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Human Antibody Titers to Epstein-Barr Virus (EBV) gp350 Correlate with Neutralization of Infectivity Better than Antibody Titers to EBV gp42 Using a Rapid Flow Cytometry-Based EBV Neutralization Assay

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Abstract

Measurement of neutralizing antibodies to Epstein-Barr virus (EBV) is important for evaluation of candidate vaccines. The current neutralization assay is based on antibody inhibition of EBV transformation of B cells and requires 6 weeks to perform. We developed a rapid, quantitative flow cytometry assay and show that neutralizing antibody titers measured by the new assay strongly correlate with antibody titers in the standard transformation-based assay. Antibodies to EBV gp350 and gp42 have been shown to block infection of B cells by EBV. Using new assays to quantify antibodies to these glycoproteins, we show for the first time that that human plasma contains high titers of antibody to gp42; these titers correlate with neutralization of EBV infectivity or transformation. Furthermore, we show that antibody titers to EBV gp350 correlate more strongly with neutralization than antibody titers to gp42. These assays should be useful in accessing antibody responses to candidate EBV vaccines.

Keywords

Epstein-Barr virus; antibody-mediated neutralization; herpesvirus; glycoprotein 350; glycoprotein 42; transformation

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Introduction

Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus that usually infects children who develop nonspecific symptoms or remain asymptomatic (Cohen, 2000). Infection of adolescents or young adults with EBV can result in infectious mononucleosis. EBV is also associated with several malignancies including Burkitt lymphoma, nasopharyngeal carcinoma, Hodgkin lymphoma, non-Hodgkin lymphoma, and post-transplant lymphoproliferative disease. EBV infection of B cells is initiated by interaction of its major surface glycoprotein gp350 with its cellular receptor CD21 (also known as CR2 or the C3d complement receptor) (Fingeroth et al., 1984; Frade et al., 1984; Nemerow et al., 1985). EBV gp42 binds to MHC class II and functions as a co-receptor for the virus in B cells (Li et al., 1997).

Antibody to EBV gp350 can neutralize infectious virus (Hoffman et al., 1980; Jackman et al., 1999; Miller et al., 1982; Moutschen et al., 2007). This observation has led to the use of gp350 as a candidate vaccine for EBV. Such vaccines have shown protection against disease in monkeys (Epstein et al., 1985; Finerty et al., 1994; Morgan et al., 1988a; Ragot et al., 1993), a trend toward protection from infection in humans (Gu et al., 1995), and protection from development of mononucleosis in humans (Sokal et al., 2007). The level of neutralizing antibody to EBV has correlated with protection from infection in some (Finerty et al., 1992), but not all (Morgan et al., 1988b; Ragot et al., 1993) vaccine studies in primates. The titer of neutralizing antibody to EBV is a useful surrogate marker for evaluating EBV vaccines. Monoclonal antibody to EBV gp42 has also been shown to neutralize virus infection of B cells (Li et al., 1995), but antibody to gp42 has not been demonstrated in human plasma or sera.

The conventional method to quantify neutralizing antibody tests the ability of antibody to inhibit EBV transformation of human peripheral blood B cells (Miller et al., 1972; Moss and Pope, 1972). This assay requires approximately 6 weeks to perform and uses peripheral blood mononuclear cells (PBMC) or umbilical cord blood mononuclear cells for EBV infection. Another assay for measuring neutralization involves the ability of antibody to inhibit Raji cell infection measured by immunofluorescent staining of the cells or counting foci of clumped cells (Pearson et al., 1970; Rocchi and Hewetson, 1973). These assays are read manually, are somewhat subjective, and are very labor intensive. A competition ELISA, based on a monoclonal antibody which has neutralizing activity, has also be used as a surrogate to estimate EBV-neutralizing activity (Wilson and Morgan, 1998). ELISA assays have also been used to measure antibodies to gp350 (Randle and Epstein, 1984); these assays measure total anti-gp350 antibodies which may include both neutralizing and non-neutralizing antibodies.

Recently neutralizing antibody assays have been developed using viruses (Biacchesi et al., 2005; Bilello et al., 2006; Earl et al., 2003; Khawplod et al., 2005) or virus particles (Pierson et al., 2006) that express green fluorescent protein (GFP). These assays are highly quantitative, reproducible, and rapid. While these assays have been used for other viruses, they have not been adapted to human herpesviruses. Here we report the use of a GFP-based infection neutralization assay to quantify the titer of neutralizing antibody to EBV and use this assay to compare EBV neutralizing titers in human plasma to the conventional transformation neutralization assay. We also compare the level of EBV neutralizing antibody in human plasma to the titer of anti-gp350 and gp42 antibodies measured by a novel immunoprecipitation assay.

Results

Neutralization of EBV using the GFP-based infection neutralization assay follows the percentage law

An assay that measures neutralization of EBV by sera, plasma, or monoclonal antibody needs to be validated for certain conditions. The percentage law, states that the titer of neutralizing

antibody is not affected by the amount of virus present if the neutralizing antibody is in excess over the virus (Andrewes and Elford, 1933; Brioen and Boeye, 1985). While the standard neutralization assay for EBV is performed using a fixed amount of virus for each assay, it is difficult to validate that the standard assay actually follows the percentage law since the assay is very time consuming and subject to a large number of variables. To determine how a GFP-based EBV neutralizing assay conforms to the percentage law, we incubated 5 serial 2-fold dilutions of B95-8/F virus (an EBV that expresses GFP) with serial dilutions of 72A1 monoclonal antibody (which targets EBV gp350 and neutralizes virus infectivity). The mixture was added to Raji cells and after 3 days the cells were fixed in paraformaldehyde and the number of GFP-expressing cells was measured by flow cytometry.

