SUPPLEMENT ARTICLE

A Single-Vector, Single-Injection Trivalent Filovirus Vaccine: Proof of Concept Study in Outbred Guinea Pigs

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The filoviruses, *Marburg marburgvirus* (MARV), *Zaire ebolavirus* (ZEBOV), and *Sudan ebolavirus* (SEBOV), cause severe and often fatal hemorrhagic fever in humans and nonhuman primates (NHPs). Monovalent recombinant vesicular stomatitis virus (rVSV)–based vaccine vectors, which encode a filovirus glycoprotein (GP) in place of the VSV glycoprotein, have shown 100% efficacy against homologous filovirus challenge in rodent and NHP studies. Here, we examined the utility of a single-vector, single-injection trivalent rVSV vector expressing MARV, ZEBOV, and SEBOV GPs to protect against MARV-, ZEBOV-, and SEBOV-induced disease in outbred Hartley guinea pigs where we observed protection from effects of all 3 filoviruses.

Keywords. cross-protection; Ebola virus; filovirus; guinea pig; Marburg virus; trivalent; vaccine; vesicular stomatitis virus.

Ebola virus (EBOV) and Marburg virus (MARV), the causative agents of Ebola and Marburg hemorrhagic fever (HF), respectively, comprise the family *Filoviridae* [1]. The *Ebolavirus* genus consists of 5 distinct species: *Zaire ebolavirus* (ZEBOV), *Sudan ebolavirus* (SEBOV), *Côte d'Ivoire* (CIEBOV) (also referred to as *Ivory Coast ebolavirus* or *Tai Forest ebolavirus*), *Bundigbugyo ebolavirus* (BEBOV), and *Reston ebolavirus* (REBOV) [1]. The *Marburgvirus* genus contains 2 lineages, one represented by a number of strains, including Angola, Ci67, and Popp, and a second lineage represented by the Ravn strain. ZEBOV, SEBOV, BEBOV, and MARV are important human pathogens that overlap in endemic areas, with case fatality rates frequently ranging up to 90% for ZEBOV and MARV, around 50%–55% for SEBOV, and 40%–66% for BEBOV (reviewed in [1]). At present, an unprecedented outbreak of ZEBOV HF, which began in late 2013/early 2014 and has yet to be controlled [2], has highlighted the need for vaccines and therapeutics that target filoviruses.

While vaccines that provide immunity against filoviruses have been investigated for several decades, currently there are no licensed vaccines available for human use; however, there are at least 8 different vaccine candidates that have shown the potential against lethal EBOV and/or MARV infection using platforms based on DNA vectors, recombinant adenovirus (rAd) vectors, combined DNA/rAd vectors, virus-like particles (VLPs), alphavirus replicons, recombinant human parainfluenza virus 3 (rHPIV3), rabies virus, and recombinant vesicular stomatitis virus (rVSV) [3]. The paradigm of filovirus vaccine development consists of an initial screen in rodents such as guinea pigs, mice, and hamsters as animal models of filovirus HF [4–9] using rodent-adapted viruses. Once utility is shown in rodents, studies in nonhuman primates (NHPs), using wild-type (wt) viruses, are required to confirm vaccine efficacy against the desired filovirus [3].

Considering the potential endemic overlap of ZEBOV, SEBOV, and MARV HF outbreaks [1] and potential for

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deliberate misuse of each agent, we sought to design and construct a single-vector rVSV vaccine vector that would simultaneously express all 3 filovirus glycoproteins (GPs). Our hypothesis was that this construct would provide cross-protection against MARV-, ZEBOV-, and SEBOV-induced disease in the outbred Hartley strain guinea pig models using guinea pig–adapted (GPA-) viruses. Consistent with this hypothesis, our initial assessment of this vaccine in guinea pigs has shown that all vaccinated animals developed antifilovirus GP immunoglobin G (IgG), had reduced circulating viremia, and were protected from GPA-MARV-, GPA-ZEBOV-, and GPA-SEBOV-induced disease.

