A Recombinant Hendra Virus G Glycoprotein Subunit Vaccine Protects Nonhuman Primates against Hendra Virus Challenge

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Hendra virus (HeV) is a zoonotic emerging virus belonging to the family Paramyxoviridae. HeV causes severe and often fatal respiratory and/or neurologic disease in both animals and humans. Currently, there are no licensed vaccines or antiviral drugs approved for human use. A number of animal models have been developed for studying HeV infection with the African green monkey (AGM) appearing to most faithfully reproduce human disease. Here, we assessed the utility of a newly developed recombinant subunit vaccine based on the HeV attachment glycoprotein (G) in the AGM model. Four AGMs were vaccinated with two doses of the HeV vaccine (sGHeV) containing alhydrogel; four AGMs received the sGHeV with alhydrogel and CpG; and four control animals did not receive the sGHeV vaccine. Animals were challenged with a high dose of infectious HeV 21 days after the boost vaccination. None of the eight specifically vaccinated animals showed any evidence of clinical illness and survived challenge. All four controls became severely ill with symptoms consistent with HeV infection and three of the four animals succumbed 8 days after exposure. Success of the recombinant subunit vaccine in AGMs provides pivotal data in supporting its further preclinical development for potential human use.

Importance

A Hendra virus attachment G glycoprotein subunit vaccine was tested in nonhuman primates to assess its ability to protect them from a lethal infection by Hendra virus. It was found that all vaccinated subjects were completely protected against subsequent Hendra virus infection and disease. The success of this new subunit vaccine in nonhuman primates now provides critical data in support of its further development for future human use.
INTRODUCTION

The henipaviruses, Hendra virus (HeV) and Nipah virus (NiV), cause severe and often fatal respiratory disease and encephalitis in horses, pigs, and humans (1-4). In contrast to all other paramyxoviruses, henipaviruses infect a broad range of species spanning six mammalian orders. Because of this broad species tropism, ease of access and propagation, potential for person-to-person transmission, high case fatality rates, and lack of approved countermeasures for human use HeV and NiV pose significant biosecurity threats and are classified as Biosafety Level (BSL)-4 pathogens.

HeV emerged in Australia in 1994 and was identified as the causative agent of an acute respiratory illness in horses (5). HeV is transmitted to horses by pteropid fruit bats, commonly known as flying foxes, with human infection occurring by close contact with infected horses (6, 7). Outbreaks of HeV have occurred in Australia on a near annual basis since the initial outbreak with all episodes involving infection of horses. In total, over 80 horses have succumbed to HeV infection with a case fatality rate of approximately 75%. There have been 7 human HeV infections recorded, most recently in 2009, of which 4 have been fatal (57%) (8). All patients initially presented with influenza-like symptoms after an incubation period of 7-16 days. While two of the patients recovered from the influenza-like illness, one developed pneumonitis and succumbed from multi-organ failure. Three different patients developed encephalitic manifestations (mild confusion, ataxia) with two of these cases progressing to seizures and resulting in death (5, 8, 9).

There are currently no vaccines or antiviral drugs approved for combating human HeV or NiV infections. Regarding treatment options for henipavirus infection, an open label ribavirin trial was performed in 140 patients during the initial outbreak of NiV in Malaysia in 1998;
however, the results of this study remain controversial (10). In addition, 3 of the 7 recorded human HeV cases were treated with ribavirin, one of which survived (8). The utility of ribavirin as a countermeasure for HeV infection was subsequently assessed in African green monkeys (AGMs) (11). While there was a small benefit in delaying death there was no survival benefit in this nonhuman primate model. Currently, the most promising post-exposure treatment for henipavirus infection appears to be an experimental human monoclonal antibody (mAb). The mAb, m102.4, targets the ephrin-B2 and -B3 receptor binding domain of the henipavirus envelope attachment glycoprotein (G) (12-16). m102.4 is a potent cross-reactive neutralizing antibody in vitro (17, 18) and had been shown to protect ferrets from lethal NiV challenge (19) and AGMs from lethal HeV challenge (20). Importantly, in 2010 m102.4 was administered to two individuals in Australia who had a significant exposure risk to HeV under a compassionate use protocol. It was also used in 2012 to treat a possible exposure to HeV in Australia and in 2013 to treat a possible laboratory exposure in the United States. To date, all of four of these individuals have no evidence of henipavirus infection.

