Vesicular Stomatitis Virus-Based Vaccine Protects Hamsters against Lethal Challenge with Andes Virus

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Andes virus (ANDV) is a highly pathogenic South American hantavirus that causes hantavirus pulmonary syndrome (HPS). A high case fatality rate, the potential for human-to-human transmission, the capacity to infect via aerosolization, and the absence of effective therapies make it imperative that a safe, fast-acting, and effective ANDV vaccine be developed. We generated and characterized a recombinant vesicular stomatitis virus (VSV) vector expressing the ANDV surface glycoprotein precursor (VSVΔG/ANDVGPC) as a possible vaccine candidate and tested its efficacy in the only lethal-disease animal model of HPS. Syrian hamsters immunized with a single injection of VSVΔG/ANDVGPC were fully protected against disease when challenged at 28, 14, 7, or 3 days postimmunization with a lethal dose of ANDV; however, the mechanism of protection seems to differ depending on when the immunization occurs. At 28 days postimmunization, a lack of detectable ANDV RNA in lung, liver, and blood tissue samples, as well as a lack of seroconversion to the ANDV nucleocapsid protein in nearly all animals, suggested largely sterile immunity. The vaccine was able to generate high levels of neutralizing anti-ANDV G\textsubscript{N}/G\textsubscript{C} antibodies, which seem to play a role as a mechanism of vaccine protection. Administration of the vaccine at 7 or 3 days before challenge also resulted in full protection but with no specific neutralizing humoral immune response, suggesting a possible role of innate responses in protection against challenge virus replication. Administration of the vaccine 24 h postchallenge was successful in protecting 90% of hamsters and again suggested the induction of a potent antiviral state by the recombinant vector as a mechanism of overall. Our data suggest the potential for the use of the VSV platform as a fast-acting and effective prophylaxis/postexposure treatment against lethal hantavirus infections.

Hantaviruses are enveloped viruses containing a trisegmented, negative-sense, single-stranded RNA genome that are members of the family Bunyaviridae, genus Hantavirus (46, 63). Hantaviruses are a closely related group of mostly rodent borne viruses that are roughly divided by their geographical locations and rodent hosts. “Old World” hantaviruses are found throughout Asia and Europe and cause a disease characterized by vascular leakage and renal involvement known as hemorrhagic fever with renal syndrome (HFRS). More than 200,000 cases of HFRS requiring hospitalization are documented annually, with lethality rates ranging from 0.1% to 15% (41, 66). “New World” hantaviruses were discovered in the Americas in 1993 (31, 47, 52). These hantaviruses were found to cause a vascular leakage syndrome characterized by massive pulmonary edema, followed by a shock syndrome known as hantavirus pulmonary syndrome (HPS) or hantavirus cardiopulmonary syndrome (HCPS). HPS occurs throughout North and South America at a much lower incidence than HFRS, although lethality rates can range as high as 30 to 50% (29, 30, 41). Transmission of the virus to humans usually occurs through the inhalation of infectious materials found in the urine, feces, and saliva of infected animals (6, 30, 46). In addition, for at least one South American hantavirus, Andes virus (ANDV), there is evidence of human-to-human transmission (11, 34, 43, 50, 70). ANDV is carried primarily by Oligoryzomys longicaudatus (long-tailed pygmy rice rat) and was first identified in a series of HPS outbreaks in Argentina and Chile in 1995 (36, 38). A lethal-disease model for HPS involving ANDV-infected Syrian hamsters was described in 2001 (26). To date, this ANDV hamster model remains the only small-animal model for the study of hantavirus disease, where the disease course closely mimics what is seen in human cases with regard to disease progression and pathology (5, 26).

A limited number of different postexposure treatment modalities and potential vaccine candidates have been evaluated for efficacy in the treatment or prevention of HPS disease (41, 62). Ribavirin appears to be an effective treatment for HFRS; however, its use in the treatment of HPS is not well documented (45, 59, 71). Vaccination approaches have been focused primarily on HFRS-causing hantaviruses and have used exclusively nonlethal infection models that do not exhibit disease manifestations similar to those in humans (23, 29, 30, 41). In the Syrian hamster ANDV HPS model, two strategies have been successful. First, passive immune transfer with serum derived from DNA-vaccinated nonhuman primates or rabbits using a vector expressing the ANDV glycoprotein precursor (GPC) protected hamsters against lethal ANDV challenge (10, 24); however, direct immunization of the hamsters with the DNA vaccine did not afford protection. Second, recombinant human adenovirus 5 (Ad5)-based vaccines expressing either an ANDV glycoprotein (G\textsubscript{N} or G\textsubscript{C}) or the nucleocapsid (N) protein protected hamsters from lethal ANDV infection following
a single immunization (60). In this study, BALB/c mice immunized with the Ad vectors developed a fairly robust CD8+ cytotoxic lymphocyte response, although CD8+ responses in hamsters were not monitored, due to a lack of available reagents. Neutralizing antibody titers in the hamsters were low. The results of these different immune strategies suggest that the exact role that humoral and cellular immunity plays in protection remains unclear.

