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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 non-pathogenic 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.

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 et al., 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

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(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 IgG1that 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% ($TCID_{50}$) 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, 10^4 $TCID_{50}$ 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 $TCID_{50}$. 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 $TCID_{50}$, 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 $TCID_{50}$, 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 200× 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 exppessing 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, 2000 $TCID_{50}$ of rVSV-EBOVgp-GFP in 0.1 ml 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) × 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 overlayed with 2 ml medium containing 1% agarose and incubated for 7-days at 37 °C. At day-6, plates were overlayed 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) × 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 overlayed 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 2000 $TCID_{50}$ of rVSV-EBOVgp-GFP were added per well. After incubation at 37 °C for 1 h, 96-well plates