In addition to the post-exposure treatments, there have been two experimental preventive vaccines against henipaviruses evaluated in animal models. A recombinant adeno-associated vaccine expressing the NiV G protein completely protected hamsters against homologous NiV challenge and protected 50% of animals against heterologous HeV infection (21). In addition, a recombinant subunit vaccine based on the HeV G protein (sG_{HeV}) completely protects small animals against lethal HeV and NiV infection (22-25) and more recently was shown to be efficacious in the robust AGM model of NiV infection (26). sG_{HeV} (amino acids 73-604) is an engineered secreted version of the full ectodomain of the G glycoprotein in which the transmembrane and cytoplasmic tail domains have been deleted from the N-terminus (27).
Importantly, this vaccine has also been shown to protect horses from lethal HeV infection and has been licensed for use by the equine industry in Australia (28). However, there has been no study to date assessing performance of this vaccine in a nonhuman primate model of HeV infection which is a necessary prerequisite prior to licensure of such a vaccine for use in humans. Here, we report for the first time the prophylactic efficacy of the sG_{HeV} vaccine in a lethal HeV AGM model that faithfully recapitulates human HeV infection.

**MATERIALS AND METHODS**

**Statistics.** Conducting animal studies in BSL-4 severely restricts the number of animal subjects, the volume of biological samples that can be obtained and the ability to repeat assays independently and thus limit statistical analysis. Consequently, data are presented as the mean or median calculated from replicate samples, not replicate assays, and error bars represent the standard deviation across replicates. Statistics were calculated for serum neutralizing antibody titers using GraphPad Prism 5 software by using a 2way ANOVA analysis comparing treatments and times between all groups using the Bonferroni method posttest.

**Viruses and cells.** HeV was obtained from a patient from the 1994 outbreak in Australia and was kindly provided by Dr. Tom Ksiazek. The virus was propagated on Vero E6 cells in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal calf serum. The titer of the HeV stock used was \( \sim 1 \times 10^7 \) pfu/ml. The HeV challenge virus stock was assessed for the presence of endotoxin using The Endosafe®-Portable Test System (PTS) (Charles River, Wilmington, MA). Virus preparations were diluted 1:10 in Limulus Amebocyte Lysate (LAL) Reagent Water (LRW) per manufacturer’s directions and endotoxin levels were tested in LAL.
Endosafe®-PTS cartridges as directed by the manufacturer. Each preparation was found to be below detectable limits while positive controls showed that the tests were valid.

**Vaccine formulation.** Production and purification of sGHeV was done as previously described (26). Two vaccines containing sGHeV and Alhydrogel (Accurate Chemical & Scientific Corporation, Westbury, NY) at a weight ratio of 1:25 were prepared with one formulation also containing CpG oligodeoxynucleotide (ODN) 2006 (InvivoGen, San Diego, CA) with a fully phosphorothioate backbone (sGHeV + alum + CpG). The vaccine without CpG was formulated as follows: 100 μg of sGHeV and 2.5 mg of aluminum ion (sGHeV + alum) per vaccinated animal. The vaccine with CpG was formulated as follows: 100 μg of sGHeV, 2.5 mg of aluminum ion, and 150 mg of ODN 2006 per vaccinated animal. For the vaccine containing both Alhydrogel and CpG the Alhydrogel and sGHeV were mixed first before ODN 2006 was added. Each vaccine dose was adjusted to 1 ml with PBS, and mixtures were incubated on a rotating wheel at room temperature for at least 2 to 3 hours before injection. Each subject received the same dose of 1 ml for prime and boost, and all vaccine doses were given via intramuscular (i.m.) injection. The dose of 100 μg was used in this study based on the protective efficacy against NiV and the prolonged level of IgG in AGMs vaccinated at this dose versus 10 and 50 μg (26).