The aim of this study was to characterize the use of the attenuated, replication-competent recombinant vesicular stomatitis virus (VSV) platform as a fast-acting, effective vaccine candidate for hantavirus disease in the lethal Syrian hamster model of HPS. VSV is a nonsegmented, negative-sense, single-stranded RNA virus that belongs to the family Rhhabdoviridae, genus Vesiculovirus (40). Its use as a molecular tool started with the establishment of the reverse-genetic system in 1995 (33). This system has been shown to be very robust, with the ability to tolerate the addition of extra transcriptional units for the expression of foreign genes (20, 32, 64). Deletion of the VSV glycoprotein (G) removes a key VSV virulence determinant (42), and replacement of VSV G with a foreign, type I transmembrane glycoprotein allows the generation of attenuated recombinant VSV carrying the foreign antigen on the particle surface. This system has been used successfully as a potential vaccine platform for several viral diseases (9, 15, 27, 28, 58, 67). VSV has been shown to generate high humoral and cellular immune responses in vivo (28, 40). Studies with non-human primates immunized with recombinant VSV expressing filovirus glycoproteins have even shown partial or full postexposure protection when the vaccine is given shortly after virus challenge (12, 17, 19). We replaced the VSV G gene with the gene encoding the ANDV GPC, which is cotranslationally cleaved into two glycoproteins, G\textsubscript{N} (formerly G1) and G\textsubscript{C} (formerly G2), to form a heterodimer. Upon successful rescue of the recombinant virus, we were able to demonstrate the fast-acting protective pre- and postexposure efficacy of the vaccine against lethal ANDV challenge in the Syrian hamster model.

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MATERIALS AND METHODS

Cells and viruses. Vero E6 (African green monkey kidney) cells and HEK 293T (human embryonic kidney) cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma-Aldrich) containing 2% fetal bovine serum (FBS) (Wisent and 2 mM t-glutamine (Gibco). The hantavirus species ANDV, strain Chile 9717869 (44) (kindly provided by C. Schmaljohann, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD), was propagated in Vero E6 cells containing 2% FBS. The titer of ANDV was determined as described previously (60). VSV containing the VSV G glycoprotein (VSV wt) were used as control viruses.

TEM of recombinant VSV. Vero E6 cells grown to approximately 90% confluence, the cells were transfected with the helper plasmids (kindly provided by J. Rose, Yale University, New Haven, CT) encoding the VSV ribonucleoprotein (RNP) (constituents (0.5 μg of pBS-VSV N, 1.25 μg of pBS-VSV P, 0.25 μg of pBS-VSV L, and 2.5 μg of pcAG-T7 polymerase) and 2 μg of the full-length genomic plasmid, all under the control of the T7 promoter (see Fig. 1A). Transfections were performed using Lipofectamine 2000 (Invitrogen). Secretion of recombinant VSV occurred 6 days posttransfection, and the supernatant was used to infect 3T3 cells (blinded passages; triple passage with fresh Vero E6 cells (90% confluence). The virus was rescued under enhanced biosafety level 2 (BSL-2) conditions (BSL-2+), and success was determined by the observation of cytopathic effect (CPE) in the cell monolayer. The rescued virus was referred to as VSV\textsubscript{G/ANDVGPC}. The ANDV GPC sequence was verified by nucleotide sequence analysis in both directions.

Transfection and rescue of recombinant VSV. Vero E6 and 293T cells were seeded together into a six-well plate at 1.6 and 1.4 dilutions, respectively. At approximately 90% confluence, the cells were transfected with the helper plasmids (kindly provided by J. Rose, Yale University, New Haven, CT) encoding the VSV G glycoprotein (VSV wt) were used as control viruses. Neutralizing antibody titers in the hamsters were low. The supernatant was collected, purified with a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel loading buffer containing 5% ammonium molybdate in water. The samples were examined at 80 kV in a model 1030 (GE Healthcare).