MATERIALS AND METHODS

rVSV-MARV-ZEBOV-SEBOV-GP Recovery and Characterization

rVSV-based viruses expressing the MARV GP as well as the ZEBOV and SEBOV GPs were created by sequentially inserting the appropriate GP complementary DNA (cDNA) into an independent transcription start/stop sequence within a rVSV construct lacking the VSV G pAK-VSVΔG-3N-SSS. A codon-optimized version of Marburg virus Musoke strain GP was inserted between the VSV N and P genes while the codon-optimized versions the ZEBOV-Mayinga strain and SEBOV-Boniface strain GPs were placed between the M and L genes (Figure 1*A*). The resulting cDNA vector was then sequenced to verify insertion sites and was then used in a standard rVSV recovery protocol [10] to recover a replicating vaccine strain named rVSV-MARV-ZEBOV-SEBOV-GP (rVSV-MZS-GP).

Low passage isolates of rVSV-MZS-GP were used to infect Vero cell monolayers at a multiplicity of infection of 0.1. At 48–55 hours post infection, media was harvested and virus was purified using standard techniques [10]. Briefly, virus was pelleted from cells through ultracentrifugation at 100 000*g*, over a 20% sucrose cushion. The purified virus was lysed and individual components were separated by size on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Individual glycoproteins were identified by immunoblotting using a ZEBOV GP recognizing antibody (6D8), and a MARV GP recognizing antibody (MBG704) (Figure 1*B*). Lack of wt-VSV contamination was established using the 8G5 anti-VSV-G antibody, and the 23H12 antibody recognizing VSV Matrix was utilized to assess the presence of viral vector components [11, 12] (Figure 1*B*).

Vaccine Efficacy in Guinea Pigs

We recently developed outbred Hartley strain guinea pig models for MARV-Angola (GPA-MARV) [13], ZEBOV-Mayinga (GPA-ZEBOV) [14], and SEBOV-Boniface (GPA-SEBOV) by serial passage of liver and/or spleen. The GPA-MARV was developed by 4 passes in Hartley guinea pigs, the GPA-ZEBOV was adapted by 4 passes in strain 13 guinea pigs and 3 passes in Hartley guinea pigs, and the GPA-SEBOV was adapted by 3 passes in Hartley guinea pigs. The resulting adapted strains all display clinical scores and give rise to plasma viremia. With the exception of GPA-SEBOV, all are uniformly lethal with death occurring 8–12 days after challenge for MARV-Angola and 7–9 days after challenge for GPA-ZEBOV. The GPA-SEBOV causes approximately 25% lethality in Hartley guinea pigs. Animal studies were performed under Biosafety Level–4 biocontainment at the Galveston National Laboratory and were approved by the University of Texas Medical Branch at Galveston Institutional Animal Care and Use Committee (IACUC) in accordance with state and federal statutes and regulations relating to experiments involving animals and the Institutional Biosafety Committee. Twenty-four female outbred Hartley strain guinea pigs (351–400 grams) were purchased from Charles River Laboratories and were (4 per group) vaccinated intramuscularly (i.m.) with approximately 1×10^7 plaque-forming units (PFUs) of rVSV-MZS-GP (3 groups) or phosphate-buffered saline (PBS) control (3 groups) (Figure 1*C* and 1*D*, arrowhead). Individual animals were infected with approximately 1,000 PFUs of GPA-filovirus by intraperitoneal (i.p.) injection (Figure 1*C* and 1*D*, asterisks). Blood was collected from all animals prevaccination (−28), day of challenge (0), and day 7 postchallenge (Figure 1*D*, arrows), and survival and clinical scores (Rough = 4, Sick = 8, Moribund = 12, Expired = 16) of the animals were followed for 28 days after which survivors were euthanized at the study endpoint per IACUC protocol.