The percentage of GFP-positive cells, which is indicative of EBV-infected cells, was plotted against the amount of 72A1 antibody added (g/ml) (Fig 1A). The effective dilution of antibody that inhibited infectivity by 50% (EDI_{50}), based on the reduction of GFP-positive cells, was calculated by regression analysis. Curves depicting the relationship between infection and amount of antibody were similar over a wide range of virus titers (Fig. 1A), and the EDI_{50} was nearly identical for the different antibody titers ranging from 1.2 to 1.8×10^{-6} g of antibody per ml (Table 1). Incubation of serial dilutions of B95-8/F with serial dilutions of a human plasma, followed by infection of Raji cells and quantification of GFP-positive cells also yielded similar curves (Fig. 1B) with a narrow EDI_{50} ranging from neutralizing dilutions of 52.58 to 59.29 (Table 1). These results indicate that the GFP-based EBV neutralization assay obeys the percentage law for both a monoclonal antibody and for human plasma.

Neutralizing titers of human plasma by GFP-based assay

The GFP-based neutralizing titration assay was used to measure EBV neutralizing titers in 31 human plasma samples; 28 were seropositive and 3 were seronegative by ELISA for viral capsid antigen (VCA). B95-8/F virus was used at a titer so that 1 to 4 % of Raji cells were infected which allowed measurements that conform to the percentage law. Serial dilutions of plasma from an EBV seropostive donor yielded a sigmoidal curve when the number of GFP-positive cells was plotted against the dilution of plasma, and the EDI_{50} was 23.35 (Fig. 2A). In contrast, plasma from an EBV seronegative donor failed to neutralize B95-8/F (Fig. 2B). Of 29 plasma samples from EBV seropositive donors, 28 were positive and 1 was negative for EBV neutralizing antibody. For the 28 positive samples the geometric mean EDI_{50} was 35.13 (95% confidence interval [CI], 19.88–62.06), the median was 31.67, and the range was 1.784 to 1309. (Fig 3A). The EBV VCA ELISA assay in the donor that had no detected neutralizing antibody was 1.45 which was in the lowest quartile of the VCA titers.

The GFP-based infection neutralization titer shows a strong correlation with the conventional transformation neutralization assay titer using human plasma

Of the 31 plasma samples tested by the GFP-based neutralization assay, sufficient volumes were available to test 28 samples in the conventional neutralization assay. Of these 28 samples, 26 were from EBV seropositive and 2 were from EBV seronegative donors. Serial dilutions of human plasma were incubated with B95-8 EBV in 96 well plates, PBMCs were added, and after 6 weeks the number of wells containing transformed B cells was counted and the effective dilution of plasma that inhibited transformation by 50% (EDT_{50}) was calculated. Both plasma samples from EBV seronegative donors were also negative in the conventional transformation neutralization assay. All 26 plasma samples from EBV seropositive donors were positive in the conventional transformation neutralization assay, including the one sample which was negative in the GFP-based infection neutralization assay. For the 26 samples positive in the transformation neutralization assay, the geometric mean EDT_{50} was 42.49 (95% CI, 20.32–88.87), the median was 22.99, and the range was 2.830 to 3158 (Fig 3A). The one sample that was negative in the GFP-based infection neutralization assay, but positive in the conventional

transformation neutralization assay had a titer of 2.830, the lowest among the positive samples in this assay.

Comparison of the results of 25 samples which were positive in both GFP-based infection and conventional transformation neutralizing assays showed a highly significant correlation between the two assays. The Spearman's rank correlation coefficient was r=0.8708 (95% CI 0.7192 to 0.9432, P<0.0001, Fig. 3B). The neutralization titers for the 25 samples were similar in the two assays with geometric means of 41.80 (95% CI 23.80–73.42) and 47.36 (95% CI 22.75–98.60) for the GFP-based infection assay and the conventional transformation assay, respectively. The medians of the neutralization titers of the 25 samples were also similar to each other (median 34.00 vs. 23.34, GFP-based infection assay vs. conventional transformation assay, respectively).

To further compare the two assays, the GFP-based infection neutralizing antibody titer was divided by the conventional transformation neutralizing antibody titer for each plasma sample. The median of this ratio was 1.083, the range was 0.1278 to 4.361, and the 10th and 90th percentiles were 0.2759 and 2.668, respectively. These results indicate that these two assays provide very similar neutralizing antibody titers.

EBV neutralization titers show a strong correlation with gp350 antibody titers measured by a novel immunoprecipitation assay

Previously we used luciferase immunoprecipitation systems (LIPS) assays to quantify antibody responses to a panel of herpes simplex virus (HSV)-1 and HSV-2 antigens (Burbelo et al., 2009). In the present study, we evaluated all 31 plasma samples using LIPS assays for anti-gp350 EBV antibodies. This assay quantifies the amount of antibody that immunoprecipitates with a gp350-*Renilla* luciferase fusion protein expressed in human Cos1 cells; thus the antigen recognized by the plasma is non-denatured gp350. All plasma samples from EBV seropositive donors were positive in the LIPS gp350 antibody assay, and plasma from the two EBV seronegative donors were below the cut-off value of the LIPS gp350 assay and were therefore negative. For the 29 plasma from EBV seropositive donors, the geometric mean was 34,909 LU (95% CI, 22931–53144), the median was 36,436 LU, and the range was 3,048 to 176,217 LU (Fig. 4A).