Gel electrophoresis and Western blot analysis of ANDV proteins. VSV particles purified from tissue culture supernatants over a 20% sucrose gradient were collected from Vero E6 cells infected with VSV\textsubscript{G/ANDVGPC} or VSV wt and were mixed with a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel loading buffer containing 5% β-mercaptoethanol. Samples were heated to 95°C for 5 and min and were then loaded onto a 10% SDS-PAGE gel. After electrophoresis, proteins were wet transferred to a Hybond-P polyvinylidene difluoride (PVDF) membrane (GE Healthcare) and were then blocked overnight at 4°C in phosphate-buffered saline (PBS) containing 5% skim milk and 0.1% Tween 20 (Sigma-Aldrich). Primary antibodies were diluted to 1:8,000 (mouse anti-G\textsubscript{C}; Austral Biologicals), 1:5,000 (mouse anti-G\textsubscript{2}; Sigma-Aldrich), and 1:2,500 (mouse anti-G\textsubscript{VSV G}; Sigma-Aldrich) and were incubated with the membrane for 2 h. The secondary antibody (a horseradish peroxidase [HRP]-conjugated anti-mouse antibody; Invitrogen) was diluted to 1:10,000 and was incubated with the membrane for 1 h. PBS–0.1% Tween 20 washing steps were carried out between and after incubations. The secondary antibody was detected and imaged using the Amersham ECL Plus detection kit and Hyperfilm ECL (GE Healthcare).

TEM of recombinant VSV. Vero E6 cells grown in T75 flasks were infected with VSV\textsubscript{G/ANDVGPC} or VSV wt at a multiplicity of infection (MOI) necessary to obtain 80% CPE within 48 h. The supernatant was collected, purified over a 20% sucrose cushion, and subsequently resuspended in and fixed with 2% paraformaldehyde (PFA) at 4°C. Fixed virus particles (20 μl) were adsorbed to carbon- and Formvar-coated grids overnight at 4°C. The grids were then transferred to 20-μl droplets of blocking buffer containing 2% (mass/vol) glycerol-free bovine serum albumin (BSA) in PBS and were irradiated at 150 W in a BioWave model microwave processor (Ted Pella, Inc.) with a cycle of 1 min on, 1 min off, and 1 min on. The grids were then washed once in blocking buffer, twice in PBS, and twice in deionized water and were subsequently negatively stained with 1% ammonium molybdate in water. The samples were examined at 80 kV in a model H7500 transmission electron microscope (TEM) (Hitachi High Technologies). Digital images were recorded using a model HR-100 charge-coupled device (CCD) camera (Advanced Microscopy Techniques).

Growth kinetics analysis of recombinant VSV. Vero E6 cells were plated in 12-well plates and were then infected with either VSV\textsubscript{G/ANDVGPC}, VSV\textsubscript{G/ ZEBOVGPC}, or VSV wt at an MOI of 0.0001. The virus was allowed to adsorb for 1 h; then the supernatant was removed, and cells were washed twice with DMEM. At set time points, the scraped cells and supernatant were collected
from 3 wells per virus, centrifuged in order to remove cells, and stored at −80°C for further analysis. Analysis was carried out by 10-fold dilutions of the samples from each time point and infection of Vero E6 cells in quadruplicate in a 96-well plate. The 50% endpoint dilutions were expressed as the TCID<sub>50</sub> (tissue culture infectious dose leading to CPE in 50% of cells) per milliliter and were calculated using the Reed-Muench method (55). The values from 3 wells per time point were averaged, and the standard error was calculated.

**Immunization of hamsters with VSV vaccine candidates followed by ANDV challenge.** Syrian hamsters (Mesocricetus auratus) (5 to 6 weeks old, female) were housed by groups in microisolator units. For immunization, hamsters were anesthetized with isoflurane. Study animals (<i>n</i> = 18; 12 animals for survival and 6 animals for virus titration) were immunized via intraperitoneal (i.p.) injection with VSVΔG/ANDVGPC by using 10<sup>7</sup> PFU of the virus diluted in 400 μl of DMEM. The control group was immunized with 10<sup>7</sup> PFU VSVΔG/ZEBOVGPC (<i>n</i> = 15; 9 animals for survival and 6 animals for virus titration), a VSV vaccine candidate that was generated, rescued, and evaluated previously (15, 28). After 28 days, the hamsters were challenged with ANDV at 100× LD<sub>50</sub> (the dose leading to death in 50% of the animals) by i.p. injection as described previously (60). For time-to-protection and postexposure studies, different groups of animals were immunized with 10<sup>7</sup> PFU of VSVΔG/ANDVGPC (study group; 10 animals per group), VSVΔG/ZEBOVGPC, or DMEM (control groups; 6 and 3 animals per group, respectively) at 14, 7, and 3 days prechallenge or at 24 and 72 h postchallenge. All groups were challenged i.p. with the same ANDV lethal dose at day zero.