**Animals.** Animal studies were performed in BSL-4 biocontainment at the Galveston National Laboratory (GNL) at the University of Texas Medical Branch (UTMB) at Galveston and were approved by the UTMB Institutional Animal Care and Use Committee. Animal research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to the principles stated in the eighth edition of the *Guide for the Care and Use of Laboratory Animals*, National Research...
Council, 2013. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Twelve adult AGMs weighing 3-6 kg (PreLabs, Hines, IL) were employed for this study. Subjects were anesthetized by i.m. injection with ketamine and vaccinated with sG_{HeV} by i.m. injection on day -42 (prime) and day -21 (boost) (Fig. 1, black and grey arrows respectively). Vaccine formulations are described above. Four subjects received two doses of vaccine containing sG_{HeV} and Alhydrogel; 4 animals received two doses of vaccine containing sG_{HeV}, Alhydrogel, and CpG; one animal received two doses of Alhydrogel only; one animal received two doses of CpG only; and two animals were not vaccinated. Animals were inoculated intratracheally (i.t.) with ~ 5x10^5 pfu of HeV in 5 ml of Dulbecco’s minimal essential medium (DMEM) (Sigma-Aldrich, St Louis, MO) 21 days after the boost vaccination (Fig. 1, *).

Animals were anesthetized for clinical examination including temperature, respiration quality, blood collection, and swabs of nasal, oral and rectal mucosa on days 0, 3, 5, 7, 10, 15, and 30 post-challenge. Subjects in the vaccine cohorts were euthanized on day 30 post-challenge whereas three of the four control subjects had to be euthanized according to approved humane end points on day 8 post-challenge. All other subjects survived until the end of the study.

**Measurement of serum or plasma HeV G specific antibodies.** AGM sera collected at indicated time points was tested for immunoglobulin (Ig) antibodies against HeV G using previously developed multiplexed microsphere assays (11). In brief, 96-well filter plates were primed with PBS. Test sera were diluted in PBS at 1:10 for pre vaccination time points and 1:10,000 at post-vaccination time points. Biotinylated goat anti-human IgM/IgG and streptavidin-phycoerythrin (strep-PE) were also diluted in PBS. Coupled microspheres (sG-HeV) were prepared by sonication for 1 minute followed by vortex mixing for 1 minute each and
then diluted in PBS. Priming liquid was removed from plate using Bio-Plex Pro II Wash Station (Bio-Rad Laboratories, Hercules, CA) and 100 μL/well containing 1500 of each coupled microsphere was added to each well. Microsphere mixture was removed by vacuum, 100 μL of diluted test sera was added to appropriate wells and incubated at room temperature for 30 minutes while shaking in the dark. Diluted test samples were removed by vacuum and 100 μL of diluted biotinylated goat anti-human (1:500) (Pierce Protein Biology Products, ThermoFisher Scientific, Waltham, MA) was added to each well and incubated as described above. Liquid was removed by vacuum and 100 μL of strep-PE (1:1000) (Qiagen Inc., Valencia, CA) was added to each well and again incubated for 30 minutes. All liquid was removed from plates with a vacuum manifold and washed twice with 300 μL of PBS, removing liquid between wash steps. Finally, 125 μL of PBS was added to each well and incubated for 2 minutes as described above. Samples were assayed for mean fluorescence intensity (MFI) across at least a 100 bead region performed on the BioPlex-200 machine and analyzed using Bio-Plex Manager Software (v 6.1) (Bio-Rad). Mean fluorescence intensity (MFI) and the standard deviation (s.d.) of fluorescence intensity across 100 beads were determined for each sample and plotted.

**HeV serum neutralization assays.** Neutralization titers were determined by a conventional serum neutralization assay. Briefly, sera were serially diluted twofold, and incubated with ~100 pfu of HeV for 1 hour at 37°C. Virus and antibodies were then added to individual wells of 6-well plates of Vero cells. Plates were stained with neutral red 2 days after infection and plaques were counted 24 hours after staining. The 50% neutralization titer was determined as the serum dilution at which at there was a 50% reduction in plaque counts versus control wells.

