Published in final edited form as:

Vaccine. 2010 December 16; 29(2): 212–220. doi:10.1016/j.vaccine.2010.10.053.

INDUCTION OF NEUTRALIZING ANTIBODIES TO HENDRA AND NIPAH GLYCOPROTEINS USING A VENEZUELAN EQUINE ENCEPHALITIS VIRUS *IN VIVO* EXPRESSION SYSTEM

Gabriel N. Defang^{a,b,c,*}, Dimple Khetawat^b, Christopher C. Broder^b, and Gerald V. Quinnan Jr.^a

^a Department of Preventive Medicine and Biometrics, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD. 20814, USA

^b Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD. 20814, USA

^c Viral and Rickettsial Diseases Department, Naval Medical Research Center, 503 Robert Grant Avenue, Silver Spring, MD. 20910

Abstract

The emergence of Hendra Virus (HeV) and Nipah Virus (NiV) which can cause fatal infections in both animals and humans has triggered a search for an effective vaccine. Here, we have explored the potential for generating an effective humoral immune response to these zoonotic pathogens using an alphavirus-based vaccine platform. Groups of mice were immunized with Venezuelan equine encephalitis virus replicon particles (VRP) encoding the attachment or fusion glycoproteins of either HeV or NiV. We demonstrate the induction of highly potent cross-reactive neutralizing antibodies to both viruses using this approach. Preliminary study suggested early enhancement in the antibody response with use of a modified version of VRP. Overall, these data suggest that the use of an alphavirus-derived vaccine platform might serve as a viable approach for development of an effective vaccine against the henipaviruses.

Keywords

Henipavirus; Alphavirus replicon; Vaccine

1 INTRODUCTION

Hendra Virus (HeV) and Nipah Virus (NiV) are important human pathogens that have emerged only recently. HeV and NiV are the prototype members of a new genus, *Henipavirus*, in the *Paramyxoviridae* family, and are also zoonotic biological safety level-4 (BSL-4) select agents (reviewed in EATON Nat Med 2006). NiV was first recognized in 1998 during an outbreak in Malaysia and was primarily transmitted to humans from infected pigs. The outbreak was responsible for 265 cases of encephalitis in people, with a nearly

*Corresponding author: Gabriel N. Defang, Ph.D., Naval Medical Research Center, Viral and Rickettsial Diseases Department, 503 Robert Grant Avenue, Silver Spring, MD. 20910, Phone: +1 301-319-9890/+1 202-498-9295, Fax: +1 301-319-7451, gdefang@med.navy.mil; gabbydefang@yahoo.com.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

40% mortality rate [1–3]. There have been more than a dozen occurrences of NiV since its initial recognition, most appearing in Bangladesh and India (REVIEWED) [4]) and again in March 2008 [5] and January 2010 [6]. Among these spillover events of NiV, the human mortality rate has been higher (~75%) along with evidence of person-to-person transmission [7–9] and direct transmission of virus from flying foxes to humans via contaminated food [10]. HeV emerged in Australia in 1994 and was identified as the cause of fatal respiratory disease in horses, which in turn was transmitted to humans causing fatal pulmonary disease [11,12], and HeV has also repeatedly caused fatal infections in horses with documented human illness and seroconversion [13]. There have been 14 recognized occurrences of HeV in Australia since 1994 with at least one occurrence per year since 2006, the most recent in May 2010. Every outbreak of HeV has involved horses as the initial infected host, causing lethal respiratory disease and encephalitis, along with a total of seven human cases arising from exposure to infected horses, among which four have been fatal and the most recent in 2009 [4,14].

NiV and HeV have been classified as category C select agents, and both can be readily isolated from natural sources [15–17], and readily grown in cell culture [18]. Being newly described, there is limited but growing knowledge about the biology of these viruses, and there are currently no approved therapeutic regimens or vaccines available for henipaviruses making them a biodefense concern. Efforts to date to develop vaccines have included the use of both recombinant poxviruses and soluble glycoprotein subunits. A recombinant vaccinia virus expressing the NiV attachment (G) and fusion (F) glycoproteins [19,20] has been shown to induce NiV-neutralizing antibodies in mouse and hamster animal models [19,20]. A canarypox virus-based vector encoding F and G glycoproteins of NiV has also been shown to protect animals against NiV challenge in a pig model [21]. Finally, a subunit vaccine approach utilizing purified soluble versions of the G glycoproteins (sG) from HeV and NiV protected cats from subsequent NiV challenge [22].

In vivo expression systems derived from Venezuelan equine encephalitis virus (VEE) have been shown to elicit protective mucosal and systemic immunity against a variety of viral diseases [23–27]. In this study we have employed a VEE-based vector, which packages genomic VEE replicon expressing a transgene into virus replicon particles (VRP). These VRP were used to induce immune responses to HeV and NiV in a murine model. Our primary objective was to determine the effectiveness of VRP for induction of antibodies that neutralize HeV and NiV. In addition, we also compared the immunogenicity of the wild-type VEE vector and a modified VEE replicon capable of prolonged expression that we constructed. The VEE-based vaccine approach takes advantage of the vector's inherent ability to deliver immunologic proteins to immune cells as well as their potential for induction of mucosal and systemic immunity. The results demonstrate the induction of potent immune responses against both HeV and NiV glycoproteins using as expression vectors two VRP variants that differed with respect to duration of transgene expression. Taken together, these findings suggest that an alphavirus-derived vaccine platform could serve as a viable approach for development of an effective vaccine against the henipaviruses.

2. MATERIALS AND METHOS

2.1 Cell cultures

The baby hamster kidney cell line, BHK-21 (ATCC, Manassas, VA.) and human embryonic kidney cell line, 293T (ATCC, Manassas, VA.), used in this study were maintained in Dulbecco's minimal essential medium (Gibco) supplemented with 10% fetal bovine serum, L-glutamine, penicillin-streptomycin (Gibco) and tylosin (Sigma). The HeLa (ATCC CCL 2) cell line was maintained in Dulbecco's modified Eagle's medium (Quality Biologicals,

Gaithersburg, MD.) supplemented with 10% cosmic calf serum (HyClone, Logan, UT.) and L-glutamine (DMEM-10). The human head and neck carcinoma PCI 13 cells were maintained in DMEM-10 supplemented with 10 nM HEPES (Quality Biologicals). All cell cultures were maintained at 37°C in humidified 5% CO₂ atmosphere.