**Viral RNA titration in blood and tissues following VSV immunization and ANDV challenge.** At 5 and 8 days after ANDV challenge, 3 hamsters per group were anesthetized, exsanguinated via cardiac puncture, and necropsied. Pieces (100 mg) of lung and liver tissue were placed in individual tubes containing 1 ml of RNAlater buffer (Qiagen) overnight at 4°C, after which they were mechanically homogenized in 600 μl of RLT lysis buffer (Qiagen), clarified by low-speed centrifugation, and then diluted to 30-mg equivalents with RLT buffer. In addition, 140 μl of cardiac blood was mixed with 560 μl lysis buffer AVL (Qiagen). RNA was extracted from samples using the RNeasy (solid tissue) or QIAamp (blood) extraction kit (Qiagen). Quantitative real-time one-step reverse transcription-PCR (RT-PCR) was conducted on RNA extracts using a Rotor-Gene RG-3000 instrument (Corbett Life Science) with ANDV S segment-specific oligonucleotides as described previously (60). All RNA samples were normalized to 200 ng of RNA per reaction.

**Humoral immune response in hamsters following VSV immunization and ANDV challenge.** Serum samples were collected from all hamsters pre-ANDV challenge (28, 14, 7, or 3 days postimmunization) by retro-orbital bleeding as well as at 45 days postchallenge by cardiac puncture of the surviving animals (study endpoint). Because antibodies against ANDV N cross-react with Sin Nombre virus (SNV) N, an indirect enzyme-linked immunosorbent assay (ELISA) using SNV N recombinantly expressed in <i>Escherichia coli</i> as an antigen (kindly provided by R. Lindsay and A. Dibernardo, National Microbiology Laboratory, Public Health Agency of Canada [PHAC], Winnipeg, MB, Canada) was used to test for anti-N antibodies as described previously (60). Identification of antibodies produced against either the ANDV GN or GC protein, using a western blotting assay, was determined following immunoblotting using commercially obtained mouse monoclonal antibodies produced against either the ANDV G or G′ protein, with VSV wt as a control. Expression of the ANDV glycoproteins was monitored by Western blotting using commercially obtained mouse monoclonal antibodies produced against either the ANDV G or G′ protein, with VSV wt as a control. Expression of the ANDV glycoproteins by VSVΔG/ANDVGPC, or expression of VSV G by VSV wt, was determined by collecting cell lysates (for intracellular expression) or 20% sucrose cushion-purified cell culture supernatants (for released virus particles) from infected Vero E6 cells. We observed expression of the ANDV glycoproteins both in the cell lysates (data not shown) and on released virus particles (Fig. 1C) similar to the expression of VSV G seen with VSV wt.

**RESULTS**

**Construction and rescue of VSV vectors expressing ANDV protein.** The cDNA encoding the ANDV GPC was amplified by RT-PCR and was inserted into the fourth coding position of the previously constructed plasmid VSVPN2AG, replacing the VSV G gene (pVSVGΔG/ANDVGPC) (Fig. 1A). Plasmids carrying the recombinant VSV genome and necessary accessory proteins were transfected into a combination of 293T and Vero E6 cells. Transfection of cells and subsequent blind passage on Vero E6 cells resulted in a new recombinant virus that has allowed us to perform immuno-EM successfully. However, sera containing ANDV-neutralizing antibodies were able to neutralize VSVΔG/ANDVGPC, with no effect on either VSVΔG/ZEBOVGPC or VSV wt, indicating the successful incorporation of the ANDV glycoproteins into these virus particles (data not shown).

The intracellular expression and incorporation of ANDV glycoproteins into VSV particles was demonstrated by Western blotting using commercially obtained mouse monoclonal antibodies produced against either the ANDV G<sub>c</sub> or G′ protein, with VSV wt as a control. Expression of the ANDV glycoproteins by VSVΔG/ANDVGPC, or expression of VSV G by VSV wt, was determined by collecting cell lysates (for intracellular expression) or 20% sucrose cushion-purified cell culture supernatants (for released virus particles) from infected Vero E6 cells. We observed expression of the ANDV glycoproteins both in the cell lysates (data not shown) and on released virus particles (Fig. 1C) similar to the expression of VSV G seen with VSV wt.

**In vitro growth kinetics using a low-MOI infection demon-
strated that VSVΔG/ANDVGPC titers at certain time points were as much as 6 log units lower than VSV wt titers, and the time to maximum viral titer was longer. The maximum titers of VSVΔG/ZEBOVGP were similar to those of VSVΔG/ANDVGPC, but before VSVΔG/ANDVGPC titers reached the maximum, they were approximately 2 log units lower than VSVΔG/ZEBOVGP titers. The data in both cases strongly suggest reduced efficiency in entry, virus replication, or budding of VSVΔG/ANDVGPC compared to those of the other viruses (Fig. 2). The maximum titers reached in all viruses corresponded to the maximum amount of CPE. Declines in titers thereafter were most likely due to a lack of viable cells for further virus replication and a degradation of infectious virus particles in the supernatant.