Comparison of neutralization titers in plasma samples which were positive by the GFP-based infection assay (28 samples) or conventional transformation assay (26 samples) with the LIPS gp350 antibody assay showed a strong correlation between the three assays. The LIPS gp350 antibody assay correlated best with the GFP-based infection neutralization assay showing a Spearman's r value of 0.8550 (95% CI, 0.7017 to 0.9326, P<0.0001) (Fig 4B); the LIPS gp350 antibody assay also showed a strong correlation with the conventional transformation neutralization assay with r=0.7559 (95% CI 0.5122 to 0.8869), P<0.0001 (Fig. 4C).

Comparison of the LIPS gp350 antibody assay with the VCA IgG ELISA assay showed a less robust correlation than with the neutralization assays with r=0.4839 (95% CI 0.1237 to 0.7314), P=0.0091 (Fig 4D).

We tested 14 additional coded plasma for antibody to gp350 and in the GFP-based neutralization assay. All 6 EBV seronegative plasma (based on VCA IgG ELISA) obtained from adults were negative in both the gp350 antibody and in the GFP-based neutralization assay. All 3 EBV seropositive plasma from adults were positive in both gp350 and the neutralization assay. Of 5 seropositive plasma from umbilical cord bloods, all were seropositive in the gp350 antibody assay and 3 of 5 were positive in the GFP-based neutralization assay. Thus, the gp350 antibody assay correlated well with the VCA ELISA assay for both seropositive and seronegative samples.

EBV neutralization titers show a less robust correlation with gp42 antibody titers than to gp350 antibody titers

Similar to the results observed for the gp350 antibody titers, all 29 plasma samples from EBV seropositive donors were positive in the LIPS gp42 antibody assay, while plasma from 2 seronegative donors were below the cut-off value of the LIPS gp42 assay. The geometric mean, median and range of the 29 plasma from EBV seropositive donors were 162334 LU (95% CI; 116710 to 225793 LU), 181750 LU, 25989 to 655407 LU, respectively (Fig. 5A). Moderate correlations were shown between LIPS gp42 antibody titers and the GFP-based infection neutralizing assay (r=0.6700, P<0.0001), the conventional transformation neutralizing assay (r=0.5405, P=0.0044), LIPS gp350 antibody titers (r=0.6103, P=0.0004), and the VCA IgG ELISA assay (r=0.4275, P=0.0233). (Fig. 5B–E).

Discussion

We have shown that a GFP-based EBV infection neutralization assay compares favorably with the conventional transformation-based neutralization assay. In addition we find that human plasma contains high titers of antibody to gp42 that correlate with neutralization of EBV infectivity or transformation; however antibody titers to EBV gp350 correlate more strongly with neutralization than antibody titers to gp42.

Quantification of EBV neutralizing antibody titers in humans is important for evaluation of candidate EBV vaccines. Conventional assays that measure antibodies which neutralize B cell transformation by EBV are very cumbersome and require lengthy periods of time (usually 6 weeks) to obtain results. Human PBMCs or cord blood mononuclear cells must be used; the latter are not readily available. These cells may be infected with adventious agents and require precautions when working with uncharacterized human specimens. PBMCs vary in their susceptibility to infection and transformation. B95-8 cells, which were derived from cottontop tamarins- an endangered species- cannot be commercially distributed. Specific aspects of the assay, particularly the variation in human cells from donor to donor, differences in fetal bovine serum, and differences in handling the cultures during the 6 weeks required for the assay may result in variation when results are compared among different laboratories.

The GFP-based infection neutralizing titration assay we report here has a number of advantages that circumvent the problems associated with the transformation assay. The GFP assay requires only 3 to 4 days and uses an established cell line- Raji- rather than heterogeneous populations of primary human cells. This assay is not highly dependent on the concentration of virus used since it conforms to the percentage law. These properties should result in less variation when performed over different times and between different laboratories. The ease in performing the assay and the shorter time period allows multiple replicates to be performed. We found that the neutralizing titers obtained by the GFP-based infection assay had a strong correlation with the conventional transformation assay. Thus, the GFP-based assay is a useful alternative to the conventional, transformation-based assay for measuring neutralizing antibody.

Borza et al. (2002) reported that EBV derived from epithelial cells infects B cells more efficiently than epithelial cells, while EBV derived from B cells infects epithelial cells more effectively. The virus used in our GFP-based assay, B95-8/F, is grown in 293/2089 human epithelial cells, while the virus used in the conventional assay, B95-8, is propagated in cotton top tamarin B cells. Since EBV is thought to be transmitted from saliva, which contains virus derived from lytically infected epithelial cells (Jiang et al., 2006), neutralization of virus produced in human epithelial cells may be more appropriate than virus produced in monkey B cells.

We found that the GFP-based infection neutralization assay conforms with the percentage law, which indicates that the titer of neutralizing antibody is independent of the quantity of virus (or viral antigen) if antibody is in excess relative to the amount of virus at informative points on the sigmoidal neutralization curve (Andrewes and Elford, 1933). Since the B95-8/F virus is derived by transfecting cells with a plasmid expressing BALF4 (glycoprotein 110 or gB), there were concerns that excessive amounts of gp110, which is required for virus fusion with B cells, in the virus preparation could interfere with the neutralization assay. Similar concerns with soluble antigens or defective virus particles have been raised with other neutralization assays (Pierson et al., 2006). As we showed in this study, the EBV GFP-based neutralization assay obeyed the percentage law over a wide range of virus titers; therefore additional gp110 generated from the transfection did not interfere with the assay.

