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High degree of correlation between Ebola virus BSL-4 neutralization assays and pseudotyped VSV BSL-2 fluorescence reduction neutralization test

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Abstract

Ebola virus (EBOV), classified as a category A agent by the CDC and NIH, requires BSL-4 containment and induces high morbidity and mortality in humans. The 2013–2015 epidemic in West Africa underscored the urgent need to develop vaccines and therapeutics to prevent and treat EBOV disease. Neutralization assays are needed to evaluate the efficacy of EBOV vaccines and antibody therapies. Pseudotyped viruses based on nonpathogenic or attenuated vectors reduce the risks involved in the evaluation of neutralizing antibodies against highly pathogenic viruses. Selectable markers, fluorescent proteins, and luciferase have been introduced into pseudotyped viruses for detection and quantitation purposes. The current study describes the development of a BSL-2 fluorescence reduction neutralization test (FRNT) using a recombinant vesicular stomatitis virus (VSV) in which the VSV-G envelope gene was replaced with the EBOV glycoprotein (GP) and green fluorescent protein (GFP) genes (rVSV-EBOVgp-GFP). Cells infected with rVSV-EBOVgp-GFP express GFP. Anti-GP neutralizing monoclonal and polyclonal antibodies blocked rVSV-EBOVgp-GFP infection preventing or reducing GFP fluorescence. The high degree of correlation between the EBOV BSL-2 FRNT and the BSL-4 plaque reduction neutralization test (PRNT), the accepted standard of EBOV neutralization tests, supports the use of the EBOV BSL-2 FRNT to evaluate neutralizing antibodies in clinical trials.

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Competing interests

KK and GK are the Inventors in a patent application related to the use of replication-competent recombinant VSV constructs to evaluate anti-filovirus neutralizing antibodies.

ACS and SB declare that they have no competing interests.

This article reflects the views of the author and should not be construed to represent the views or policies of the FDA.

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Keywords

Filoviridae; immune response; neutralizing antibodies; replication-competent recombinant vesicular stomatitis virus; glycoprotein; green fluorescence protein

1. Introduction

Ebola virus (EBOV) is a *Filoviridae* that causes high morbidity and mortality rates in humans (Baize et al., 2014). The 2013–2015 EBOV epidemic in West Africa underscores the urgent need to develop vaccines and therapeutic interventions to prevent and control outbreaks of this deadly virus. The analysis of samples from preclinical studies and clinical trials is difficult because EBOV requires high biosafety level (BSL) containment when using samples that may contain or require live EBOV for testing. Protective humoral and cellular immune responses directed to the EBOV glycoprotein (GP) are necessary and sufficient to induce protection against lethal challenge in animal models (Bradfute and Bavari, 2011; Hevey et al., 1998; Marzi and Feldmann, 2014; Sullivan et al., 2000). Passive immunization with anti-GP neutralizing monoclonal antibodies (Olinger et al., 2012; Qiu et al., 2012) or high-titer monkey immunoglobulin preparations (Dye et al., 2012) administered concomitant with or a few days after infection protected monkeys against EBOV lethal challenge. There is a significant need for tests to evaluate anti-EBOV neutralizing antibodies in clinical trials that could be performed under lower BSL laboratory conditions (BSL-1 or BSL-2). Here, virus neutralization was evaluated by an EBOV BSL-2 fluorescence reduction neutralization test (FRNT) based on a recombinant vesicular stomatitis virus (VSV) in which the VSV-G envelope gene was replaced with the EBOV glycoprotein (GP) and green fluorescence protein (GFP) genes (rVSV-EBOVgp-GFP). The current study demonstrated that the EBOV BSL-2 FRNT correlates with the EBOV BSL-4 plaque reduction neutralization test (PRNT), which is based on the standardized plaque assay for EBOV (Moe, Lambert, and Lupton, 1981; Shurtleff et al., 2012), which is the accepted standard assay for the determination of EBOV neutralizing antibodies. Our data indicate that the EBOV BSL-2 FRNT could be used to evaluate samples from preclinical studies and clinical trials of EBOV vaccines and therapeutics.