Immunization with a VSV vector expressing ANDV GPC protects hamsters from lethal challenge. The LD_{50} for the ANDV hamster model was previously determined to be 1.54 focus-forming units (FFU) (60). In all hamster experiments, a dose of 100× LD_{50} (equal to 154 FFU) was used as the challenge dose. Hamsters (two groups of either 18 or 15 animals) were immunized using a single inoculation of 10^{5} PFU of VSVΔG/ANDVGPC or VSVΔG/ZEBOVGP via the i.p. route. Animals were challenged 28 days postimmunization. To determine level of ANDV replication, 3 hamsters in each group were euthanized at days 5 and 8 postchallenge (a total of

FIG. 1. Rescue and detection of recombinant VSV vectors expressing ANDV GPC. (A) Cartoon of cloning strategy and reverse-genetics system. The ANDV GPC coding region was amplified by RT-PCR and was inserted into the fourth position of the full-length recombinant VSV backbone, replacing VSV G (VSVΔG/ANDVGPC). Recombinant viruses expressing ANDV Gn and Gc on the particle surfaces were rescued following cotransfection of plasmids encoding the recombinant VSV genome, the N, P, and L genes under the control of the T7 promoter, and a T7 RNA polymerase expression vector under the control of the chicken β-actin promoter into 293T/Vero E6 cells. (B) Electron microscopy of recombinant VSV particles. Vero E6 cells were infected with VSVΔG/ANDVGPC or VSV wt for 48 h. Released purified (with a 20% sucrose cushion) virus particles were fixed with 2% paraformaldehyde and were analyzed by TEM imaging. (C) Detection of glycoprotein expression. ANDV Gn and Gc, and VSV G were detected by Western blotting from 20% sucrose cushion-purified virions by use of monoclonal antibodies specific to each protein. N, VSV nucleoprotein; P, VSV phosphoprotein; M, VSV matrix protein; GPC, Andes virus glycoprotein precursor; L, VSV RNA-dependent RNA polymerase (large protein); pVSVΔG/ANDVGPC, plasmid containing the full-length genome of VSV with Andes virus GPC; pBS, pBlueScript plasmid; pCAG, pCAGGS plasmid; VSVΔG/ANDVGPC, recombinant VSV expressing the Andes virus glycoprotein precursor; Gn, amino-terminal part of the Andes virus glycoprotein; Gc, carboxy-terminal part of the Andes virus glycoprotein; VSV wt, recombinant wild-type VSV; G, VSV glycoprotein.

FIG. 2. VSV growth kinetics. Vero E6 cells were infected with either VSVΔG/ANDVGPC, recombinant VSV expressing the Zaire Ebola virus glycoprotein (VSVΔG/ZEBOVGP), or VSV wt at an MOI of 0.0001. Supernatants were collected at various time points, and the TCID_{50}/ml was calculated for each sample. Each error bar represents the standard error of the mean for 3 individual samples, each with 4 replicate wells.
6 hamsters from each group) for blood and organ collection. Control animals began to show clinical signs of ANDV infection (lethargy, hunched posture, and breathing abnormalities) beginning at 8 days after challenge, and all animals had end-stage disease (severe respiratory distress) by day 11. VSVΔG/ANDVGPC-immunized animals never showed any clinical signs of infection, and all survived lethal ANDV challenge (Fig. 3A). Blood, lung, and liver samples collected from 3 hamsters in each group at days 5 and 8 postchallenge were analyzed for the presence of viral RNA by use of real-time quantitative RT-PCR directed against the ANDV S segment. Control animals had significant amounts of ANDV RNA at day 5, which increased by approximately one-half log unit by day 8. Lung tissue had the highest detectable RNA load, followed by liver and blood. In contrast, for animals immunized with VSVΔG/ANDVGPC, no ANDV RNA was detected in any of the tissues collected (Fig. 3B). These results are consistent with complete (“sterile”) protection against ANDV replication in the VSVΔG/ANDVGPC-immunized animals.

**Humoral immune responses in hamsters following immunization and challenge.** Serum samples were collected from immunized hamsters immediately before challenge (28 days postimmunization) and from the surviving animals 45 days postchallenge. In order to determine the levels of ANDV N-specific antibodies in the hamsters, we used a cross-reactive N-specific ELISA. As expected, both groups of hamsters were uniformly negative in their prechallenge bleed for N-specific antibodies. Postchallenge bleeds of the surviving animals showed borderline (2 of 12 animals), very low (1 of 12 animals), or undetectable (9 of 12 animals) levels of N antibodies (Fig. 4A), supporting the absence of ANDV replication in the vast majority of the immunized animals and thus largely supporting complete protection against ANDV infection, as demonstrated above by RT-PCR (Fig. 3B).