EBV gp350 and gp42 do not have homologs in other human herpesviruses and both glycoproteins are important for virus infection. EBV gp350 is expressed in the virus envelope and on virus-infected cells (Beisel et al., 1985; Johannsen et al., 2004), and antibody to gp350 is known to neutralize virus infectivity (Hoffman et al., 1980; Jackman et al., 1999; Miller et al., 1982; Moutschen et al., 2007). EBV gp350 is important for attachment to cells (Tanner et al., 1987). We found that the level of antibody to gp350 based on the LIPS assay showed a strong correlation to both the GFP-based and transformation-based neutralizing antibody titers (r=0.8550 and 0.7559, respectively, P <0.0001). The LIPS assay measures antibodies that immunoprecipitate gp350 expressed in human cells in non-denaturing conditions. Many assays for gp350 antibodies are ELISA-based and rely on antibody binding to small peptides bound to a plastic well (Randle and Epstein, 1984). A previous study showed that EBV neutralizing antibodies recognize conformation-dependent epitopes (Zhang and Marcus-Sekura, 1993). Therefore antibodies that detect gp350 using synthetic peptides on a plate by ELISA may be less likely to correlate with neutralizing titers than antibodies that recognize gp350 protein expressed in mammalian cells by immunoprecipitation in the LIPS assay.

EBV gp42 is present on the surface of infected cells and in the virion envelope (Johannsen et al., 2004). EBV gp42 is important for fusion of virus with the cell (Kirschner et al., 2007; Miller and Hutt-Fletcher et al., 1988). EBV produced in epithelial cells contains higher levels of gp42 than EBV made in B cells, and the former virus infects B cells more efficiently than the latter (Borza et al., 2002). Thus, the amount of gp42 in EBV may determine tropism of the virus for different types of cells. Antibody to gp42 can neutralize EBV infectivity of B cells (Li et al., 1995) and virus lacking gp42 is unable to infect B cells (Wang and Hutt-Fletcher, 1998).

We found while that antibody titers to gp42 based on the LIPS assay correlated with neutralization of virus by the conventional transformation and the infection GFP-based assays, the correlation with the neutralization assays was less robust than for that observed with the gp350 antibody assay. These results suggest that antibody to EBV gp350 may be a more important contributor to EBV neutralizing activity in human plasma than antibody to gp42. Nevertheless antibodies produced to glycoproteins that are important for different stages of infectivity, attachment (gp350) and fusion (gp42), both correlate with neutralization of infectivity.

The neutralization titers measured by the GFP assay measure the ability of antibody to prevent infection of B cells, while the conventional neutralization assay with B95-8 virus measures antibodies that inhibit EBV-induced transformation. EBV has a very high transformation efficiency and can transform 3 to 10 % of B cells in culture (Henderson et al., 1977; Sugden and Mark, 1977). Thus, while these assays measure somewhat different properties of the virus, the efficiency of EBV transformation likely explains the strong correlation of the results of the two assays.

In summary, we report the development of two novel assays whose activities correlate well with the conventional transformation based neutralizing assay. While the LIPS assay for gp350 does not distinguish between neutralizing and non-neutralizing antibodies, it can be done rapidly, has a wide dynamic range, and the results from the assay strongly correlated with the conventional neutralizing assay. The GFP-based infection neutralization assay is highly quantitative and easy to perform. This assay may ultimately replace the more cumbersome transformation neutralization assay and obviate the need to obtain primary human mononuclear cells and to wait several weeks for the results.

Materials and methods

Cells

B95-8 cells (an EBV-transformed cotton top tamarin cell line), Raji cells (an EBV-positive human Burkitt lymphoma cell line), and HB168 cells (a mouse hybridoma that expresses anti-gp350 neutralizing antibody (American Type Culture Collection, Manassas, VA) were propagated in culture media (RPMI 1640 with 10% fetal bovine serum [FBS], 100 U/mL penicillin, 100 ug/mL streptomycin and 2mM L-glutamine). 293/2089 cells (Delecluse et al., 1998), which contain the B95-8/F virus genome, were kindly provided by Henri-Jacques Delecluse and Bill Sugden and were cultured in DMEM with 10% FBS, L-glutamine, penicillin, streptomycin, and 50uM hygromycin.

Blood was obtained from healthy donors at the Warren G. Magnuson Clinical Center at the National Institutes of Health under an Institutional Review Board approved protocol and PBMCs were isolated from buffy coat preparations using Ficoll-Paque Plus (GE Healthcare BioSciences AB, Uppsala, Sweden). EBV-infected B cells were propagated in transformation media (IMDM with Glutamax [Invitrogen, Carlsbad, CA] in 20% fetal bovine serum, L-glutamine, penicillin, and streptomycin). Plasma was obtained from blood bank donors at the National Institutes of Health and from healthy donors at the Center for Cell and Gene Therapy, Baylor College of Medicine under protocols approved by Institutional Review Boards at both institutions.

Viruses and virus titrations—EBV B95-8 was obtained from B95-8 cells after incubation with 20ng/mL 12-*O*-tetradecanoyl-phorbol-13-acetate and 3mM butyric acid (Sigma-Aldrich, St. Louis, MO). After 72 hrs, the cells were collected by centrifugation (300 × g), the supernatant was transferred to 4°C, and the cell pellet was frozen and thawed 3 times, added back to the supernatant, and the mixture was centrifuged at 300 × g. The supernatant was passed through a 0.45 um MCE membrane filter (Millipore, Billerica, MA) and then underwent ultracentrifugation at 10,000 × g for 1 hr. The pellet was resuspended in RPMI with 10% FBS and the concentrated EBV B95-8 virus stock was stored at −70°C until use.

B95-8/F virus, which expresses GFP, was prepared from 293/2089 cells (Delecluse et al., 1998). 293/2089 cells were transfected with plasmids p509 and p2670 expressing EBV BZLF1 and EBV BALF4 (Neuhierl et al., 2002), respectively, using lipofectamine 2000 (Invitrogen). At 72 hrs after transfection, medium was collected and passed through a 0.80 um MCE membrane filter, and the B95-8/F virus stock was stored at 4°C for several weeks or −70°C for long term storage.