2. Material and methods

2.1 Cells, viruses, and antibodies

Vero E6 cells were obtained from the American Type Culture Collection (ATCC), grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Wild-type (wt) Indiana vesicular stomatitis virus (VSV) and VSV-G-deleted replication-competent recombinant VSV containing the Mayinga EBOV GP gene (rVSV-EBOVgp) (Konduru et al., 2011) and also the GFP gene (rVSV-EBOVgp-GFP) (Konduru et al., 2016) were derived using the VSV reverse genetics system (Schnell et al., 1996). Viruses were grown in Vero E6 cells, passage three times, stored at -80°C as working stocks, and used to perform all subsequent BSL-2 neutralization assays. The cell culture adapted Mayinga EBOV and virus infected Vero E6 cells were handled under BSL-4 maximum

containment at the United States Army Medical Research Institute of Infectious Diseases (USAMRIID).

The human neutralizing monoclonal antibody (mAb) KZ52 (Parren et al., 2002) (Integrated BioTherapeutics, Inc.) and anti-FLAG tag mAb M2 (Sigma-Aldrich) were used in the neutralization assays.

Pre-challenge serum samples were collected from five guinea pigs vaccinated with a recombinant protein containing the extracellular portion of EBOV GP fused to the Fc fragment of human IgG1 that survived EBOV lethal challenge, and five guinea pigs vaccinated with a FLAG-tagged Fc fragment (FLAG-Fc), which did not survive EBOV lethal challenge (Konduru et al., 2016). The animal research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations following the principles stated in the Guide for the Care and Use of Laboratory Animals, 8th Edition, National Research Council, 2011. The animal facility is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. The USAMRIID Institutional Animal Care and Use Committee (IACUC) approved the animal protocol. The World Health Organization (WHO) human anti-EBOV plasma interim international reference reagent 15/220 (Wilkinson et al., 2015; Wilkinson et al., 2017) and a matching normal blood donor plasma were obtained from the National Institute of Biological Standards and Control, UK.

2.2 Virus titration by endpoint dilution assay

Virus titers were determined by an endpoint dilution assay in 96-well plates containing Vero E6 cells using 10-fold serial dilutions in octuplicate wells. Cytopathic effect (CPE) was assessed 4 days post-infection under the microscope and viral titers were calculated as tissue culture infectious doses 50% (TCID₅₀) per ml using the ID50 program server https://www.ncbi.nlm.nih.gov/CBBresearch/Spouge/html_ncbi/html/id50/id50.cgi (Spouge, 1992).

For the neutralization assay, 10⁴ TCID₅₀ of rVSV-EBOVgp, rVSV-EBOVgp-GFP, or wt VSV in 0.1 ml of cell culture medium were incubated with 2 µg of neutralizing mAb KZ52, 2 µg of negative control M2 mAb, or medium (mock) for 1 h at 37°C. Neutralization mixtures were titrated by the endpoint dilution assays in Vero E6 monolayers.

2.3 One-step virus growth analysis

Vero E6 cell sub-confluent (80%) monolayers in 6-well plates were infected with rVSV-EBOVgp, rVSV-EBOVgp-GFP, or wt VSV at an MOI of 10 TCID₅₀. After 1 h adsorption at 37°C, cells were washed three times with medium, 1 ml of medium was added to each well, and plates were incubated at 37°C. Supernatants from duplicate wells were harvested at different time post-infection (p.i.), clarified by centrifugation, and stored at -80°C. Viral titers were determined by the endpoint dilution assay in Vero E6 cells.

2.4 Fluorescence microscopy analysis

Confluent Vero E6 monolayers were infected with rVSV-EBOVgp or rVSV-EBOVgp-GFP at a MOI of 0.1 TCID₅₀, incubated at 37°C for 24 h, and observed under a microscope. To

analyze the kinetics of GFP expression, Vero E6 cell monolayers grown in 8-well Permanox chamber slides (Nunc) were infected with rVSV-EBOVgp-GFP at a MOI of 0.1 TCID₅₀, the virus was absorbed for 1 h, and cells were washed, fresh medium was added, incubated at 37°C for different times post-infection, fixed with 2% paraformaldehyde. Coverslips were mounted with medium containing DAPI (Invitrogen) as a nuclear counterstain. Phase contrast and fluorescence micrographs were taken with an inverted Zeiss Axiovert 200 microscope at 200X magnification.

For 96-well plates, GFP fluorescence was scored under the fluorescence microscopy at 26 h p.i. as + (100% of cells expressing GFP), +/- (25% of cells expressing GFP), or - (no GFP fluorescence).