2.2 VEE Replicon construct

The VEE constructs pRepX (VEE replicon vector) and pCV have been previously described [23]. They were kindly provided by J. Smith, U.S. Army Medical Research Institute of Infectious Diseases, Frederick, Md. The pGpM has been previously described [28]. It is a modified version of the pGP [23] with two back mutations: E1 (272A/T) and E2 (209E/K) that facilitate lymphoid trafficking and immunogenicity of VRP [28]. All PCR amplified genes from donor plasmids that were cloned into pRepX, were amplified by primers designed to introduce a ClaI recognition sequence followed by a 16-nucleotide VEE promoter sequence at the 5' end of the gene, and a ClaI recognition sequence at the 3' end [23,28]. All PCR amplification reactions were performed using rTth DNA polymerase (Applied Biosystems, Foster City, CA.). A mutation was introduced into the Nsp2 region of pRepX by site-directed mutagenesis (Stratagene Quick Change) according to manufacturer's instructions. Proline (P)⁷¹³ was replaced by glycine (G). Replicon constructs with the resulting G mutation are designated starting with mV. The pRepX-R2gp160 (also named V-R2gp160) and its corresponding R2 envelope gene has been previously described [28,29].

The V-HeVF, V-HeVG, V-NiVF and V-NiVG were constructed as VEE replicon vectors encoding the corresponding fusion (F) and attachment (G) glycoprotein of HeV and NiV. The constructs were made by ClaI restriction cloning of PCR amplified coding sequences of the corresponding envelope glycoprotein into pRepX. The original vaccinia virus promoter-driven expression vector, pMC02, with the corresponding F and G coding sequences have been previously described [30]. The modified VEE replicon construct carrying a single G mutation in the Nsp2 region was generated by replacing an ApaI/NotI digested fragment of the mutant pRepX with a similarly digested fragment from a wild-type VEE replicon construct encoding the Hendra G glycoprotein (HeVG) transgene. The modified VRP has shown a more sustained expression of transgene compared to wild-type based on laboratory experience.

2.3 Generation of packaged VEE Replicon Particles

The two-helper system developed by Pushko et al. was employed in the preparation of VRP. Replicon particle preparation has previously been described [28]. Replicon plasmids V-R2gp160, V-HeVF, V-HeVG, mV-HeVG, V-NiVF, and V-NiVG together with helper plasmids pGpM, and pCV were linearized with NotI and *in vivo* transcribed using T7 RNA polymerase (Ambion, Austin, TX.) according to manufacturer's instructions. The resulting transcripts of individual replicon constructs were separately combined with transcripts of pGpM and pCV, and each mixture was used to transfect 2×10⁷ BHK-21 cells by electroporation using a Bio-Rad Gene Pulser (Bio-Rad, Hercules, CA.). The cells were seeded into 75-cm² tissue culture flasks and fed with medium. Cells transfected with wild-type constructs were maintained at 37°C for 27 h in 5% CO₂ atmosphere. Those transfected with replicon constructs carrying a single Nsp2 mutation were maintained at 35°C. Medium from each flask was harvested and then clarified by centrifugation at 10,000 rpm for 30 min at 4°C in a Beckman L5-5E ultracentrifuge. The clarified medium was transferred to a 35-ml centrifuge tube, underlayered with 5 ml of 20% sucrose in phosphate-buffered saline (PBS), and ultracentrifuged at 24,000 rpm for 3 h to pellet the particles. The medium and sucrose were removed, the pellet was covered with 0.5 ml Dulbecco's phosphate-buffered saline containing Ca²⁺ and Mg²⁺ (D-PBS) and 0.1% fetal bovine serum (FBS) at 4°C overnight and then scraped off into D-PBS, and aliquots were stored at -70°C until needed.

Infectivity of replicon particle preparations and infectious particle concentrations were determined by an immunofluorescence assay (IFA), as has been previously described [28]. BHK-21 cells were seeded at a density of 2×10^4 /well into wells of 16-well LabTek tissue culture slides (Nalge Nunc International), incubated overnight at the appropriate temperature of either 35°C or 37°C in 5% CO₂ atmosphere. Wells were inoculated with 50 µl aliquots of serial 10-fold dilutions of replicon particle preparations in D-PBS with 0.1% FBS. Medium was then added in 150 µl aliquots. After 24 h, the cells were fixed with cold acetone at -20°C for 20 min, air dried, and stored at -20°C. For IFA, the cells were rehydrated in PBS with 0.1% bovine serum albumin (BSA), and blocked with PBS containing 7.5% BSA for 15 min. Cells were probed with the appropriate anti-serum (either globulin fraction of human HIV-1 immune serum [31], soluble HeVG (sHeVG)-specific rabbit immune serum [30], NiVF- or HeVF-specific rabbit anti-sera [30]) or negative serum, each diluted 1:200 in PBS containing 0.1% BSA. Sera were applied for 1 h at room temperature. The wells were washed twice and the assays were developed by using the corresponding goat anti-human, or goat anti-rabbit immunoglobulin G (IgG)-fluorescein isothiocyanate (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD.). The numbers of infected cells per well were determined by fluorescence microscopy, and the volume inoculated and dilution of replicon particles applied to the particular well were used to calculate the concentration of infectious particles in the starting inoculum.

A safety test was performed on each preparation to test for replication competence. Confluent BHK-21 cell culture in six-well plates were inoculated with 1:10 dilutions of replicon particle suspensions, allowed to adsorb for 1 h, washed three times with PBS, fed with medium, and incubated at 37°C for 24 h. The medium was harvested, filtered through 0.45µm-pore-size filters, and stored at -70°C. After three serial blind passages, the medium was inoculated into wells of tissue culture slides for IFA. All VRP preparations used for immunization passed the safety test; with no replication competent virus detected by IFA.

2.4 Detection of in vitro protein expression by VEE replicon constructs

Western immunoblot analysis was used to test for protein expression by the various replicon constructs. BHK-21 cells were either transfected with replicon RNA as described above or infected with VRP encoding the gene of interest. After 24 to 72 h, medium was collected, centrifuged to remove cell debris, and HaltTM protease inhibitor cocktail (PIERCE, Rockford, IL.) was added. The cells were washed with cold PBS, lysed and cell extracts prepared using M-PER[®] Mammalian Protein Extraction Reagent (PIERCE, Rockford, IL.). Cell extracts were briefly centrifuged to remove cell debris and HaltTM protease inhibitor cocktail (PIERCE, Rockford, IL.) was added. Samples were prepared by boiling in sample buffer containing 2-mercaptoethanol. Proteins in samples of cell extracts and medium were resolved by sodium dodecyl sulfate 4–12% polyacrylamide gel electrophoresis (SDS-PAGE). Following transfer to nitrocellulose membrane the blot was probed with HIV-1 positive human serum, sHeVG rabbit antiserum, NiVF_{cytoplasmic tail (cyt)}-specific rabbit antiserum, or HeVF₂-specific rabbit antiserum as appropriate. The blot was then incubated with alkaline phosphate-conjugated goat anti-human (or anti-rabbit) IgG (Bio-Rad) and developed.