To titrate B95-8 virus, 10-fold serial dilutions of virus (beginning at 50 ul of undiluted virus) were incubated with 50 ul of 1×10^5 PBMCs in each well of a round bottom 96-well plate at 37°C for 1 hr. Each dilution of virus was done in 6 replicates. The plates were centrifuged at 300 × g for 5 min, the media was removed, and transformation medium containing 0.5ug/mL of cyclosporine A was added. After 1 week, 50 ul of media containing 0.5 ug/ml cyclosporine A was added, and after 3 and 5 weeks media was added without cyclosporine A. The number

of wells containing transformed B cells was determined at week 6 and the tissue culture transforming dose that resulted in transformants in 50% of the wells ($TCTD_{50}$) was calculated using the method of Reed and Muench (1938).

To titrate B95-8/F virus, 25 ul of virus stock was incubated with 75 ul of 1×10^5 Raji cells for 1 hr in each well of a 96 well round bottom plate. The cells were then washed twice by centrifuging the plates at 300 × g for 5 min, and replacing the media with 100 ul of fresh culture media and then culturing the cells at 37°C. After 3 days the proportion of GFP-positive cells was measured by flow cytometry. The following equation was used as a starting point for estimating infected cells:

$$\text{Green Raji Unit (GRU/ml)} = \text{number of GFP positive cells/total number of Raji cells} \times \text{total number of cells} \times \text{dilution factor}$$

We assumed that the number of EBV-infected (i.e. green) Raji cells follows a Poisson distribution, so that the probability that a well has a least one positive cell is $1-e^a$ where e is the base of the natural logarithm and a is the parameter for the Poisson distribution. This Poisson distribution assumption has been used previously for calculating the number of herpesvirus genomes in infected cells (Hoshino et al., 2008). 36An estimate of a is obtained by equating the number of GFP positive cells (G)/total number of Raji cells (n) to $1-e^a$. Solving this equation for a results in $a=-\ln(1-G/n)$. Therefore we revised the original equation for Green Raji Units and calculated the number of EBV-infected cells using the following equation:

$$\text{Green Raji Unit (GRU)/mL} = -\ln(1 - [\text{number of GFP positive cells/total number of Raji cells}]) \times \text{total number of cells} \times \text{dilution factor}$$

The dilution factor in the equation above was 1000/25 since 25 ul of virus was placed in a well and the number of Green Raji units are expressed per ml (1000 ul). Each well contained 10^5 cells in a total volume of 100 ul. Each titration was performed in 3 to 6 replicates.

EBV neutralizing monoclonal antibody, ELISA assay, and human plasma

EBV neutralizing monoclonal antibody 72A1 was obtained from the media of B168 cells and purified by protein G column chromatography and resuspended in 2 × PBS. Human plasma samples were heat-inactivated at 56°C for 30 min to inactivate complement and clotting factors and then tested for anti-EBV VCA IgG and anti-EBV nuclear antigen (recombinant EBNA-1) IgG by ELISA (Bio-Rad Laboratories, Hercules, CA).

Of 31 plasma samples, 26 were positive for anti-VCA IgG, 2 were weakly positive and 3 were negative. Of the 31 plasma samples, 21 were positive for anti-EBNA IgG, 2 were weakly positive, 7 were negative and 1 was not tested because of limited amounts of plasma. Of 5 samples which had weakly positive or negative results in the anti-VCA assay, 3 were positive for anti-EBNA and only 2 samples were negative in both the anti-VCA and anti-EBNA assays. Thus 29 plasma were defined as EBV seropositive (EBV VCA IgG and/or EBNA IgG positive), and two were deemed seronegative (EBV VCA and EBNA IgG negative). These samples were subsequently tested for EBV neutralizing activity, gp350, and gp42 antibody titers. An additional 14 coded human serum samples were tested in the neutralization, gp350, gp42, and VCA IgG ELISA antibody assays.

Luciferase immunoprecipitation system (LIPS) assay

A fusion protein containing the EBV gp350 gene linked to the *Renilla* luciferase gene was constructed in the mammalian expression vector pREN3S. The extracellular domain of EBV

gp350, including the first methionine codon, from B95-8 viral DNA was amplified by PCR using forward primer 5′-AAAAGATCTACCATGGAGGCAGCCTTGCTTGTGTGTC-3′ and reverse primer 5′-AAAAGATCTGGAGGTTTGAGAATCTGGGCTGGGACGT-3′. The PCR product was digested with BglII and cloned into the corresponding BglII site of pREN3S. The resulting plasmid contains the extracellular domain of EBV gp350 with a carboxyl terminal *Renilla* luciferase gene. An EBV gp42-*Renilla* luciferase gene fusion protein was constructed by PCR amplification of the full-length EBV gp42 open reading frame using forward primer 5′-GAGAGATCTGTTTCATTTAAGCAGGTG-3′ and reverse primer 5′-GAGCTCGAGTTAGCTATTTGATCTTTGAC-3′. The PCR product was digested with BglII and XhoI and cloned into the BamHI and XhoI sites of pREN2 (Burbelo et al., 2005). The resulting plasmid contains full length EBV gp42 with an amino terminal Renilla luciferase gene. Since gp42 is normally cleaved at its amino terminus around residue 40 (Ressing et al., 2005) and there was concern that the amino terminal Renilla luciferase moiety could be cleaved off, we verified that the construct remained intact by immunoprecipitation with human serum or monoclonal antibody to gp42. The gp42 monoclonal antibody pulled down even more of the Renilla luciferase-gp42 protein than did EBV-positive human serum, confirming that the Renilla luciferase-gp42 construct remained intact (data not shown). The EBV inserts in both the *Renilla* luciferase gp42 and gp350 constructs were confirmed by sequence analysis.