2.5 EBOV BSL-2 fluorescence reduction neutralization test (EBOV BSL-2 FRNT) in 6-well plates

To assess virus neutralization in 6-well plates by flow cytometry, 2,000 TCID₅₀ of rVSV-EBOVgp-GFP in 0.1ml cell culture medium were treated with different amounts of KZ52 mAb (0, 0.1, 0.5, or 1 µg) for 1 h at 37°C. Vero E6 cells in duplicate 6-well plates were infected or not with the neutralization mixtures and incubated for 14–16 h at 37°C. Cell were detached with 0.5 mM EDTA in PBS, washed once with 2% FBS in PBS, and fixed with 2% paraformaldehyde. One million cells of each well were analyzed for GFP fluorescence in a FACSCanto II instrument (BD Biosciences). The percent of neutralization was calculated as 100 - (number of GFP fluorescent cells in serum-treated wells/number of GFP fluorescent cells in mock-treated wells) X 100.

2.6 EBOV BSL-4 plaque reduction neutralization test (EBOV BSL-4 PRNT)

EBOV BSL-4 PRNT was performed as previously described (Shurtleff et al., 2012). Briefly, 100 PFU of cell-culture adapted Zaire EBOV were treated with four-fold serial dilutions of guinea pigs sera in the presence of 5% guinea pig complement for 1 h at 37°C. Vero E6 cells in 6-well plates were infected in duplicates with the neutralization mixtures for 1 h at 37°C the inoculum was removed, and monolayers were overlaid with 2 ml medium containing 1% agarose and incubated for 7-days at 37°C. At day-6, plates were overlaid with 4% neutral red solution. The visible plaques were counted manually from duplicate wells for each sample at day-7. The whole assay was performed at the USAMRIID BSL-4 laboratory. The percent of neutralization was calculated as 100 - (number of PFU in serum-treated wells/number of PFU in mock-treated wells) X 100.

2.7 EBOV BSL-2 Plaque Reduction Neutralization Test (BSL-2 PRNT)

EBOV BSL-2 PRNT was performed as previously described (Konduru et al., 2011). Briefly, four-fold serial dilutions of guinea pig serum were mixed with 100 PFU of rVSV-EBOVgp-GFP in the presence of 5% guinea pig complement and incubated for 1 h at 37°C. Vero E6 cell monolayers in 6-well plates were infected with the neutralization mixtures in duplicates and incubated for 1 h at 37°C. The inocula was aspirated, and cell monolayers were overlaid with medium containing 1% bacto-agar and incubated at 37°C for 48 h. The agar overlay was carefully removed from the wells, cell monolayers were fixed with 10% trichloroacetic acid, and plates were stained with 1% crystal violet for 30 min. The visible

plaques were counted manually from duplicate wells for each sample. The percent of neutralization was calculated as in the EBOV BSL-4 PRNT assay.

2.8 EBOV BSL-2 FRNT in 96-well plates

Levels of anti-EBOV GP neutralizing antibodies in guinea pig sera or human plasma were determined in 96-well plates containing Vero E6 cell monolayers. Plates with low levels of autofluorescence and DMEM without phenol red were used to grow cells and perform dilutions to reduce the fluorescence background of the assay. Two- or four-fold serial dilutions of guinea pig serum (1:25 starting dilution) or human plasma (1:50 starting dilution) were performed by duplicates to octuplicates in 96-well plates, and 2,000 TCID₅₀ of rVSV-EBOVgp-GFP were added per well. After incubation at 37°C for 1 h, 96-well plates containing 70% confluent Vero E6 cell monolayers were inoculated with the neutralization mixtures. After incubation at 37°C for 26 h, GFP fluorescence was determined in an automated plate reader using top light source, 485/20 nm excitation filter, and 528/20 nm emission filter. Mean fluorescence intensity (MFI) from duplicate to octuplicate wells were used to calculate the mean percent of neutralization as $100 - (\text{MFI in serum-treated wells} / \text{MFI in mock-treated wells}) \times 100$.

2.9 Statistical analysis

Virus titers were calculated using the ID50 program developed by John L. Spouge (National Center for Biotechnology Information, NIH). Statistical significance between two means or groups was determined by unpaired Student's t-test using the Prism 6 program (GraphPad Software, Inc.). Regression analysis and determination of coefficient (R^2), Pearson's correlation analysis and determination of coefficient (r), and sigmoidal curve fitting (y axis = MFI, x axis = log dilution) were also performed using the Prism 6 program.