2.5 Measurement of immunogen-specific antibody response

Antibody response in serum was measured by enzyme-linked immunosorbent assay (ELISA) using the ELISA starter accessory kit (Bethyl Laboratories, Inc.). For detection of HIV-1 specific antibody responses, Nunc MaxiSorp C bottom well plates (Bethyl Laboratories, Inc.) were coated with 100µl (5ng)/well of HIV-1 strain R2 gp120 or gp140 purified protein diluted in coating buffer (Sigma Chemical). For detection of HeV and NiV G-specific antibody responses, plates were coated with 50µl (50ng)/well of purified sHeVG

or sNiVG protein diluted in coating buffer. All purified proteins used as coating antigens were produced using a recombinant vaccinia virus expression system and purified using protein purification techniques as described previously [28,32,33]. For detection of HeV and NiV F-specific responses, cell lysates of BHK-21 cells transfected 36 h prior with replicon construct encoding the respective F glycoprotein were used to coat plates after dilution in coating buffer. Coated plates were incubated overnight at 4°C. After blocking with Postcoat solution (Sigma Chemical) for 1 h at room temperature, plates were washed, 100 µl of serially diluted mouse or rabbit sera in sample/conjugate diluent (Sigma Chemicals) were added to the wells and the plates were held at room temperature for 1 h. Sera were tested in duplicate. Positive and negative control sera were included in each assay. Reaction mixtures were further developed by adding 100 µl of biotinylated anti-mouse or –rabbit IgG and horseradish peroxidase streptavidin (Vector Laboratories Inc., CA.), each diluted 1:5000, to each well. Plates were incubated at room temperature for 1 h, washed, and a 100-µl aliquot of TMB Peroxidase Substrate & Peroxidase Solution B (Kirkegaard & Perry) was added to each well. Plates were held in the dark at room temperature for 30 min. Color development was stopped by addition of 50 µl of 2 M H₂SO₄ to each well. The optical density at 450 nm was measured in a Bio-Rad Model 680 microplate reader. Linear regression curves were plotted for each serum sample, and the titers calculated as the inverse of the highest serum dilution that produced an optical density twice that of the negative control serum.

2.6 Generation of Henipavirus pseudovirions and Neutralization assays

Neutralization assays using HIV-1 pseudotyped viruses have been widely employed. They tend to be highly reproducible and quantitative [28,31,34–40]. The pseudotyped virus assays can facilitate testing the neutralizing activity of immune sera and monoclonal antibodies against otherwise BSL-3 and 4 restricted agents. Pseudoviruses were prepared by FuGENE® 6 Transfection Reagent (Roche)-mediated co-transfection of 60–80% confluent 293T cells in 25-cm² flask with plasmids pNL4-3.luc.E-R- and pCAGG-Henipavirus_{envelope} (Khetawat and Broder unpublished). For homotypic NiV and HeV pseudoviruses; pCAGG-NiVF and –NiVG or pCAGG-HeVF and –HeVG were used respectively. Following 24 h of incubation, fresh medium was added and cells were further incubated for 24 h at 37°C in 5% CO₂ atmosphere. Pseudovirus-containing medium was collected, centrifuged at 1700 rpm for 5 min to remove cell debris, and then passaged through 0.45µm pore size filters (Millipore, Bedford, MA.) prior to use in neutralization assays.

Pseudotyped virus neutralization assays were performed by preincubation of 25 µl of two-fold dilutions of mouse sera with 25 µl of pseudovirus suspension for 1 h at 4°C in wells of 96-well, white-walled, flat-bottomed tissue culture plates (Costar, Corning, NY.). 150 µl suspensions of 1×10⁴ 293T cells were added to all wells, plates were incubated at 37°C in 5% CO₂ atmosphere for 3 days, then washed with PBS and lysed for 30 min in a shaker with 15 µl of Luciferase Assay System cell lysis buffer (Promega, Madison, WI.). Next, Luciferase Assay System reporter lysis buffer (Promega) was added, and luciferase activity was measured using a MicroLumat *Plus* Luminometer (Wallac, Gaithersburg, MD.). Neutralization titers were determined based on relative luminescence units (RLU). Neutralization endpoint was calculated as the highest serum dilution at which mean luminescence from test samples were reduced by at least 90% compared to non-neutralized controls. All test sera were run in triplicate in at least two independent experiments. Positive and negative controls were included in each assay.

2.7 Cell fusion assays

NiV and HeV glycoprotein-mediated cell fusion reporter gene assay has previously been described and used extensively [30,33,41–43]. It is an adaptation of a previously described reporter gene assay based on gene expression using the recombinant vaccinia virus system

[44,45]. In this system, in addition to expression of viral envelope glycoproteins and viral receptors on effector and target cell populations respectively, one cell population also expresses a T7 RNA polymerase while the other expresses a T7 promoter-driven *E. coli lacZ* cassette. β -galactosidase (β -Gal) is synthesized only in fused cells [44,46]. Briefly, vaccinia virus encoded proteins were produced by infecting cells at an MOI of 10 and incubating infected cells at 31°C overnight [45]. HeLa cells were infected with recombinant vaccinia viruses encoding F and G glycoproteins of either HeV or NiV, along with a recombinant vaccinia virus encoding T7 RNA polymerase (effector cells). PCI (target cells) infected with the reporter vaccinia virus, vCB21R, encoding *E. coli lacZ* were mixed with effector cells in duplicate wells of a 96-well plate and incubated at 37°C. The ratio of envelope glycoprotein expressing effector cells to target cells was 1:1 (2×10^5 total cells per well; 200 μ l total volume). Cytosine arabinoside (40 μ g/ml) was added to the fusion reaction mixture to reduce nonspecific β -Gal production [45]. For quantitative analysis, Nonidet P-40 was added (0.5% final) at 2.5 h, and aliquots of lysates were assayed for β -Gal at ambient temperature with the substrate chlorophenol red-D-galactopyranoside (Roche). For testing fusion inhibition by immune sera, serial dilutions of mouse sera were prepared and added to effector cell populations 30 min prior to the addition of the target cell population. Positive and negative controls were included in each assay and fusion results were calculated and expressed as rates of β -Gal activity (change in optical density at 570 nm per minute $\times$ 1,000) [44]. The 50% inhibitory endpoints were determined by comparing the rates of β -Gal activity in test sample wells at each dilution to the rate of β -Gal activity in negative control wells. The highest dilution at which the test serum inhibited fusion to less than 50% of the control was considered the endpoint. The difference in scale (rate of β -Gal activity) between HeV-mediated and NiV-mediated fusion is due to the inherent ability of HeV glycoproteins to mediate fusion more efficiently than NiV glycoproteins. The sensitivities of both assays are thought to be comparable.