Cos1 cells were transfected with plasmids containing EBV gp350 or gp42 *Renilla* luciferase fusion proteins and activity of lysates was determined by measuring light units (LU) using a luminometer as described previously (Burbelo et al., 2009). Human plasma or sera were diluted 1:10 and 10 ul was added to 1×10^7 light units (LU) of transfected Cos1 cell extract, immunoprecipitations were performed with addition of protein A/G beads, and LU were determined by a plate luminometer. A cut-off threshold limit was derived from the mean value plus 2 standard deviations of background LU. All LU data shown represent the average of two independent experiments.

Conventional EBV transformation neutralizing titration assay

25 ul of undiluted and 2-fold serial dilutions of human plasma or sera were incubated with 25 ul of 10 $TCID_{50}$ of EBV B95-8 in round bottom wells of a 96 well plate at 37°C for 2 hrs. 50 ul of 1×10^5 PBMC was added for 1 hr and the plates were then centrifuged at 300 × g for 5 min, the media was removed, and transformation media containing 0.5ug/mL of cyclosporine A was added. After 1 week 50 ul of media containing 0.5 ug/ml cyclosporine A was added, and after 3 and 5 weeks 50 uL of transformation media was added without cyclosporine A. Each assay was performed in 6 replicates. The number of transformed wells was determined at week 6 and the effective dilution of plasma that inhibited transformation by 50% (EDT_{50}) was calculated based on the method of Reed and Muench (1938).

GFP-based EBV infection neutralizing titration assay

Human sera, human plasma, media, or 72A1 monoclonal antibody was serially diluted in 2-fold steps (from undiluted to 18 serial dilutions) and 25uL of the diluted antibody was added to wells of a 96 well plate in triplicate. 25 ul of B95-8/F virus was added to each well and incubated for 2 hrs. 50ul of 1×10^5 Raji cells were added and incubated for 1 hour at 37°C, the cells were washed twice by centrifuging the plates at 300 × g for 5 min and replacing the media, and incubated for 3 days at 37°C. The plate was then centrifuged, the cells were washed once with PBS, the PBS was removed, and the cells were fixed in 2% paraformaldehyde in PBS.

GFP-expressing cells were quantified using a FACSCalibur Flow Cytometer (BD Biosciences, San Jose, CA, USA) and FlowJo software (Tree Star Inc., Ashland, OR). The effective dilution of antibody that inhibited infectivity by 50% (EDI_{50}) based on reduction of the number of GFP-

positive cells was calculated by non-linear regression analysis using GraphPad PRISM software. Neutralizing antibody was considered absent when the software program failed to fit the results to an appropriate regression curve.

Statistical analyses

Correlations between GFP-based neutralization assay, conventional neutralizing assay, gp350 antibody, gp42 antibody titers, and VCA IgG ELISA were evaluated by Spearman's rank test using GraphPad PRISM Software (San Diego, CA).

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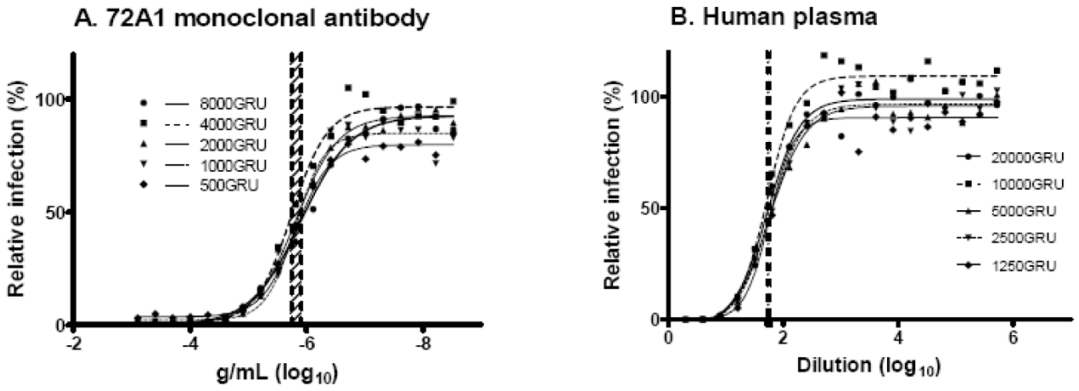

Fig. 1.

Neutralization of EBV B95-8/F infection by 72A1 monoclonal antibody (A) and human plasma (B). Relative infection (percentage) was determined by dividing the number of GFP-positive (infected) cells in the presence of antibody or plasma by the number of GFP-positive cells in the absence of antibody or plasma and converting to percentage for each antibody concentration (g/ml) or plasma dilution point (dilution). Each experiment was done in triplicate and non-linear regression analysis was performed. Symbols show the means at each point, lines show regression curves, and shaded columns represent the range of EDI_{50} values in each experiment. Regardless of the amount of virus input, measured in green Raji units [GRU], the EDI_{50} is constant.