**Specimen collection and processing in HeV-infected AGMs.** Blood was collected in EDTA or serum Vacutainers (Becton Dickinson, Franklin Lakes, NJ). Nasal, oral, and rectal swabs were
collected in 1 ml of Dulbecco’s minimal essential medium (DMEM) (Sigma-Aldrich) and vortexed for 30 seconds. Immediately following sampling, 100μl of blood or 100μl DMEM from individual swab samples was added to 600μl of AVL viral lysis buffer (Qiagen) for RNA extraction. For tissues, approximately 100 mg was stored in 1 ml RNAlater (Qiagen) for 7 days to stabilize RNA. RNAlater was completely removed, and tissues were homogenized in 600μl RLT buffer (Qiagen) in a 2-ml cryovial using tissue lyser (Qiagen) and stainless steel beads. The tissues sampled included conjunctiva, tonsil, oro/nasopharynx, nasal mucosa, trachea, right bronchus, left bronchus, right lung upper lobe, right lung middle lobe, right lung lower lobe, right lung upper lobe, right lung middle lobe, right lung lower lobe, bronchial lymph node (LN), heart, liver, spleen, kidney, adrenal gland, pancreas, jejunum, colon transversum, brain (frontal and cerebellum), brain stem, cervical spinal cord, pituitary gland, mandibular LN, salivary gland LN, inguinal LN, axillary LN, mesenteric LN, urinary bladder, testes or ovaries, and femoral bone marrow. All blood samples and swabs were inactivated in AVL viral lysis buffer, and tissue samples were homogenized and inactivated in RLT buffer prior to removal from the BSL-4 laboratory. Subsequently, RNA was isolated from blood and swabs using the QIAamp viral RNA kit (Qiagen) and from tissues using the RNeasy minikit (Qiagen) according to the manufacturer’s instructions supplied with each kit.

**Hematology and serum biochemistry.** Total white blood cell counts, white blood cell differentials, red blood cell counts, platelet counts, hematocrit values, total hemoglobin concentrations, mean cell volumes, mean corpuscular volumes, and mean corpuscular hemoglobin concentrations were analyzed from blood collected in tubes containing EDTA using a laser based hematologic analyzer (Beckman Coulter, Brea, CA). Serum samples were tested for concentrations of albumin, amylase, alanine aminotransferase (ALT), aspartate
aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT), glucose, cholesterol, total protein, total bilirubin (TBIL), blood urea nitrogen (BUN), creatine (CRE), and C-reactive protein (CRP) by using a Piccolo point-of-care analyzer and Biochemistry Panel Plus analyzer discs (Abaxis, Sunnyvale, CA).

**Histopathology and immunohistochemistry.** Necropsy was performed on all subjects. Tissue samples of all major organs were collected for histopathologic and immunohistochemical examination and were immersion-fixed in 10% neutral buffered formalin for at least 21 days in BSL-4. Subsequently, formalin was changed; specimens were removed from BSL-4, processed in BSL-2 by conventional methods and embedded in paraffin and sectioned at 5 μm thickness. For immunohistochemistry, specific anti-HeV immunoreactivity was detected using an anti-HeV N protein rabbit primary antibody at a 1:5000 dilution for 30 minutes. The tissue sections were processed for immunohistochemistry using the Dako Autostainer (Dako, Carpinteria, CA). Secondary antibody used was biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) at 1:200 for 30 minutes followed by Dako LSAB2 streptavidin-HP (Dako) for 15 minutes. Slides were developed with Dako DAB chromagen (Dako) for 5 minutes and counterstained with hematoxylin for one minute. Non-immune rabbit IgG was used as a negative staining control.

**Detection of HeV load.** RNA was isolated from nasal, oral, and rectal swabs, blood, or tissues and analyzed using primers/probe targeting the N gene of HeV for quantitative real-time PCR (qRT-PCR) (11) with the probe used here being 6-carboxyfluorescein (6FAM)-5’ TCCTGAGTCTGCTGAGGGCGGT 3’-6 carboxytetramethylrhodamine (TAMRA) (Life Technologies, Carlsbad, CA). HeV RNA was detected using the CFX96 detection system (Bio-Rad) in One-step probe qRT-PCR kits (Qiagen) with the following cycle conditions: 50°C for 10
minutes, 95°C for 10 seconds, and 40 cycles of 95°C for 10 seconds and 59°C for 30 seconds. Threshold cycle (CT) values representing HeV genomes were analyzed with CFX Manager Software, and data are shown as genome equivalents (GEq). To create the GEq standard, RNA from HeV challenge stocks was extracted and the number of HeV genomes was calculated using Avogadro’s number and the molecular weight of the HeV genome. Virus titration was performed by plaque assay with Vero cells from all blood samples. Briefly, increasing 10-fold dilutions of the samples were adsorbed to Vero cell monolayers in duplicate wells (200 μl); the limit of detection was 25 pfu/ml.