Virus Titration by Plaque Assay

Filovirus titration was performed by conventional plaque assay on Vero E6 cells from plasma collected from guinea pigs at day 7 postinfection. In brief, increasing 10-fold dilutions of the samples were adsorbed to Vero E6 monolayers in duplicate wells (200 μL); the limit of detection was 25 PFUs/mL.

Humoral Immune Response to Vaccination

Enzyme-linked immunosorbent assay (ELISA), using recombinant GPdTM purified protein (Integrated BioTherapeutics, Inc) for the appropriate filovirus, was used to detect cross-reactive IgG. Species-specific GPdTM were diluted to an optimal working concentration of 0.08 μg/mL and used to coat the 96-well ELISA plates (Nunc). The serum samples were assayed at 2-fold dilutions, starting at a 1:100 dilution in ELISA diluent (1% heat inactivated fetal bovine serum [HI-FBS], 1X PBS, and 0.2% Tween-20). Samples were incubated for 1 hour at room temperature (r.t.), removed, and plates were washed. Wells were then incubated at r.t. for 1 hour with goat anti–guinea pig IgG conjugated to horseradish peroxidase (Fitzgerald Industries International) at a 1:7000 dilution. These wells were washed and then incubated with 2,2′-azine-di(3ethylbenzthiazoline-6-sulfonate) peroxidase substrate system (KPL) and read for dilution endpoints at 405 nm on a microplate reader (Molecular Devices Emax system).