In order to further determine the role of the humoral immune response in the protection of these animals, we tested sera for the presence of neutralizing antibodies by using a PRNT80 assay against VSVΔG/ANDVGPC. It has been shown previously that serum antibodies will neutralize pseudotyped, nonreplicating VSV expressing the hantavirus surface glycoproteins in a manner equivalent to the neutralization of native hantaviruses, but more rapidly (49). To further validate our assay, we compared the neutralization of our virus, VSVΔG/ANDVGPC, with hamster serum containing anti-GΔ/GC antibodies to the neutralization of native ANDV and found that the titers were either identical or within ±1 dilution factor, supporting the notion that the neutralizing response is specific to the incorporated glycoprotein and is not a nonspecific response to the VSV vector (data not shown). The control group showed no detectable ANDV GΔ/GC-neutralizing antibodies prechallenge, whereas the VSVΔG/ANDVGPC group demonstrated relatively high levels of neutralizing antibodies, indicating a strong humoral neutralizing immune response following a single VSVΔG/ANDVGPC vaccination (Fig. 4B). The titers of neutralizing antibodies dropped 45 days after challenge (Fig. 4B), suggesting the absence of a boost effect following ANDV challenge and thus supporting the notion that a single VSVΔG/ANDVGPC vaccination conferred complete immunity on almost all animals.

**Time to protection and postexposure treatment.** After determining that a single immunization with VSVΔG/ANDVGPC 28 days before challenge was able to completely protect all hamsters from lethal ANDV challenge, we wanted
to determine if a single-dose immunization with the vaccine was protective when administered either closer to the challenge time point or postchallenge. Groups of hamsters (10 animals per study group [VSVΔG/ANDVGPC] and 6 or 3 animals per control group [VSVΔG/ZEBOVGP or DMEM, respectively] for each time point) were immunized once at 14, 7, or 3 days prior to challenge (Fig. 5A), or at 1, 3, or 5 days postchallenge (Fig. 5B). Hamsters were challenged in a manner and at a dose identical to those in previous experiments. Hamsters were 100% protected from challenge when immunized with VSVΔG/ANDVGPC at any of the time points prior to challenge; unexpectedly, 50% of hamsters that received the control VSVΔG/ZEBOVGP vaccine at day 7 and 83% of those receiving VSVΔG/ZEBOVGP at day 3 were also protected (Fig. 5A). In the postchallenge immunization experiment, 90% of hamsters receiving VSVΔG/ANDVGPC at 1 day postexposure were protected, but none survived when treated 3 days postexposure. Interestingly, all hamsters receiving the control VSVΔG/ZEBOVGP vaccine were protected when treated at 1 day and 67% were protected when treated at 3 days postexposure (Fig. 5B). All animals treated at 5 days postchallenge, as well as all control (DMEM-treated) animals, died between days 9 and 11.

Serum samples were collected immediately prior to challenge, as well as 45 days postchallenge from all surviving animals in each group (5 animals per time point), and the levels of N-specific and glycoprotein neutralizing antibodies in the hamsters were determined using the cross-reactive N-specific ELISA (measure for challenge virus replication) and PRNT_{50} assay, respectively. Data from the −28-day-time point (Fig. 4) were included for comparison. Hamsters immunized at 28, 7, or 3 days prior to challenge showed low or undetectable levels of ANDV N-specific antibodies 45 days postchallenge, indicating mostly sterile protection with no or limited ANDV replication (Fig. 6A). All hamsters immunized once 14 days prior to challenge had high levels of ANDV N-specific antibodies, indicating protection from disease but not from ANDV replication, possibly suggesting that the vaccine-induced adaptive immune response is no longer sufficient to prevent virus replication at this point (Fig. 6A). Prechallenge sera from hamsters immunized with VSVΔG/ANDVGPC showed high levels of neutralizing antibodies at 28 days postimmunization and moderate levels at 14 days; however, no neutralizing antibodies were detected at 7 or 3 days (Fig. 6B). At 45 days postchallenge, all surviving animals showed similar neutralizing antibody titers (Fig. 6B). These data suggest that while neutralizing antibodies may play a role in protection from lethal challenge at 28 and 14 days postimmunization, they do not seem to play a role for immunizations occurring 7 or 3 days prior to challenge.

Detection of hamster innate immune responses. After demonstrating that some hamsters receiving the VSVΔG/ZEBOVGP control vaccine survived when immunized 7 or 3 days prior to challenge, and considering the fact that no nonneutralizing or neutralizing antibodies were detected in those animals prior to challenge, we wanted to determine if non-glycoprotein-specific innate responses were the primary determinant of protection in these cases. Unfortunately, very few reagents to test this are commercially available, and those designed for other rodent species exhibit no cross-reactivity. Our group has previously established a quantitative real-time RT-PCR assay to monitor hamster cytokine mRNA levels (74). We chose 3 distinct genes that represented different components of the innate immune response—Mx-2, STAT-1, and IFN-γ—and determined their transcript levels in spleen and lung tissues compared to those in the tissues of DMEM-immunized control hamsters. We found that the VSVΔG/ZEBOVGP-immunized hamsters typically exhibited much higher levels of cytokine expression in both tissues tested and the maximum was seen at 1 day postimmunization. After dem-
entire period. These data show that the VSVΔG/ZEBOVGP vaccine seems to stimulate the innate immune system more quickly and robustly than the VSVΔG/ANDVGPC vaccine, which may explain the survival rates seen in the VSVΔG/ZEBOVGP control groups at 7 and 3 days postimmunization (Fig. 5A), as well as at 1 and 3 days postexposure (Fig. 5B).