2.8 Mouse immunization

Animals were used with approval of the Institutional Animal Care and Use Committee. Six- to eight-week-old female C3H/He mice (Jackson Laboratories) were used for immunogenicity studies. All mice were immunized by footpad inoculation of 50- μ l suspensions of VRP. Mice were bled from the tail vein.

2.9 Statistical analysis

For comparing animal responses among and between various immunization groups, a one-way ANOVA with Bonferroni's Multiple Comparison Test was performed using GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego, CA.

3. RESULTS

3.1 Expression of HeV and NiV glycoproteins

Packaged VRP were initially tested for their ability to functionally express encoded envelope proteins of HeV and NiV. BHK-21 cells were mock infected, or were simultaneously co-infected with V-HeVF and V-HeVG or V-HeVF and mV-HeVG or V-NiVF and V-NiVG combinations. Cell lysates were prepared 24 h postinfection and transgene expression analyzed by SDS-PAGE and Western blot. Using cross-reactive anti-NiVF and anti-sHeVG antibodies, respectively, expression of the F and G glycoproteins of both viruses by the corresponding wild-type or mutant VRP were confirmed. A $\sim$ 60 kDa F₀ protein and a $\sim$ 70–75 kDa HeVG protein were detected for both henipaviruses, consistent with previously published data (Figure 1) [30]. To determine whether the VRP-expressed proteins were biologically functional a syncytia formation assay was performed. Here, V-HeVF and V-HeVG or V-NiVF and V-NiVG combinations were used to co-infect 293T

cells which naturally express the henipavirus receptor ephrinB2 [41] and incubated overnight. Syncytia were observed in all cases where F and G were co-expressed and no cell-cell fusion was observed in mock infected cells, or in control cells expressing NiV or HeV F alone (Figure 2).

3.2 Immunogenicity of G glycoprotein-expressing VRP

To evaluate the induction of binding and neutralizing antibody by G glycoprotein-expressing VRP, immunogenicity studies were conducted in C3H/He mice. Included in the immunization regimen were wild-type VRP expressing HeVG or NiVG (V-HeVG and V-NiVG respectively), and a modified prolonged-expression VRP expressing HeVG (mV-HeVG) (see Table 1). The inclusion of mV-HeVG allowed for comparison with V-HeVG for the magnitude of immune response elicited. We hypothesized that both wild-type and modified VRP would induce strong immune responses. While we hypothesize that an even greater response will be seen with the more prolonged-expression VRP, the results reported here are preliminary in that regard.

Groups of five mice were immunized by footpad inoculation with either 1.2×10^6 IU of V-HeVG or mV-HeVG or 3.1×10^5 IU of V-NiVG, or 2.7×10^6 IU V-R2gp160 (VRP encoding a nonrelevant HIV-1 envelope glycoprotein.) (Table 1). A non-immunized control group was also maintained throughout the study. A lower inoculation dose of V-NiVG was due to the lower titers achieved of V-NiVG preparations. However, it is also possible that we may have underestimated the titers of V-NiVG, since the titrations of VRP were performed using anti-sHeVG antibodies. Mice in each group received three doses, one each at weeks 0, 5 and 18. All mice were bled two weeks after each dose.

There were no detectable henipavirus G-specific antibodies in sera of mice immunized with V-R2gp160 which served as an irrelevant control. Following the second immunization, there was a significant increase in geometric mean (GM) titers in all three groups (V-HeVG, mV-HeVG and V-NiVG). Titers were significantly higher after two doses in the mouse group that received the modified VRP, mV-HeVG ($p < 0.05$), compared to V-HeVG group (Figure 3A), but titers were comparable in these two groups after the third dose. Titers in the V-NiVG group were near maximal after two doses (Figure 3B).

The sera from these mice were analyzed for their ability to inhibit cell-cell fusion [30,33,41–43]. V-NiVG immune sera post-first and -second dose inhibited NiV-mediated fusion by 50% at dilutions up to 1:1600, to similar degrees (Figure 4A). Comparable inhibition of NiV-mediated fusion was observed with V-HeVG and mV-HeVG immune sera. After the first and second doses, sera from V-HeVG and mV-HeVG groups were able to inhibit HeV mediated fusion by 50% at dilutions greater than 1:6400 (Figure 4B). The 50% inhibition titers for post-second dose sera from mice in the V-HeVG group were less than two fold higher compared to post-first dose sera of mice in the mV-HeVG group. Following second immunization mV-HeVG sera inhibited slightly more than V-HeVG sera although this difference was not statistically significant. V-NiVG immune sera (post-second dose) inhibited HeV-mediated fusion although at a lower potency compared to V-HeVG immune sera. Further, the cross reactivity observed with HeV and NiV immune sera with regards to their ability to inhibit NiV- and HeV-mediated cell-cell fusion respectively (Figure 4A and B), was consistent with previous reports [30,33].

The neutralizing activity of the various sera was then tested using a henipavirus pseudotyped reporter-gene assay. The sera were assayed using the pNL4-3.luc.E-R-reporter-gene encoding retroviruses pseudotyped with NiVF + NiVG glycoprotein (Figure 5A) and HeVF + HeVG glycoproteins (Figure 5B). Sera from the HeVG and NiVG-immunized groups were able to mediate 90% neutralization of Nipah (NiVF + NiVG) and Hendra (HeVF +

HeVG) pseudotyped viruses at high titers (Figure 5A and B). The 90% neutralization titers were significantly higher ($p < 0.001$) after second dose, compared to post first dose titers. The NiVG-immune sera were less potent in neutralizing heterologous virus in comparison to V-HeVG- and mV-HeVG immune sera which neutralized homologous and heterologous virus with high potency. There were no significant differences in the neutralizing activity of V-HeVG and mV-HeVG immune sera following each of three immunizations. No significant increases in neutralization titers were observed in any groups after administration of the third vaccine dose. Sera from V-R2gp160-immunized control mice did not exhibit neutralizing activity.

3.3 Immunogenicity of F glycoprotein-expressing VRP

To address the significance of F-specific immune responses, mouse immunization experiments were also carried out. Here, groups of five mice were immunized by footpad inoculation with either V-HeVF or V-NiVF or V-R2gp160 (Table 2). Mice in each group received three doses, one each at weeks 0, 4, and 28. No henipavirus F-specific antibodies were detected in sera of mice immunized with V-R2gp160. Significant increases in HeVF- and NiVF-specific IgG titers, as measured by ELISA, were found in mice immunized with V-HeVF and V-NiVF, respectively, ($P < 0.001$) following second immunization, with no further increase after the third dose (Figure 6A and B).

