complement-supplemented high-throughput EBV-GFP SVK-CR2 FFA-based neutralization assay has been developed for evaluating humoral responses to EBV during epidemiologic studies, selection, and follow-up of participants in EBV vaccine trials.

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