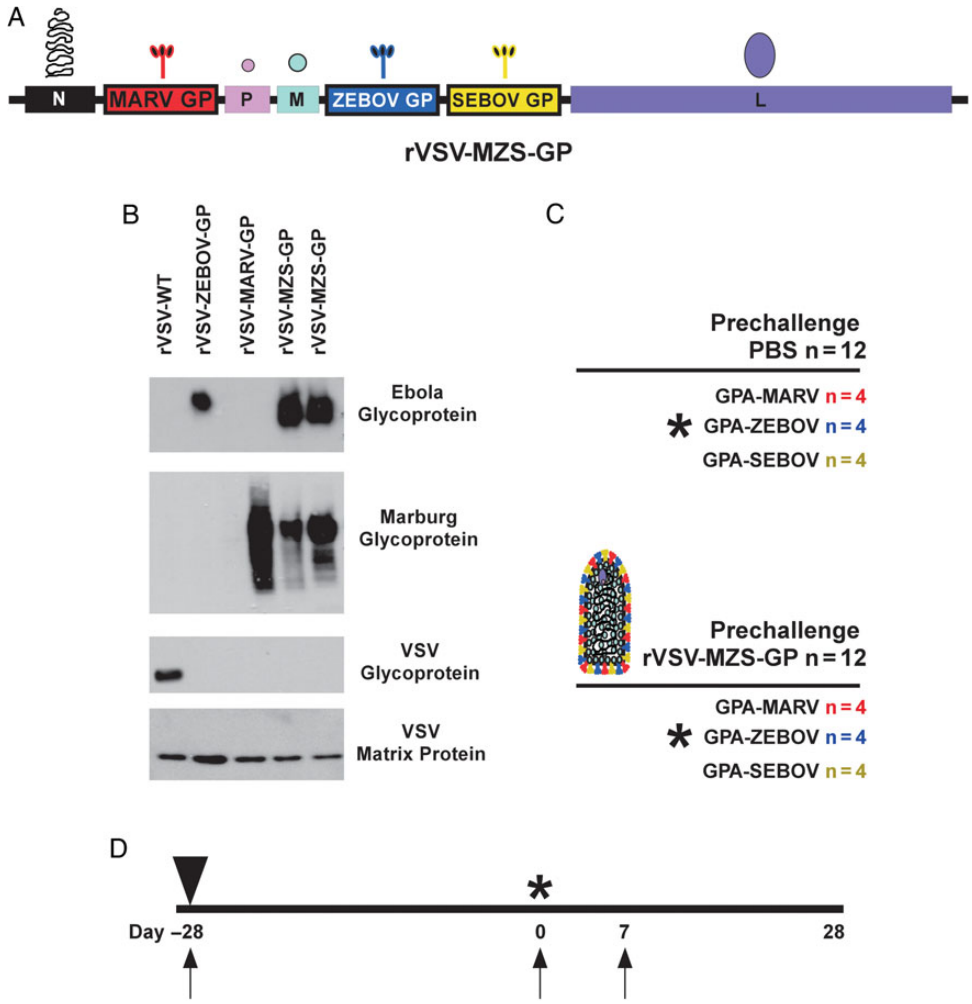

Figure 1. *A*, Diagram of rVSV genome, rVSV-MZS-GP, vaccine used in this study. N, nucleoprotein; P, phosphoprotein; M, matrix protein; GP, filovirus glycoprotein (MARV [red], ZEBOV [blue], or SEBOV [yellow]); L, large polymerase protein. *B*, Western blot of sucrose cushion purified virions; first lane: rVSV-wt (matrix protein and VSV G control); second lane: rVSV-ZEBOV-GP (EBOV GP control); third lane: rVSV-MARV-GP (MARV GP control): and lanes 4 and 5: rVSV-MZS-GP showing lack of VSV G with VSV matrix protein, and multiple filovirus GPs associated with virions. *C*, PBS and rVSV-MZS-GP vaccine groups and the challenge groups within each group. *D*, Flow chart showing the days of vaccination (arrowhead), days of sampling (arrows), and day of challenge (*). Abbreviations: GP, glycoprotein; GPA, guinea pig–adapted; MARV, *Marburg marburgvirus*; MZS, MARV-ZEBOV-SEBOV; PBS, phosphate-buffered saline; rVSV, recombinant vesicular stomatitis virus; SEBOV, *Sudan ebolavirus*; VSV, vesicular stomatitis virus; wt, wild-type; ZEBOV, *Zaire ebolavirus*.

RESULTS AND DISCUSSION

Previously, vaccination studies in guinea pigs revealed that monovalent rVSV-filovirus-GP vectors (eg, rVSV-ZEBOV) do not provide protection against heterologous virus challenge (e.g. SEBOV) [3]. Bivalent rVSV vaccine vectors expressing multiple filovirus antigens improved cross-protection [15]. To further examine the potential for cross-protection of the rVSV-filovirus-GP vaccine platform, we developed, recovered, and characterized an rVSV vaccine vector expressing the MARV, ZEBOV, and SEBOV GPs from 3 separate open reading frames; rVSV-MZS-GP (Figure 1*A*). Western blot analysis of sucrose cushion purified virions showed that all 3 GPs were expressed and incorporated into the envelopes of rVSV-MZS-GP virions (Figure 1*B*). To our knowledge, this is the first rVSV vaccine vector recovered that expresses multiple filovirus GPs from 1 vector.

To test the ability of the trivalent rVSV-MZS-GP to provide protection against multiple filoviruses, 2 groups of 12 guinea pigs each were injected with either PBS or rVSV-MZS-GP (Figure 1*C* and 1*D*, arrowhead). Examination of the animals vaccinated with rVSV-MZS-GP 28 days postinjection revealed detectable levels of circulating anti-MARV, anti-ZEBOV, and anti-SEBOV GP IgG (Figure 2*A*, 0), while there were no detectable levels of circulating antifilovirus GP IgG in the PBS vaccinated animals (data not shown). The levels of circulating IgG