The 90% neutralization titers against NiVF + NiVG and HeVF + HeVG pseudotyped viruses are shown in Figures 7A and 7B respectively. Sera of mice in both V-HeVF and V-NiVF groups developed considerable neutralizing activity against both viruses. Significant increases in serum neutralization titers were detected in both groups following the second immunization, and the HeVF-immune sera had the most potent neutralizing activity against homologous and heterologous virus. No significant increases were observed after administration of the third dose and no detectable neutralizing titers were seen in the V-R2gp160 or in the non-immunized control group.

4. DISCUSSION

Because of the high morbidity and mortality associated with HeV and NiV infections coupled with their unusually broad species tropisms, the development of effective countermeasures against these agents are of high priority and are currently ongoing. We demonstrated in this report that HeV and NiV immunogens developed using an alphavirus vectored system induced cross-reactive, potent henipavirus neutralizing antibodies.

Neutralizing antibodies produced against enveloped viruses including paramyxoviruses target the major envelope glycoproteins [47,48]. It is for this reason that vaccine approaches to induce protective immune responses to henipaviruses utilize the F and G envelope glycoproteins as immunogens. Henipavirus vaccine approaches that have been reported to date have utilized recombinant vaccinia or canarypox vectors, or soluble G glycoprotein subunits to induce protective immune responses to NiV, and to some degree HeV [19,21,22,33,49,50]. This is the first report describing the use of an alphavirus-derived vector system to induce immune responses to HeV and NiV. There are several advantages in using a VEE replicon vector platform, including their targeting of dendritic cells, high level protein expression, minimal preexisting immunity to VEE in the general population, and well documented record of induction of protective immune responses in different animal models to various viral agents [23,51–57].

Optimal induction of neutralizing antibody responses against HeV and NiV probably requires presentation of biologically functional proteins to immune cells. We confirmed the functional nature of expressed F and G proteins by demonstrating induction of syncytium

formation in 293T cells co-infected with VRP encoding F and G of either HeV or NiV. This effect documents the ability of the two expressed proteins to interact functionally with each other as well as with target cell membranes to induce membrane fusion [30,58]. These are important functions of the native proteins on the surface of infectious virus particles.

To assay for neutralizing antibodies in mouse sera, we employed both cell-cell fusion-inhibition and henipavirus F and G pseudotyped virus neutralization assays. We demonstrated in mouse immunization experiments that all VRP encoding the corresponding antigens were able to induce F- or G-specific binding antibody as well as neutralizing antibody responses in mice. In fact, only two doses of VRP were needed to induce maximum neutralizing effects in all mouse groups. Our attempts to enhance the magnitude of the humoral immune response to HeVG immunogen using modified prolonged-expression VRP, mV-HeVG, suggested modest increases in antibody production with no significant increase in neutralizing titers (Figure 3A, 5A and B); the difference merits further study. It appears that maximal neutralizing titers were reached following administration of two doses of either V-HeVG or mV-HeVG, although absence of a boost following the third dosage of VRP might have been due to anti-vector immunity induced by the first two immunizations. In vaccinations involving NiV immunogens, NiVG induced higher titers of neutralizing antibodies compared to NiVF. These observations, which indicate higher immunogenicity of the attachment protein compared to the fusion protein, are consistent with those of previous immunogenicity studies involving NiV and other paramyxoviruses [19–21, 59, 60]. Relatively low anti-NiVF neutralizing titers have nonetheless been shown to correlate with moderate protection in two different animal challenge studies [19, 21]. In contrast, the neutralizing activity of HeVF was comparable to that seen with HeVG based on our study results (Figure 5B and 7B). The potency of the cross-reactive neutralizing activity of NiVF and G immune sera was inferior to that seen with HeVF and G immune sera. Interestingly, anti-HeVF heterologous neutralization titers were much higher compared to anti-NiVF homologous titers based on the Nipah pseudovirus neutralization assay (Figure 7A), although the observed difference could have occurred if there were actually more infectious particles in the HeVF immunization dose.

Taken together, our data demonstrate that the F or G glycoprotein of either virus is sufficient to induce potent, cross-reactive neutralizing antibodies. These results also suggest the possibility of developing a single vaccine against both HeV and NiV based on HeV immunogens. Recent vaccination studies using either HeVG or NiVG immunogen alone demonstrated protection in a feline model against challenge with NiV [22,50]. Protection against HeV challenge using this vaccine approach is still to be established. Future studies using small animal and non-human primate [57] infection models are needed to determine whether individual F protein immunizations or HeVF and HeVG combination vaccines protect against henipavirus infections, and whether the protective effects induced are homologous and heterologous.

Acknowledgments

This work was supported by NIH grants AI037438, AI054715 and the Middle Atlantic Regional Center of Excellence (MARCE) for Biodefense and Emerging Infectious Disease Research, NIH AI057168.

The views expressed in this article are solely those of the authors, and do not represent the official views or opinions of the Department of Defense, Department of the Navy or Uniformed Services University of the Health Sciences.