3. Results

3.1 Characterization of recombinant VSV constructs containing GP

Replication-competent recombinant vesicular stomatitis virus (VSV) constructs in which the VSV-G envelope was replaced with the EBOV GP, termed rVSV-EBOVgp (Konduru et al., 2011; Ou et al., 2012), or by the same GP followed by the green fluorescence protein (GFP) gene, termed rVSV-EBOVgp-GFP (Konduru et al., 2016), were used to assess immune responses. Vero E6 cells infected with rVSV-EBOVgp-GFP but not the parental rVSV-EBOVgp expressed GFP as assessed by fluorescence microscopy (Fig. 1A). Human anti-GP monoclonal antibody KZ52 neutralized rVSV-EBOVgp and rVSV-EBOVgp-GFP but not wt VSV (Fig. 1B) indicating that the recombinant VSV particles infected cells via GP on the virus surface. A one-step growth curve analysis of cells infected with a MOI of 10 TCID₅₀ showed that the growth kinetics of rVSV-EBOVgp and rVSV-EBOVgp-GFP was similar in Vero E6 cells but slower than that of wt VSV (Fig. 1C), consistent with the different cell entry process of the wt and recombinant VSV. rVSV-EBOVgp-GFP was stable, and expression of GFP did not change during 10 serial passages in Vero E6 cells (data not shown).

3.2 Analysis of GFP expression in rVSV-EBOVgp-GFP infected cells

The expression of GFP in cells infected with rVSV-EBOVgp-GFP was assessed to develop an EBOV pseudotype neutralization assay based on GFP fluorescence. A low MOI of rVSV-EBOVgp-GFP was used to prevent saturation of the GFP expression and maintain a linear range in the fluorescence signal. At a MOI of 0.1 TCID₅₀, expression of GFP in Vero E6 cells infected with rVSV-EBOVgp-GFP was first detected by fluorescence microscopy in a few cells at 4 h p.i. (Fig. 2A). The number of GFP fluorescent single cells increased at 8 h p.i. likely due to a second round of virus infection. The formation of small fluorescent foci indicated that multiple rounds of infection occurred at 12 h p.i. All cells expressed GFP and showed CPE at 24 h p.i. (data not shown). Based on these kinetics of GFP expression, flow cytometry was used to quantitate the number of GFP-expressing cells after overnight infection. At a MOI of 0.002 TCID₅₀, 4.24% of the cells infected with rVSV-EBOVgp-GFP expressed GFP at 16 h p.i. compared to a very low fluorescence background in uninfected cells (Fig. 2B). To analyze the effect of neutralizing antibodies on GFP fluorescence, rVSV-EBOVgp-GFP was treated with different amounts of KZ52 mAb for 1 h at 37°C before infecting Vero E6 cells. Flow cytometry analysis at 16 h p.i. showed that the KZ52 mAb treatment reduced the number of fluorescent cells in a dose-dependent manner (Fig. 2B) indicating that rVSV-EBOVgp-GFP could be used to evaluate EBOV neutralizing antibodies in a fluorescence reduction neutralization test (FRNT).

3.3 Correlation of EBOV BSL-4 and BSL-2 neutralization assays

A correlation of the EBOV BSL-4 PRNT, which is based on neutralization of live EBOV, with the EBOV BSL-2 PRNT and FRNT assays, which are based on neutralization of rVSV-EBOVgp-GFP, was performed to evaluate the relevance of the EBOV pseudotype neutralization assays. To do so, pre-challenge serum samples were used from five guinea pigs that were vaccinated with EBOVgp-Fc, a recombinant protein containing the extracellular portion of EBOV GP fused to the Fc fragment of human IgG1, that survived EBOV lethal challenge (Konduru et al., 2016). Serum samples from five guinea pigs from the same study that were vaccinated with control FLAG-Fc and did not survive the EBOV lethal challenge were used as negative controls. Sera from guinea pigs vaccinated with EBOVgp-Fc neutralized EBOV in a dose-dependent manner as assessed by the EBOV BSL-4 PRNT (Fig. 3A). Using the same sera samples, the EBOV BSL-2 PRNT, an assay based in plaque reduction of rVSV-EBOVgp-GFP (Fig. 3B), and the BSL-2 EBOV FRNT, based on reduction of GFP fluorescence in rVSV-EBOVgp-GFP infected cells (Fig. 3C) showed similar neutralization patterns. Sera from the FLAG-Fc control animals resulted in a background reduction of virus infectivity of approximately 20% (Fig. 3A–C). Regression analysis showed a highly significant ($P < 0.0001$) linear relationship between the BSL-4 PRNT and BSL-2 PRNT, the BSL-4 PRNT and BSL-2 FRNT, and the BSL-2 PRNT and EBOV BSL-2 FRNT assays (Table 1). Pearson's correlation analysis also showed a highly significant degree of correlation between the BSL4 and BSL2 assays ($P < 0.0001$).