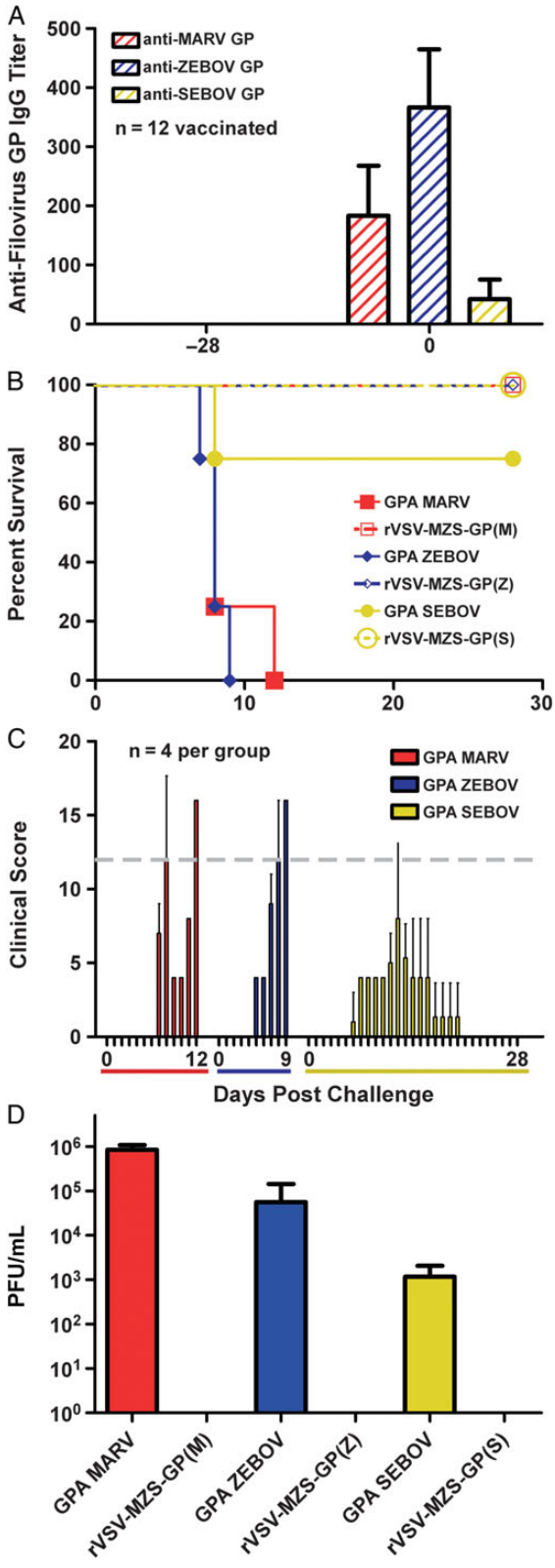

Figure 2. *A*, Reciprocal endpoint dilution titers for IgG against MARV GP (red hatch), ZEBOV GP (blue hatch), and SEBOV GP (yellow hatch) as determined from serum samples in each group at 28 and 0 days before challenge. Negative PBS only IgG titers not shown. *B*, Kaplan–Meier survival curve for each group post-GPA-filovirus challenge. GPA-MARV (solid red), GPA-ZEBOV (solid blue), and GPA-SEBOV (solid yellow) represent PBS-only control groups. rVSV-MZS-GP (M, Z, or S) represent vaccinated groups with respective challenge virus. *C*, Average clinical scores for the PBS-only vaccinated groups; negative clinical scores for rVSV-MZS-GP not depicted. Dashed line depicts euthanasia criteria. *D*, PFUs/mL isolated from serum at day 7 postchallenge for all groups. Limit of detection 25 PFUs/mL. Error bars represent standard deviation of the mean. Abbreviations: GP, glycoprotein; GPA, guinea pig–adapted; IgG, immunoglobin G; MARV, *Marburg marburgvirus*; MZS, MARV-ZEBOV-SEBOV; PFUs, plaque-forming units; PBS, phosphate-buffered saline; rVSV, recombinant vesicular stomatitis virus; SEBOV, *Sudan ebolavirus*; ZEBOV, *Zaire ebolavirus*.

against the MARV GP and ZEBOV GP were similar to those observed for boosted guinea pigs expressing SEBOV GP in a bivalent rVSV vector [15], while the circulating anti-SEBOV GP IgG was the lowest out of the 3 filovirus antigens from the rVSV-MZS-GP vector. Currently, it is not understood whether this is due to antigenic preference or to the location of the SEBOV GP gene at the end of the transcription/translation gradient in this vector. Further characterization of transcriptional gradient position and circulating IgG response in the rVSV-trivalent filovirus-GP vector is warranted.