DISCUSSION

Although the incidence of HPS cases in North and South America remains low in comparison to that of HFRS cases in Europe and Asia, the significantly higher case fatality rate and the lack of any licensed vaccines or effective treatments for the disease suggest an urgent need for the development of such measures (29, 30, 41, 66). The hantavirus glycoproteins are typically thought to be the principal targets of neutralizing antibodies (1, 8, 39, 51), and although a few potential vaccine candidates have been evaluated with various degrees of success, largely in infection rather than disease models (2, 7, 10, 21, 24, 25, 35, 56, 60, 72, 73), the mechanism of protection against hantavirus infections remain unidentified. Here we constructed an attenuated, replication-competent recombinant VSV expressing the ANDV GPC in order to investigate its efficacy as a hantavirus vaccine in the Syrian hamster HPS disease model and to gain further insight into the mechanism of protection against lethal ANDV infection.

The VSV vector expressing the ANDV GPC (Fig. 1) represents a unique attenuated replication-competent VSV construct that expresses a glycoprotein from a bunyavirus, although replication-incompetent VSV particles have previously been pseudotyped with glycoproteins from “Old World” (35, 49) and “New World” (54, 60) hantaviruses. Bunyaviruses are generally believed to mature and bud from the Golgi apparatus, including the incorporation of the glycoproteins into virus particles (63). This is in contrast to the VSV egress pathway, where glycoprotein incorporation and particle budding occur at the plasma membrane (40). Thus, the ability to rescue the VSV containing the ANDV glycoproteins seems to indicate that at least a portion of the ANDV glycoproteins are transported to the plasma membrane, a mechanism similar to what has been reported before for the glycoproteins of different “Old World” (Hantaan, Seoul) and “New World” (Black Creek Canal) hantaviruses (49, 53). The incorporation of a foreign transmembrane glycoprotein into VSV particles is likely further reduced by a less optimal interaction of these foreign proteins with VSV structural proteins, such as the M protein, as has been shown for the incorporation of other type I transmembrane glycoproteins into VSV particles in the absence of VSV G (15).

The recombinant VSVΔG/ANDVGPC was able to fully protect hamsters with a single dose of vaccine (Fig. 3A and B). Concerns have been raised about the use of replication-competent VSV vaccines, particularly in immunosuppressed patients, such as those treated with immunosuppressive drugs or infected with human immunodeficiency virus (HIV). However, rhesus macaques infected with simian-human immunodeficiency virus (SHIV) showed no adverse effect following immunization with a replication-competent VSV expressing the Ebola virus glycoprotein (VSVΔG/ZEBOVGP), and the vaccine was still able to protect 66% of the highly immunocom-
promised animals against lethal Ebola virus challenge (16). In addition, the vaccine dose of replication-competent vaccines is expected to be much lower (approximately 3 log units) and thus less toxic than that of the replication-incompetent Ad5 platform, based on recent studies that used both platforms as Ebola virus vaccines (18, 22, 28, 69). Another advantage of the VSV vaccines is the negligible level of preexisting neutralizing antibodies against VSV G in the world population (40). Furthermore, G-neutralizing antibodies are unlikely to affect VSV vaccine efficacy if these vaccines are based on VSV G deletion vectors, as in our study here. Finally, there seems to be a lack of serious pathogenicity in humans associated with VSV infections (4), making VSV altogether a very suitable candidate for a vaccine platform.

A previous vaccination strategy using DNA vaccination of hamsters with ANDV M-segment cDNA was unable to produce an immune response that afforded protection against lethal ANDV challenge (10). However, the injection of the same DNA vaccine into nonhuman primates or rabbits was able to generate a humoral immune response that, when passively transferred into hamsters, fully protected them from lethal challenge (24). This is partially consistent with our data, which suggest that the production of a strong neutralizing antibody response may be sufficient to protect from lethal disease when individuals are immunized at 28 or 14 days before challenge. We found that high levels of neutralizing antibodies were produced at 28 days after immunization, with low to moderate levels produced at 14 days. However, no neutralizing antibodies were detected prior to challenge at either 7 or 3 days after immunization (Fig. 6B). Humoral immune responses have also been viewed as important for protection in other hantavirus studies using small-animal infection models (25, 51) as well as mild clinical cases of HPS in humans (3). In contrast, the recent success with the Ad5-based vaccines expressing G\textsubscript{c} alone or in combination with G\textsubscript{c} in \textit{trans} seems to be attributed mainly to a strong cellular immune response (mainly CD8\textsuperscript{+} cytotoxic lymphocytes), as observed in BALB/c mice (60). Unfortunately, no Ad5 expressing the full-length ANDV GPC was investigated, preventing us from making a direct comparison of the immune response between the Ad5 and VSV platforms. Further, immune reagents with which to examine the hamster adaptive cellular responses have been sorely lacking, currently limiting the examination of these responses in both the Ad5 (60) and VSV (this study) vaccine studies.