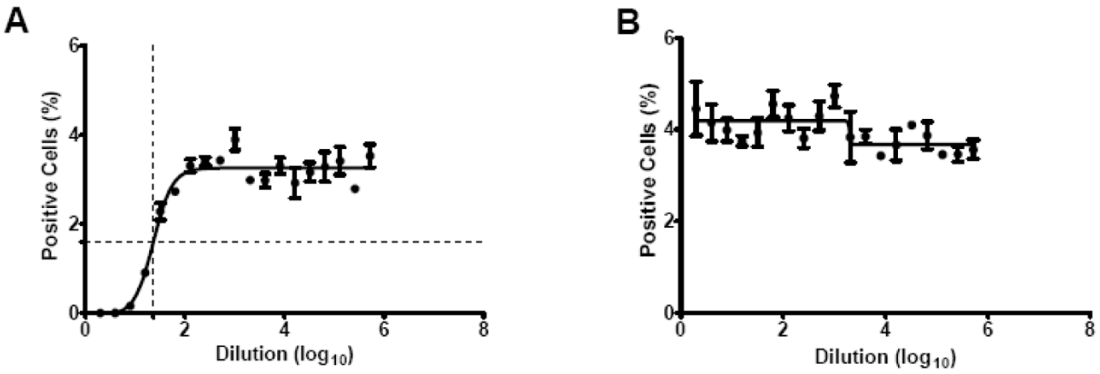

Fig. 2.

GFP-based infection neutralizing titration assay results for plasma samples from EBV seropositive (A) and EBV seronegative donors (B). Non-linear regression analysis was applied to each sample result. The dotted horizontal line in (A) is the percent of positive cells at 50% inhibition by the plasma, and the x intercept of the vertical dotted line is the dilution of antibody that inhibits infection by 50% (EDI_{50}). For this plasma the EDI_{50} is $10^{1.368} = 23.35$. Samples were deemed negative (B) if the statistical software (Graphpad PRISM) failed to fit the data to a sigmoidal regression curve. Closed circles represent the average of triplicate values, and error bars show the range of percentage of positive cells at each dilution point.

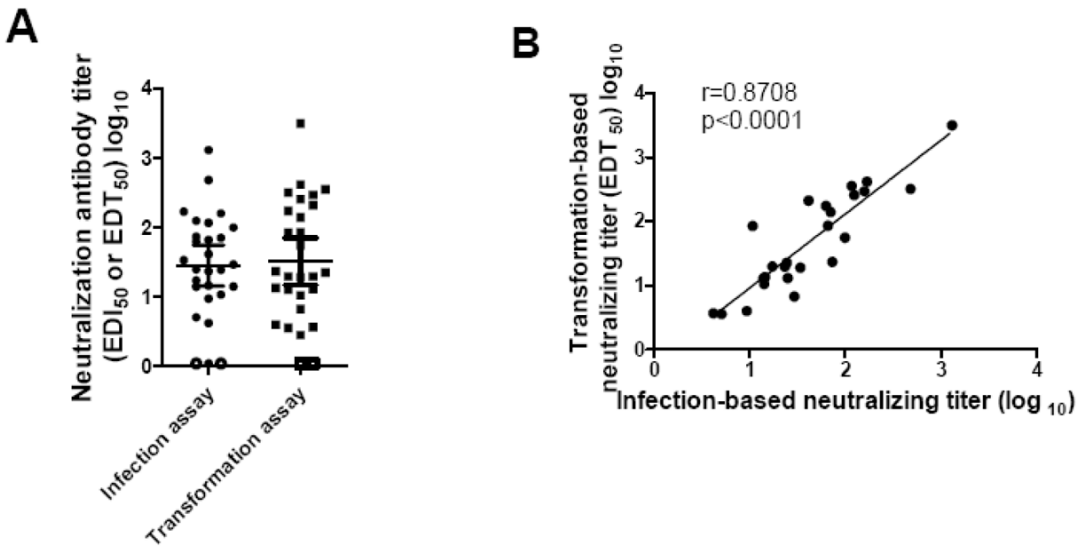

Fig. 3.

Comparison of GFP-based infection neutralization and conventional transformation neutralization assays. (A) Effective dilution of human plasma that inhibited infectivity by 50% (EDI_{50}) and transformation by 50% (EDT50) using the GFP infection-based and transformation-based neutralizing assay. Horizontal bars indicate means, vertical bars indicate 95% CI, closed circles or squares indicate EBV seropositive plasma, and open circles or squares indicate EBV seronegative plasma. (B) Correlation between GFP-based infection and conventional transformation neutralizing assay. Neutralization titers of 25 samples positive in both GFP-based infection and conventional transformation-based assay were plotted.