References

1. Chua KB, Goh KJ, Wong KT, Kamarulzaman A, Tan PS, Ksiazek TG, et al. Fatal encephalitis due to Nipah virus among pig-farmers in Malaysia. *Lancet*. 1999; 354(9186):1257–9. [PubMed: 10520635]
2. Chua KB, Bellini WJ, Rota PA, Harcourt BH, Tamin A, Lam SK, et al. Nipah virus, a newly emergent deadly paramyxovirus. *Science*. 2000; 288:1432–1435. [PubMed: 10827955]
3. Lam SK, Chua KB. Nipah virus encephalitis outbreak in Malaysia. *Clin Infect Dis*. 2002; 34(2):S48–51. [PubMed: 11938496]
4. Bishop, KA.; Broder, CC. Hendra and Nipah: Lethal Zoonotic Paramyxoviruses. In: Scheld, WM.; Hammer, SM.; Hughes, JM., editors. *Emerging Infections*. American Society for Microbiology; Washington, D.C: 2008. p. 155-187.
5. Pro-Med. International Society for Infectious Diseases; 2008. NIPAH VIRUS, FATAL - BANGLADESH (DHAKA).
6. Pro-MED. International Society for Infectious Diseases; 2010. Nipah virus, fatal - Bangladesh: (Faridpur).
7. Gurley ES, Montgomery JM, Hossain MJ, Bell M, Azad AK, Islam MR, et al. Person-to-person transmission of Nipah virus in a Bangladeshi community. *Emerg Infect Dis*. 2007; 13(7):1031–7. [PubMed: 18214175]
8. Harit AK, Ichhpujani RL, Gupta S, Gill KS, Lal S, Ganguly NK, et al. Nipah/Hendra virus outbreak in Siliguri, West Bengal, India in 2001. *Indian J Med Res*. 2006; 123(4):553–60. [PubMed: 16783047]
9. Homaira N, Rahman M, Hossain MJ, Epstein JH, Sultana R, Khan MS, et al. Nipah virus outbreak with person-to-person transmission in a district of Bangladesh, 2007. *Epidemiol Infect*. 2010; 138(11):1630–6. [PubMed: 20380769]
10. Luby SP, Rahman M, Hossain MJ, Blum LS, Husain MM, Gurley E, et al. Foodborne transmission of Nipah virus, Bangladesh. *Emerg Infect Dis*. 2006; 12(12):1888–94. [PubMed: 17326940]
11. Field H, Young P, Yob JM, Mills J, Hall L, Mackenzie J. The natural history of Hendra and Nipah viruses. *Microbes Infect*. 2001; 3(4):307–14. [PubMed: 11334748]
12. Westbury HA. Hendra virus disease in horses. *Rev Sci Tech*. 2000; 19(1):151–9. [PubMed: 11189712]
13. Hanna JN, McBride WJ, Brookes DL, Shield J, Taylor CT, Smith IL, et al. Hendra virus infection in a veterinarian. *Med J Aust*. 2006; 185(10):562–4. [PubMed: 17115969]
14. Playford EG, McCall B, Smith G, Slinko V, Allen G, Smith I, et al. Human Hendra virus encephalitis associated with equine outbreak, Australia, 2008. *Emerg Infect Dis*. 2010; 16(2):219–23. [PubMed: 20113550]
15. Chua KB, Koh CL, Hooi PS, Wee KF, Khong JH, Chua BH, et al. Isolation of Nipah virus from Malaysian Island flying-foxes. *Microbes Infect*. 2002; 4(2):145–51. [PubMed: 11880045]
16. Reynes JM, Counor D, Ong S, Faure C, Seng V, Molia S, et al. Nipah virus in Lyle's flying foxes, Cambodia. *Emerging Infectious Diseases*. 2005; 11(7):1042–1047. [PubMed: 16022778]
17. Wacharapluesadee S, Lumlertdacha B, Boongird K, Wanghongsa S, Chanhom L, Rollin P, et al. Bat Nipah virus, Thailand. *Emerg Infect Dis*. 2005; 11(12):1949–51. [PubMed: 16485487]
18. Crameri G, Wang LF, Morrissy C, White J, Eaton BT. A rapid immune plaque assay for the detection of Hendra and Nipah viruses and anti-virus antibodies. *J Virol Methods*. 2002; 99(1–2): 41–51. [PubMed: 11684302]
19. Guillaume V, Contamin H, Loth P, Georges-Courbot MC, Lefeuvre A, Marianneau P, et al. Nipah Virus: Vaccination and Passive Protection Studies in a Hamster Model. *Journal of Virology*. 2004; 78(2):834–840. [PubMed: 14694115]
20. Tamin A, Harcourt BH, Ksiazek TG, Rollin PE, Bellini WJ, Rota PA. Functional Properties of the Fusion and Attachment Glycoproteins of Nipah Virus. *Virology*. 2002; 296(1):190–200. [PubMed: 12036330]
21. Weingartl HM, Berhane Y, Caswell JL, Loosmore S, Audonnet JC, Roth JA, et al. Recombinant Nipah Virus Vaccines Protect Pigs against Challenge. *Journal of Virology*. 2006; 80(16):7929–7938. [PubMed: 16873250]

22. Mungall BA, Middleton D, Crameri G, Bingham J, Halpin K, Russell G, et al. Feline model of acute nipah virus infection and protection with a soluble glycoprotein-based subunit vaccine. *J Virol.* 2006; 80(24):12293–302. [PubMed: 17005664]
23. Pushko P, Parker M, Ludwig GV, Davis NL, Johnston RE, Smith JF. Replicon-helper systems from attenuated Venezuelan equine encephalitis virus: expression of heterologous genes in vitro and immunization against heterologous pathogens in vivo. *Virology.* 1997; 239(2):389–401. [PubMed: 9434729]
24. Johnston RE, Johnson PR, Connell MJ, Montefiori DC, West A, Collier ML, et al. Vaccination of macaques with SIV immunogens delivered by Venezuelan equine encephalitis virus replicon particle vectors followed by a mucosal challenge with SIVsmE660. *Vaccine.* 2005; 23(42):4969–79. [PubMed: 16005121]
25. Schultz-Cherry S, Dybing JK, Davis NL, Williamson C, Suarez DL, Johnston R, et al. Influenza virus (A/HK/156/97) hemagglutinin expressed by an alphavirus replicon system protects chickens against lethal infection with Hong Kong-origin H5N1 viruses. *Virology.* 2000; 278(1):55–9. [PubMed: 11112481]
26. Balasuriya UB, Heidner HW, Davis NL, Wagner HM, Hullinger PJ, Hedges JF, et al. Alphavirus replicon particles expressing the two major envelope proteins of equine arteritis virus induce high level protection against challenge with virulent virus in vaccinated horses. *Vaccine.* 2002; 20(11–12):1609–17. [PubMed: 11858869]
27. Gupta S, Janani R, Bin Q. Characterization of HIV-gag-specific IFN γ -expressing cells following protective mucosal immunization with alphavirus replicon particles. *J Virol.* 2005; 79(11):7135–45. [PubMed: 15890953]
28. Dong M, Zhang PF, Grieder F, Lee J, Krishnamurthy G, VanCott T, et al. Induction of primary virus-cross-reactive human immunodeficiency virus type 1-neutralizing antibodies in small animals by using an alphavirus-derived in vivo expression system. *J Virol.* 2003; 77(5):3119–30. [PubMed: 12584337]
29. Quinnan GV, Zhang PF, Fu DW, Dong M, Alter HJ. Expression and Characterization of HIV Type 1 Envelope Protein Associated with a Broadly Reactive Neutralizing Antibody Response. *AIDS Research and Human Retroviruses.* 1999; 15(6):561–570. [PubMed: 10221533]
30. Bossart KN, Wang LF, Flora MN, Chua KB, Lam SK, Eaton BT, et al. Membrane fusion tropism and heterotypic functional activities of the Nipah virus and Hendra virus envelope glycoproteins. *J Virol.* 2002; 76(22):11186–98. [PubMed: 12388678]
31. Park EJ, Quinnan GV Jr. Both neutralization resistance and high infectivity phenotypes are caused by mutations of interacting residues in the human immunodeficiency virus type 1 gp41 leucine zipper and the gp120 receptor- and coreceptor-binding domains. *J Virol.* 1999; 73(7):5707–13. [PubMed: 10364321]
32. Quinnan GV Jr, Yu XF, Lewis MG, Zhang PF, Sutter G, Silvera P, et al. Protection of rhesus monkeys against infection with minimally pathogenic simian-human immunodeficiency virus: correlations with neutralizing antibodies and cytotoxic T cells. *J Virol.* 2005; 79(6):3358–69. [PubMed: 15731230]
33. Bossart KN, Crameri G, Dimitrov AS, Mungall BA, Feng YR, Patch JR, et al. Receptor binding, fusion inhibition, and induction of cross-reactive neutralizing antibodies by a soluble G glycoprotein of Hendra virus. *J Virol.* 2005; 79(11):6690–702. [PubMed: 15890907]
34. Leavitt M, Park EJ, Sidorov IA, Dimitrov DS, Quinnan GV Jr. Concordant modulation of neutralization resistance and high infectivity of the primary human immunodeficiency virus type 1 MN strain and definition of a potential gp41 binding site in gp120. *J Virol.* 2003; 77(1):560–70. [PubMed: 12477860]
35. Park EJ, Gorny MK, Zolla-Pazner S, Quinnan GV Jr. A global neutralization resistance phenotype of human immunodeficiency virus type 1 is determined by distinct mechanisms mediating enhanced infectivity and conformational change of the envelope complex. *J Virol.* 2000; 74(9):4183–91. [PubMed: 10756031]
36. Park EJ, Vujcic LK, Anand R, Theodore TS, Quinnan GV Jr. Mutations in both gp120 and gp41 are responsible for the broad neutralization resistance of variant human immunodeficiency virus type 1 MN to antibodies directed at V3 and non-V3 epitopes. *J Virol.* 1998; 72(9):7099–107. [PubMed: 9696803]