3.4 Migration of EBOV BSL-2 FRNT into a 96-well plate format

To further simplify the assay, the EBOV BSL-2 FRNT was migrated to a 96-well plate format that allowed the easy handling of multiple dilutions and several replicates/dilution.

The assay in Vero E6 cells using 2,000 TCID₅₀/well of rVSV-EBOVgp-GFP provided the highest neutralization ratio in the 96-well plate format (Fig. 4A), conditions that were used for further experimentation. Analysis of EBOV BSL-2 FRNT percent neutralization in the 96-well plate format (Fig. 4B, upper panel) revealed a pattern similar to the 6-well format (Fig. 3C), which was also verified by fluorescence microscopy analysis (Fig. 4B, lower panel). Regression analysis of the EBOV BSL-2 FRNT in the 96-well plates versus the 6-well plate format showed a very significant linear relationship ($P<0.0001$, $r^2=0.96$) (Fig. 4C) and correlation ($P<0.0001$, $r=0.98$) indicating the equivalence of both formats. Plotting of the MFI versus the log of the sera dilution resulted in well-fitted sigmoidal curves (Fig. 4D) for sera from guinea pig vaccinated with EBOVgp-Fc whereas sera from FLAG-Fc vaccinated animals resulted in flat neutralization curves. Based on the sigmoidal curves, the dilution of each guinea pig serum that reduced 50% (FRNT50) and 80% (FRNT80) GFP-fluorescence were determined, which ranged from 441 to 579 and 281 to 388, respectively.

3.5 Analysis of EBOV neutralizing antibodies in human convalescent plasma by EBOV BSL-2 FRNT

Samples from the WHO human anti-EBOV plasma interim international reference reagent 15/220 obtained from an Ebola virus convalescent patient (Wilkinson et al., 2015; Wilkinson et al., 2017) and a matching blood donor plasma were used to assess the performance of the EBOV BSL-2 FRNT in the 96-well plate format (Fig. 5). Non-linear regression analysis showed that the convalescent plasma neutralized rVSV-EBOVgp-GFP in a dose dependent manner resulting in a characteristic sigmoidal curve whereas the blood donor plasma did not affect infectivity of the virus. The FRNT50 and FRNT80 of the WHO reagent 15/220 were calculated as 283.9 and 116.1, respectively.

4. Discussion

Ebola virus neutralization assays based on antibody-mediated reduction of plaque forming units (Shurtleff et al., 2012), endpoint dilution titers (Smither et al., 2013), and fluorescence or luciferase signal using engineered EBOV constructs (Ebihara et al., 2007; Hoenen et al., 2013; Panchal et al., 2010; Towner et al., 2005) have been developed but are time-consuming, labor-intensive, and require BSL-4 facilities. Therefore, a robust and simple EBOV neutralization assay based on rVSV-EBOVgp-GFP, a VSV-G deleted recombinant VSV containing the GP and GFP genes that can be performed in 26 h under BSL-2 conditions was developed in this work. A high degree of correlation was observed between the EBOV BSL-2 FRNT based on GFP-fluorescence reduction and the EBOV BSL-4 PRNT based on plaque reduction, which is the recognized standard for EBOV neutralization assays. This correlation validated the use of the EBOV BSL-2 FRNT to determine levels of anti-EBOV neutralizing antibodies in clinical trials.