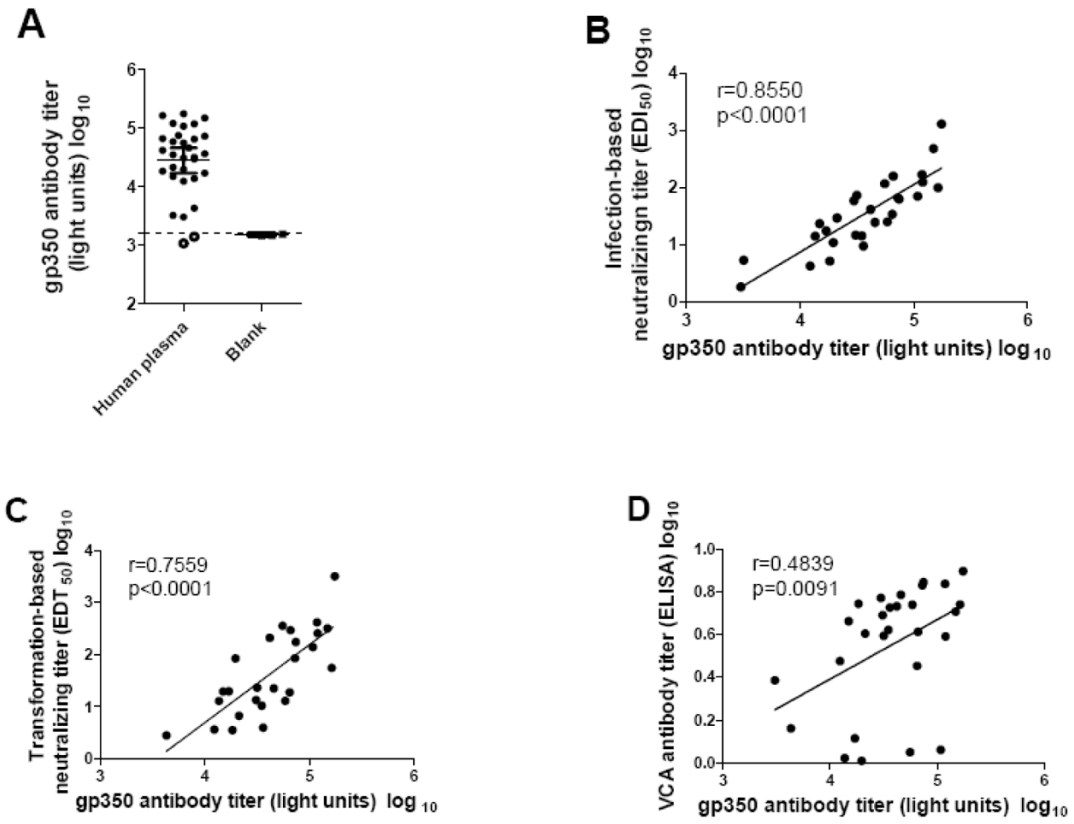

Fig. 4.

Comparison of gp350 antibody titers with GFP-based infection neutralization, conventional transformation neutralization, and VCA antibody assays. (A) Anti-gp350 antibody titers by LIPS assay for EBV seropositive (closed circles) or seronegative (open circles) human plasma samples. Cut off value is shown as horizontal dotted line, which was determined as the mean + 2 SD of blank signal (closed squares). Correlation between gp350 antibody titer and GFP-based infection neutralization assay (B), conventional transformation-based neutralization assay (C), and EBV VCA IgG ELISA (D) for human plasma samples.

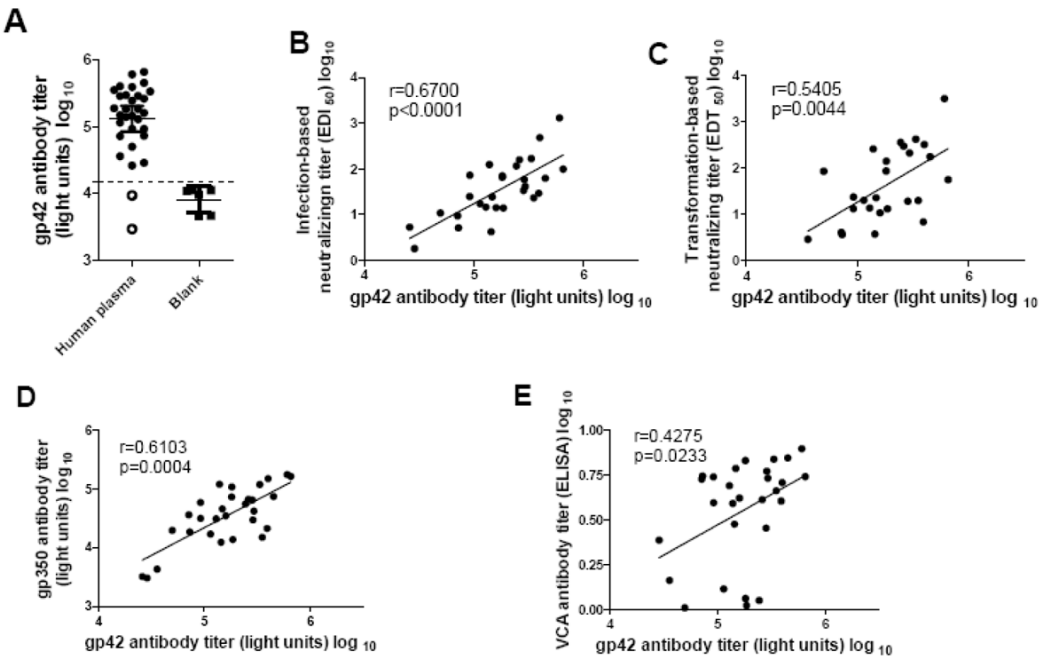

Fig. 5.

Comparison of gp42 antibody titers with GFP-based infection neutralization, conventional transformation neutralization, and VCA antibody assays. (A) Anti-gp42 antibody titers by LIPS assay for EBV seropositive (closed circles) or seronegative (open circles) human plasma samples. Cut off value is shown as horizontal dotted line, which was determined as the mean + 2 SD of blank signal (closed squares). Correlation between gp42 antibody titers and GFP-based infection neutralization assay (B), conventional transformation-based neutralization assay (C), gp350 antibody assay, (D) and EBV VCA IgG ELISA (E) for human plasma samples.

TABLE 1

Dilution of EBV gp350 antibody 72A1 that inhibits EBV infectivity by 50% (EDI_{50}) with 95% confidence intervals. GRU are Green Raji Units (see text).

Virus titer (GRU)	**EBV gp350 monoclonal antibody 72A1 (gm/mL) EDI_{50} ($\times 10^{-6}$)**	**95% Confidence interval ($\times 10^{-6}$)**
8,000	1.23	1.02 – 1.49
4,000	1.80	1.51 – 2.15
2,000	1.52	1.25 – 1.87
1,000	1.56	1.30 – 1.87
500	1.53	1.21 – 1.93
Virus titer	Human plasma (times dilution)	
(GRU)	EDI_{50}	95% Confidence interval
20,000	53.87	44.77 – 64.81
10,000	52.58	42.83 – 64.55
5,000	58.18	46.08 – 73.46
2,500	59.29	45.65 – 77.02
1,250	57.09	46.56 – 69.99