RESULTS

HeV challenge of vaccinated AGMs. We previously described the development of a lethal AGM model for HeV infection with observed clinical signs and pathology highly consistent with HeV-mediated disease reported in humans (11). Clinical signs in this model include severe depression, respiratory disease leading to acute respiratory distress, neurologic disease, reduced activity, and a time to death ranging from 8 to 10 days. The purpose of the current study was to determine whether vaccination with sG HeV could prevent HeV infection and illness in AGMs. Additionally, we tested whether protection from HeV infection required vaccination with sG HeV plus two adjuvants (sG HeV + alum + CpG) or a single adjuvant (sG HeV + alum). A timeline of the vaccination regimen, HeV challenge, and specimen collection is shown in Fig. 1A. All four control subjects showed disease consistent with historical controls (11) including loss of appetite, depression, decreased activity, and labored breathing (Table 1). Three of the four control AGMs (R335, O7521, R372) developed acute respiratory distress with one subject also developing hind limb paresis; all three of these animals succumbed on day 8 after exposure (Fig. 1B and Table 1).
While the remaining control animal (O7498) was clinically ill for a prolonged period of time the animal began to recover on post-challenge day 14 and survived until the study endpoint. In contrast, none of the vaccinated animals showed any evidence of clinical illness and all survived (Fig. 1B and Table 1).

**HeV load.** To determine if there was HeV replication in animals post-challenge, shedding of virus was assessed by qRT-PCR on nasal, oral, and rectal swabs (Fig. 2A, B, and C respectively) with viremia also screened by qRT-PCR on whole blood samples (Fig. 2D). HeV genome equivalents were observed in all swab and blood samples for two of the control animals (R335 and O7521, Fig. 2) while we detected HeV genome equivalents for another control animal (R372) in oral swabs on day 7 post-challenge (Fig. 2B, red). The surviving control animal (O7498) had no detectable HeV RNA in any sample. Likewise, none of the specifically vaccinated animals had any detectable HeV RNA in any sample (negative data not shown). HeV RNA was also detected systemically in the tissues of control animals R335 and O7521 (Fig. 3, green and black respectively), whereas HeV RNA was only detected in the respiratory tissues, the axillary LN, and femoral bone marrow for control animal O7521 (Fig. 3, red). HeV RNA was not detected in tissues of control animal O7498 or in any of the tissues of any of the specifically vaccinated animals (negative data not shown). Detection of HeV RNA in tissues (Fig. 3), swabs, and blood (Fig. 2) correlated with outcome and gross pathology (Fig. 1B and Table 1) for each animal.

**Histopathological and immunohistochemical analysis of HeV-infected AGMs.** Histopathology from the three control animals that succumbed to HeV challenge was mostly consistent with previous findings in henipavirus-infected AGM (11, 20, 26, 29) with lesions from control animals R335 and O7521 being more prominent than control animal R372. Noteworthy
lesions included interstitial pneumonia, necrosis and hemorrhage of the splenic white pulp, and variable syncytial cell formation in lymphoid tissues. Alveolar spaces were filled with edema fluid, fibrin, foamy alveolar macrophages, and cellular debris. Glomerular tufts were hypercellular and congested and syncytial cells were occasionally noted within the endothelium of the glomerular tufts. Strong immunoreactivity for HeV antigen was present within the alveolar capillary endothelium and alveolar macrophages and also within scattered mononuclear cells in the subcapsular and medullary sinuses of various lymph nodes (Fig. 4). Strong immunoreactivity for HeV antigen was also present in the kidney within segmental regions of the endothelium of the glomerular tufts (Fig. 4). Neuropathology was not as prominent in the control animals as observed in HeV-infected AGMs in a previous study (11). Nonetheless, HeV antigen was a notable finding within the endothelium of the brain stem (Fig. 4). Representative tissue sections from the control animals are shown in Fig. 4. Examination of tissue sections from vaccinated subjects revealed only normal tissue architecture. Importantly, HeV antigen was not detected in any tissue of any sGHeV-vaccinated subject using immunohistochemical techniques (Fig. 4). At the study endpoint, the tissue architecture was also normal in the control AGM that survived (O7498); HeV antigen was also undetectable in tissues from this animal.