The EBOV BSL-2 FRNT assay was further developed as a 96-well plate format to allow the use of multiple two-fold serum dilutions, several replicates/dilution to increase the accuracy of the test, and reading of the assay in a 96-well automated fluorescence plate reader. In this format, the FRNT could be used for high-throughput screening of samples from preclinical and clinical trials. The EBOV BSL-2 FRNT assay was used in preclinical studies to evaluate

vaccine candidates using sera samples from non-human primates (unpublished data). In a recent WHO collaborative study to assess the suitability of an EBOV antibody standard (Wilkinson et al., 2015; Wilkinson et al., 2017), the EBOV BSL-2 FRNT described herein performed similarly to an endpoint dilution assay using EBOV. In contrast, other neutralization assays in the same WHO study based on GP pseudotypes provided erratic results, and a luciferase-based replication-restricted recombinant VSV assay resulted in 10 to 100-fold higher EBOV neutralizing antibody titers than the EBOV BSL-4 endpoint assays and the EBOV BSL-2 FRNT. Therefore, the EBOV BSL-2 FRNT described in this paper constitutes a robust assay that performs similarly to EBOV BSL-4 neutralization assays and could be implemented in the analysis of EBOV medical countermeasures and epidemiological studies.

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Highlights

- Neutralization assay based on VSV construct containing the EBOV GP and the GFP gene
- GFP is expressed in infected cells
- EBOV neutralizing antibodies reduce GFP fluorescence in a dose-dependent manner
- BSL2 Fluorescence Reduction Neutralization Test (FRNT) yield results in 26 h
- High degree correlation of BSL2 FRNT and BSL4 Plaque Reduction Neutralization Test (PRNT)

Fig. 1.

Characterization of EBOV GP pseudotyped VSV. (A) Expression of GFP in infected cells. Vero E6 cells were infected with rVSV-EBOVgp or rVSV-EBOVgp-GFP for 24 h, and expression of GFP was visualized under the microscope. Phase contrast and fluorescence micrographs were obtained in a Zeiss Axiovert 200 inverted microscope at 200X. (B) Neutralization of EBOV GP pseudotyped VSV with human anti-GP KZ52 mAb. rVSV-EBOVgp, rVSV-EBOVgp-GFP, or control wt VSV were treated with medium (Mock), negative control mouse M2 mAb raised against the FLAG tag (Anti-FLAG), or human anti-GP KZ52 mAb. Neutralization reactions were titrated on 96-well plates containing Vero E6 cells by an endpoint assays using octuplicate wells/dilution. Data are mean virus titers (log₁₀ TCID₅₀/ml) and lines are standard errors. (C) Growth kinetics of EBOV GP pseudotyped

VSV. Vero E6 cells were infected with rVSV- EBOVgp, rVSV-EBOVgp-GFP, or wt VSV at a MOI of 10 TCID₅₀. Cell supernatants were collected at different times p.i. and virus titers were determined as in B. Differences between mean titers were determined by t-Student test; ** highly significant differences ($P < 0.01$). Data are representative of three independent experiments.

Fig. 2.

Kinetics of GFP-expression and neutralization of rVSV-EBOVgp-GFP. **(A)** Time course of expression of GFP in Vero E6 cells infected with rVSV-EBOVgp-GFP. Monolayers of Vero E6 cells were infected with rVSV-EBOVgp-GFP at a MOI of 0.1 TCID₅₀, incubated at 37°C for 0, 4, 8, and 12 h p.i., cells nuclei were stained with DAPI (blue) and monolayers were observed under a microscope. Expression of GFP (green) was detected in cells showing CPE (rounded cells). Fluorescence micrographs were taken with an inverted Zeiss Axiovert 200 microscope at 200 X. **(B)** Reduction of GFP expression due to neutralization of rVSV-EBOVgp-GFP with human KZ52 mAb. rVSV-EBOVgp-GFP (2,000 TCID₅₀) was treated with different amounts of KZ52 mAb (0, 0.1, 0.5, or 1 µg), incubated for 1 h at 37°C. Vero E6 cell monolayers infected or not (mock infected) with the virus-mAb mixtures were incubated for 16 h at 37°C, detached, and GFP fluorescence was analyzed by flow cytometry. Heat map dot-plots represent GFP fluorescence versus forward scatter (FSC) in 10⁶ counted cells. Cells expressing GFP were gated (magenta square gates) and quantitated as percent of total cells (numbers within gates). Reduction in the percent of GFP-positive cells due to neutralization with KZ52 mAb (% Neutralization) is shown in the bottom panel (blue numbers). Data are representative of three independent experiments.

Fig. 3.