37. Quinnan GV Jr, Zhang PF, Fu DW, Dong M, Alter HJ. Expression and characterization of HIV type 1 envelope protein associated with a broadly reactive neutralizing antibody response. *AIDS Res Hum Retroviruses*. 1999; 15(6):561–70. [PubMed: 10221533]
38. Quinnan GV Jr, Zhang PF, Fu DW, Dong M, Margolick JB. Evolution of neutralizing antibody response against HIV type 1 virions and pseudovirions in multicenter AIDS cohort study participants. *AIDS Res Hum Retroviruses*. 1998; 14(11):939–49. [PubMed: 9686640]
39. Zhang PF, Bouma P, Park EJ, Margolick JB, Robinson JE, Zolla-Pazner S, et al. A variable region 3 (V3) mutation determines a global neutralization phenotype and CD4-independent infectivity of a human immunodeficiency virus type 1 envelope associated with a broadly cross-reactive, primary virus-neutralizing antibody response. *J Virol*. 2002; 76(2):644–55. [PubMed: 11752155]
40. Zhang PF, Chen X, Fu DW, Margolick JB, Quinnan GV Jr. Primary virus envelope cross-reactivity of the broadening neutralizing antibody response during early chronic human immunodeficiency virus type 1 infection. *J Virol*. 1999; 73(6):5225–30. [PubMed: 10233993]
41. Bonaparte MI, Dimitrov AS, Bossart KN, Crameri G, Mungall BA, Bishop KA, et al. Ephrin-B2 ligand is a functional receptor for Hendra virus and Nipah virus. *Proc Natl Acad Sci U S A*. 2005; 102(30):10652–7. [PubMed: 15998730]
42. Bossart KN, Broder CC. Viral Glycoprotein-Mediated Cell Fusion Assays Using Vaccinia Virus Vectors. *Methods Mol Biol*. 2004; 269:309–332. [PubMed: 15114023]
43. Bossart KN, Mungall BA, Crameri G, Wang LF, Eaton BT, Broder CC. Inhibition of Henipavirus fusion and infection by heptad-derived peptides of the Nipah virus fusion glycoprotein. *Virology*. 2005; 2:57. [PubMed: 16026621]
44. Nussbaum O, Broder CC, Berger EA. Fusogenic mechanisms of enveloped-virus glycoproteins analyzed by a novel recombinant vaccinia virus-based assay quantitating cell fusion-dependent reporter gene activation. *Journal of Virology*. 1994; 68(9):5411–5422. [PubMed: 8057423]
45. Berger EA, Nussbaum O, Broder CC. HIV envelope glycoprotein/CD4 interactions: studies using recombinant vaccinia virus vectors. *HIV: a practical approach*. 1995; 2:123–145.
46. Broder CC, Berger EA. Fusogenic Selectivity of the Envelope Glycoprotein is a Major Determinant of Human Immunodeficiency Virus Type 1 Tropism for CD4+ T-Cell Lines vs. Primary Macrophages. *Proceedings of the National Academy of Sciences*. 1995; 92(19):9004–9008.
47. Pantaleo G, Koup RA. Correlates of immune protection in HIV-1 infection: what we know, what we don't know, what we should know. *Nature Medicine*. 2004; 10:806–810.
48. Griffin DE. Immune responses during measles virus infection. *Curr Top Microbiol Immunol*. 1995; 191:117–34. [PubMed: 7789155]
49. Zhu Z, Dimitrov AS, Bossart KN, Crameri G, Bishop KA, Choudhry V, et al. Potent neutralization of Hendra and Nipah viruses by human monoclonal antibodies. *J Virol*. 2006; 80(2):891–9. [PubMed: 16378991]
50. McEachern JA, Bingham J, Crameri G, Green DJ, Hancock TJ, Middleton D, et al. A recombinant subunit vaccine formulation protects against lethal Nipah virus challenge in cats. *Vaccine*. 2008; 26(31):3842–3852. [PubMed: 18556094]
51. Charles PC, Brown KW, Davis NL, Hart MK, Johnston RE. Mucosal Immunity Induced by Parenteral Immunization with a Live Attenuated Venezuelan Equine Encephalitis Virus Vaccine Candidate. *Virology*. 1997; 228(2):153–160. [PubMed: 9123821]
52. Grieder FB, Davis NL, Aronson JF, Charles PC, Sellon DC, Suzuki K, et al. Specific restrictions in the progression of Venezuelan equine encephalitis virus-induced disease resulting from single amino acid changes in the glycoproteins. *Virology*. 1995; 206(2):994–1006. [PubMed: 7856110]
53. Davis NL, Brown KW, Johnston RE. A viral vaccine vector that expresses foreign genes in lymph nodes and protects against mucosal challenge. *Journal of Virology*. 1996; 70(6):3781–3787. [PubMed: 8648713]
54. Davis NL I, Caley J, Brown KW, Betts MR, Irlbeck DM, McGrath KM, et al. Vaccination of Macaques against Pathogenic Simian Immunodeficiency Virus with Venezuelan Equine Encephalitis Virus Replicon Particles. *Journal of Virology*. 2000; 74(1):371–378. [PubMed: 10590126]