Twenty-eight days after PBS or rVSV-MZS-GP injection, 4 animals from each group were challenged with either GPA-MARV, GPA-ZEBOV, or GPA-SEBOV (Figure 1*C* and 1*D*, asterisks). After GPA-filovirus challenge, the guinea pigs were observed for survival, clinical scores, and viremia. All the rVSV-MZS-GP–vaccinated animals survived GPA-ZEBOV, GPA-SEBOV, and GPA-MARV challenge (4/4 for all groups) (Figure 2*B*), displayed no clinical signs of infection (Figure 2*C*, negative data not shown), and had no detectable viremia at day 7 postchallenge by plaque assay for all groups (Figure 2*D*). PBS-only vaccinated guinea pigs succumbed to challenge in the GPA-MARV group (0/4 survival) and GPA-ZEBOV group (0/4 survival), while the GPA-SEBOV group lost 1 animal at day 8 postchallenge (3/4 survival) (Figure 2*B*). Each PBS-only vaccinated group also had clinical scores (Figure 2*C*) and day 7 postchallenge circulating viremia (Figure 2*D*). While the GPA-MARV and GPA-ZEBOV protection data are stronger from a survival standpoint, the current state of the GPA-SEBOV model reduces any protection from challenge claims from the present study. To date, a 100% lethal GPA-SEBOV model does not exist; however, the current GPA-SEBOV used in this study was able to cause disease in 4/4 animals (Figure 2*C*, yellow) and all animals (4/4) had circulating viremia at day 7 postchallenge (Figure 2*D*). Our data clearly show that vaccination with rVSV-MZS-GP prevents GPA-SEBOV-induced disease and reduces viremia below detectable levels, suggesting that this vector will provide significant if not total protection against a lethal SEBOV challenge.

In summary, we have created a trivalent rVSV-MZS-GP vaccine vector that expresses multiple filovirus GPs; induces

circulating IgG against MARV-GP, ZEBOV-GP, and SEBOV-GP; protects against GPA-MARV-, GPA-ZEBOV-, and GPA-SEBOV-induced disease; and reduces viremia at day 7 postchallenge below detectable levels. The MARV and ZEBOV data are the first to show 100% protection from GPA-MARV and GPA-ZEBOV using a single-vector, single-injection filovirus vaccine. These data combined with the reduction in the GPA-SEBOV data as discussed above offer a glimpse into the potential for the use of rVSV-MZS-GP as a vaccine to protect against MARV, ZEBOV, and SEBOV in areas of endemic overlap with the additional potential to reduce manufacture burden of broad-spectrum filovirus vaccines. Along with the obvious need to strengthen the GPA-SEBOV model (though it is currently capable of screening against disease and viremia), the data presented here against all 3 filoviruses is encouraging; however, the rVSV-MZS-GP needs further testing of the utility in the cynomolgus macaque models for MARV, ZEBOV, and SEBOV. Additionally, while not absolutely necessary for filovirus vaccine development, a single-vector, single-injection vaccine with broad-spectrum protection against filoviruses would be advantageous for the manufacture of a vaccine against multiple viruses and for attempts to vaccinate at-risk populations where 1 vaccination versus multiple would be more practical.

Notes

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Conflicts of interest. T. W. G. and J. H. C. claim intellectual property regarding multivalent rVSV vaccine vectors against filovirus infections. T. W. G. has a patent US 61/014,626 pending to Boston University, a patent US 61/014,669 pending to Boston University, and a patent US 61/070,748 pending to Boston University. J. H. C. has a patent US 61/070,748 pending to Boston University. All other authors report no potential conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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