Interestingly, the humoral immune response does not seem to be important for protection if the vaccine is administered within a week prior to challenge, an application that fully protected the animals from disease and resulted in seemingly limited challenge virus replication (Fig. 5A and 6A). We propose that a strong nonspecific VSV-mediated innate response is the important mechanism here (40, 57, 58). It has been shown that hantaviruses are sensitive to innate responses, particularly interferons, prior to the establishment of infection but become insensitive to treatment with interferons once infection is established (68). Both VSV\textDelta G/ANDVGPC and VSV\textDelta G/ZEBOVGP were shown to stimulate the production of Mx-2, STAT-1, and IFN-\gamma during this 7-day period (Fig. 7). The strong induction of innate immune responses by VSV\textDelta G/ZEBOVGP may explain why some control hamsters survived when immunized at 3 or 7 days before challenge. However, the fact that all VSV\textDelta G/ANDVGPC-immunized animals survived during the same period suggests that a glycoprotein-specific adaptive immune response may also be occurring.

Previous investigations have shown that recombinant VSV expressing either the Ebola virus or the Marburg virus glyco-
protein administered postexposure provided full or partial protection from lethal challenge (12, 17, 19). Therefore, we also tested the use of VSVΔG/ANDVGPC as a postexposure treatment modality, which resulted in 90% survival if animals were treated 24 h after lethal ANDV challenge (Fig. 5B). Interestingly, the VSVΔG/ZEBOVGP vaccine resulted in a higher survival rate of 100% when administered 24 h postexposure and in 67% survival when administered 3 days postexposure (Fig. 5B). Although we have provided evidence for the role of neutralizing antibodies in the protection of hamsters immunized prophylactically (Fig. 4B and 6B), a pivotal role of the humoral immune response as the mechanism for postexposure treatment is unlikely. The innate immune response was thought to be, at least in part, essential for the success of the VSV platform in postexposure treatment for filovirus infections, keeping virus loads at levels that allow the adaptive immune response, which otherwise would be developed too late to clear the virus (12). The fact that we see protection from the control vaccine (VSVΔG/ZEBOVGP) also supports the induction of a nonspecific innate immune response following immunization with VSV vectors, and this protection is similar to what we saw at 3 and 7 days postimmunization. This was not tested with or compared to wild-type VSV, because this virus causes an acute infection with a lethal outcome in the Syrian hamster model, even at very low doses (13, 14). The higher survival rates seen in the groups receiving VSVΔG/ZEBOVGP than in those receiving VSVΔG/ANDVGPC may be due either to the quicker replication of that virus (Fig. 2) or to the significant difference in tropism of the glycoproteins. ZEBOV glycoproteins have a cell tropism primarily for macrophages, monocytes, and dendritic cells, which may be important in mounting an early immune response (61), whereas the ANDV glycoproteins target primarily endothelial cells (63). This is in agreement with our real-time RT-PCR hamster cytokine data, where VSVΔG/ZEBOVGP stimulated the innate immune system more quickly and robustly than VSVΔG/ANDVGPC (Fig. 7). However, the nonspecificity of protection differs from the nature of the protection seen against filovirus challenge in the nonhuman primate model, where a homologous VSV vaccine was required in order to achieve successful postexposure pro-

![Graphs](VOL. 85, 2011 VSV-BASED HANTAVIRUS VACCINE 12789)
tection (12, 17, 19). As an alternative theory, one could argue that the recombinant VSV vaccine might interfere with, and thus control, ANDV replication, allowing only subclinical or mild infections. A similar concept has been developed for other vaccines given simultaneously with the challenge virus, resulting in a subclinical infection that allows the development of a protective humoral immune response (48), and this was detected in our studies (Fig. 6B). This, however, needs to be experimentally verified once proper reagents and tools for the study of immune responses other than antibodies in hamsters become available.

In summary, our studies have demonstrated the potential of a replication-competent VSV vector expressing the ANDV GPC for both prophylaxis and postexposure treatment of ANDV infection. Future studies will focus on further unraveling the mechanism of protection of this promising vaccine candidate.

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