55. Pushko P, Bray M, Ludwig GV, Parker M, Schmaljohn A, Sanchez A, et al. Recombinant RNA replicons derived from attenuated Venezuelan equine encephalitis virus protect guinea pigs and mice from Ebola hemorrhagic fever virus. *Vaccine*. 2000; 19(1):142–53. [PubMed: 10924796]
56. Kinney RM, Esposito JJ, Mathews JH, Johnson BJ, Roehrig JT, Barrett AD, et al. Recombinant vaccinia virus/Venezuelan equine encephalitis (VEE) virus protects mice from peripheral VEE virus challenge. *Journal of Virology*. 1988; 62(12):4697–4702. [PubMed: 3184276]
57. Jahrling PB, Stephenson EH. Protective efficacies of live attenuated and formaldehyde-inactivated Venezuelan equine encephalitis virus vaccines against aerosol challenge in hamsters. *Journal of Clinical Microbiology*. 1984; 19(3):429–431. [PubMed: 6715512]
58. Baker KA, Dutch RE, Lamb RA, Jardetzky TS. Structural basis for paramyxovirus-mediated membrane fusion. *Mol Cell*. 1999; 3(3):309–319. [PubMed: 10198633]
59. Brideau RJ, Oien NL, Lehman DJ, Homa FL, Wathen MW. Protection of cotton rats against human parainfluenza virus type 3 by vaccination with a chimeric FHN subunit glycoprotein. *Journal of General Virology*. 1993; 74(3):471–477. [PubMed: 8383179]
60. Drillien R, Spehner D, Kirn A, Giraudon P, Buckland R, Wild F, et al. Protection of Mice from Fatal Measles Encephalitis by Vaccination with Vaccinia Virus Recombinants Encoding Either the Hemagglutinin or the Fusion Protein. *Proceedings of the National Academy of Sciences*. 1988; 85(4):1252–1256.

Figure 1. Identification of F and G proteins of HeV and NiV expressed in BHK-21 cells
 Cell lysates were prepared from BHK-21 cells 24 h postinfection with the different VRPs. Cell lysates were separated by 4 – 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred electrophoretically to a nitrocellulose membrane, and probed with rabbit anti-NiVF antibody (A), or anti-sHeVG antibody (B).

Figure 2. Syncytium formation in HEK 293T cells transiently expressing F and G glycoproteins of HeV or NiV

HEK 293T cells were mock infected (A), or co-infected with V-NiVF + V-HeVF (B), V-NiVF + V-NiVG (C), or V-HeVF + V-HeVG (D). Photomicrographs were taken 36 h after transfection at 150× magnification. Arrows indicate areas of cell syncytium.

Figure 3. Antibody response in mice immunized by footpad inoculation with VRP encoding HeVG (A) or NiVG (B)

Groups of C3H/He mice ($n = 6$) were immunized on weeks 0, 5, and 18 with 1.2×10^6 IU or 3.1×10^5 IU of replicon particles encoding HeVG or NiVG respectively. Control mice received 2.7×10^6 IU of V-R2gp160. HeVG- and NiVG-specific antibody titers were measured by ELISA on weeks 2 (post-first dose, empty bar), 7 (post-second dose, checkered bar), and 20 (post-third dose, solid bar). Bars show GM titers $\pm$ SD. * Titers were significantly higher after two doses in the mouse group that received mV-HeVG ($P < 0.05$) compared to the V-HeVG group.

Figure 4. Quantitative NiV- and HeV-mediated fusion inhibition assay

HeLa cells were infected with recombinant vaccinia viruses encoding either NiV F and G or HeV F and G, along with recombinant vaccinia viruses encoding T7 RNA polymerase (effector cells). PCI target cells were infected with the reporter vaccinia virus vCB21R, encoding *E. coli lacZ*. NiV (A), or HeV (B) glycoprotein-expressing cells (10^5) were mixed with PCI target cells (10^5) preincubated with various sera from VRP-immunized mice or non-immunized mice, in duplicate wells of a 96-well plate. After 3 h at 37°C, Nonidet P-40 was added and β -Gal activity was quantified. Results are shown for V-NiVG (◇, ◆), V-HeVG (□, ■), mV-HeVG (○, ●), and controls (Δ). Open symbols are post-first dose and closed symbols are post-second dose.

Figure 5. Serum 90%-neutralization titers from mice immunized by footpad inoculation with various VRPs

Sera were assessed for their ability to neutralize Nipah pseudovirus (A) or Hendra pseudovirus (B) infection of HEK 293T cells using a pseudovirion neutralization assay. Data represent 90% neutralization titers measured using sera collected on weeks 2 (post-first dose, empty bar), 7 (post-second dose, checked bar), and 20 (post-third dose, solid bar). Responses were expressed as GM titers $\pm$ SD.

Figure 6. Antibody response in mice immunized by footpad inoculation with replicon constructs encoding HeVF (A), or NiVF (B)

Groups of C3H/He mice (n = 6) were immunized on weeks 0, 4, and 28. Control mice received VRP encoding HIV-1 R2gp160 Env. HeVF- and NiVF-specific serum IgG titers were measured by ELISA on weeks 2 (post-first dose, empty bar), 6 (post-second dose, checkered bar), and 30 (post-third dose, solid bar). Bars show GM titers $\pm$ SD.

Figure 7. Serum 90%-neutralization titers from mice immunized by footpad inoculation with various VRPs encoding NiVF or HeVF glycoprotein
 Sera were assessed for their ability to neutralize Nipah pseudovirus (A), or Hendra pseudovirus (B) infection of HEK 293T cells using a pseudovirion neutralization assay. 90% neutralization titers were measured using sera collected on weeks 2 (post-first dose, empty bar), 6 (post-second dose, checkered bar), and 30 (post-third dose, solid bar). Responses were expressed as GM titers $\pm$ SD.

TABLE 1
Design of Study of Henipavirus G

Immunization of Mice with Wild-Type (V) or Mutant (mV) VRP Expressing HeVG or NiVG.

Replicon	Transgene	Dose (IU) ^a	Group Designation
Wild Type	HeVG	1.2×10^6	V-HeVG
Mutant	HeVG	1.2×10^6	mV-HeVG
Wild type	NiVG	3.1×10^5	V-NiVG
Wild type	R2gp160	2.7×10^6	V-R2gp160
None	N/A	N/A	Control

^aInfectious units.

TABLE 2
Design of Study of Henipavirus F

Immunization of Mice with Wild-Type VRP Expressing HeVF or NiVF.

Replicon	Transgene	Dose (IU) ^a	Group Designation
Wild Type	HeVF	50 μ l VRPs ^b	V-HeVF
Wild Type	NiVF	50 μ l VRPs ^b	V-NiVF
Wild type	R2gp160	1×10^6	V-R2gp160
None	N/A	N/A	Control

^a Infectious units.

^b VRP dose for each individual mouse per group was the same, ensured by using a fixed homogenous pool of VRP preparation for all immunizations; replicon particle titers could not be calculated due to lack of a suitable antibody for use in immunofluorescent assays.