Comparison of BSL-4 and BSL-2 neutralization assays. EBOV or rVSV-EBOVgp- GFP were treated with 4-fold dilutions of sera from guinea pigs vaccinated with EBOVgp-Fc that survived EBOV lethal challenge (n=5) or guinea pigs vaccinated with negative control FLAG-Fc that did not survive the challenge (n=5). After incubation at 37°C for 1 h, Vero E6 cell monolayers in 6-well plates were infected with the neutralization mixtures in duplicate wells. **(A)** BSL-4 plaque reduction neutralization test (EBOV BSL-4 PRNT). EBOV plaques were counted at 7 days p.i. and compared to a mock-treated control. **(B)** BSL-2 plaque reduction neutralization test (EBOV BSL-2 PRNT). rVSV-EBOVgp-GFP plaques were counted at 2 days p.i. and compared to a mock-treated control. **(C)** BSL-2 fluorescence reduction neutralization test (EBOV BSL-2 FRNT). Vero E6 cells were detached at 16 h p.i. and GFP-fluorescence was determined by flow cytometry and compared to a mock-treated control. Data are mean percent neutralization of duplicate wells, standard errors are shown as bars. Differences between means were evaluated by unpaired Student's t-test; ** highly significant ($P < 0.01$). Data are representative of three independent experiments.

Fig. 4.

Migration of the EBOV BSL-2 FRNT to a 96-well plate format. **(A)** Optimization of the concentration of rVSV-EBOVgp-GFP. Different amounts of rVSV-EBOVgp-GFP were treated with a 1/100 dilution of pooled sera from two guinea pigs vaccinated with EBOVgp-Fc or FLAG-Fc. Neutralization reactions were titrated on 96-well plates containing Vero E6 cells by an endpoint assays using octuplicate wells/dilution. Data are GFP mean fluorescence intensity (MFI) of infected cells and lines are standard errors. **(B)** Neutralization of rVSV-EBOVgp-GFP in a 96-well plate format. rVSV-EBOVgp-GFP (2,000 TCID₅₀) was treated with four-fold serial serum dilutions of guinea pigs (n=5) vaccinated with EBOVgp-Fc or FLAG-Fc for 1 h at 37°C. Vero E6 cells in 96-well plates were infected with each dilution in duplicates. After 26 h of incubation at 37°C, GFP fluorescence was determined in a plate reader (upper panel) as percent neutralization or in a fluorescence microscope (under the graph) as the estimated number of GFP fluorescent cells scored as 100% (+), 25% (+/-), or 0% (-). **(C)** Comparison of the 6-well and 96-well plate format EBOV BSL-2 FRNT. Regression analysis of percent neutralization of rVSV-EBOVgp-GFP by guinea pig sera (n=5) in the 6-well format from Fig. 3C versus the 96-well plate format from (B). **(D)** Dose response neutralization analysis of individual guinea pig serum samples. Non-linear regression analysis of rVSV-EBOVgp-GFP neutralization data

from (B) for each guinea pig serum sample (n=5). Data are GFP mean fluorescence intensity (MFI) at each serum dilution for each guinea pig (EBOVgp-Fc#1 to 5 and FLAG- Fc#1 to 5). Differences between two means were evaluated by unpaired Student's t-test; ** highly significant ($P<0.01$). Data are representative of three independent experiments.

Fig. 5.

Analysis of anti-EBOV neutralizing antibodies in human convalescent plasma by EBOV BSL-2 FRNT. rVSV-EBOVgp-GFP was treated with two-fold dilutions of plasma from a convalescent patient (WHO human anti-EBOV plasma interim international reference reagent 15/220) or a matching blood donor for 1 h at 37°C. Vero E6 cell monolayers in 96-well plates were infected with the virus-plasma mixtures in octuplicate wells and incubated at 37°C for 24 h. Plates were read in a plate reader for GFP fluorescence. Data are plotted as a non-linear regression of GFP mean fluorescence intensity (MFI) versus the log of the plasma dilution, and are representative of three independent experiments.

Table 1

Regression analysis of BSL-4 and BSL-2 neutralization assays.

Comparison EBOV neutralization assays ^a	Linear regression equation (Y)	Regression Coefficient (R ²)	Pearson's Correlation (r)	Significance (P)
BSL-4 PRNT vs BSL-2 PRNT	0.9783x +0.8022	0.9225	0.96	<0.0001
BSL-4 PRNT vs BSL-2 FRNT	0.9662x +2.5237	0.9408	0.96	<0.0001
BSL-2 PRNT vs BSL-2 FRNT	1.0105x -1.3048	0.9504	0.93	<0.0001

^aRegression analysis using data from Fig. 3.