**Humoral immune response to HeV G.** The AGMs employed in this study were vaccinated with sGHeV with adjuvant; therefore, we were interested in measuring the humoral immune response to HeV G and serum neutralizing titers induced in all animals. Circulating Ig antibodies specific for HeV G in serum were measured by microsphere assay as done previously (11). As expected, we did not detect HeV G specific Ig in sera before prime vaccination (day -42) but we were able to detect Ig directed against HeV G in sera of the sGHeV-vaccinated cohorts on the day of boost (day -21) along with an increase in titer after boost (day 0) as well as
detectable HeV G specific Ig after challenge (Fig. 5, teal and orange). This pattern was also seen when we tested the circulating neutralizing antibody titers in sera against HeV with both specifically vaccinated groups producing good neutralizing antibody titers with higher levels detected after boost (Table 2).

DISCUSSION

A substantial increase in the number of independent spillover events of HeV between bats and horses occurred in Australia in 2011 (30). Notably, the first case of HeV seroconversion in a dog in Australia was also reported during these episodes (31). These latest findings, which have since repeated, have had a major impact on equine veterinary practices (32). Specifically, Australian veterinarians reduced or ceased equine medicine in order to avoid contracting HeV. In order to address this problem the Australian Government supported the commercial development of the sG_{HeV} vaccine Equivac® HeV for use in horses (28, 32). The Equivac® HeV vaccine was launched late in 2012 and is currently in use in Australia with more than 150,000 horses vaccinated to date.

While significant progress on a veterinary vaccine for HeV has been made, the development of effective human vaccines and antiviral drugs for high consequence pathogens such as HeV and NiV has been a much slower and complicated process. In particular, the restriction of infectious HeV work to BSL-4 containment has hampered vaccine development progress. In addition, conventional clinical trials with vaccines or antiviral therapies against viruses such as HeV are not practical or possible. To address the development of countermeasures for exotic pathogens such as HeV the U.S. Food and Drug Administration (FDA) implemented the Animal Efficacy Rule in 2002. This rule specifically applies to the
development of countermeasures when human efficacy studies are not possible or ethical. In brief, this rule permits the evaluation of vaccines or therapeutics using data generated from studies performed in animal models that faithfully recapitulate human disease. At this time the ferret and AGM appear to be the models that most accurately reflect human HeV infection. The protective efficacy of sG\textsubscript{HeV} has been evaluated in the ferret (24) and AGM (26) models of NiV-mediated infection and in the ferret (25) and now in the AGM model of HeV-mediated disease. Additionally, the durability of the sG\textsubscript{HeV} vaccine has been tested in ferrets one year post-vaccination against NiV challenge with promising results (24). In these studies, all vaccinated animals developed high levels of antigen-specific serum Ig (Fig. 5) and neutralizing antibodies before challenge (Table 2) which were comparable to the levels of antigen-specific Ig and neutralizing antibody levels seen against HeV in a previous study (26). While both vaccine formulations in the current study resulted in HeV G-specific Ig and significant neutralizing antibody titers against HeV, the cohort of AGMs receiving the sG\textsubscript{HeV} + alum + CpG vaccine formulation had a significantly (P <0.05) higher neutralizing antibody titer by Bonferroni posttest on day 0 (Table 2). Nevertheless, the present findings have clearly demonstrated that sG\textsubscript{HeV} + alum alone is also capable of providing complete protection from HeV challenge.

As HeV and NiV replicate and cause severe pathology in the lung, and CpG motifs have been shown to elicit Th1 and mucosal immunity regardless of immunization route (33-35), the most recent sG vaccine studies have employed and explored several CpG adjuvants. Additionally, several CpG motifs have also entered into human clinical trials, which could facilitate future regulatory processes for this adjuvant in general. Host responses to CpG are highly sequence and species specific. The CpG adjuvant used in feline and ferret sG vaccine trials had been employed previously in humans (36-38) and cats. Mucosal IgA was detected in
vaccinated and protected animals (23) suggesting the CpG may have contributed to mucosal immunity. As vaccine studies transitioned to nonhuman primates, a slightly different CpG motif (26) was selected that was shown to be an effective adjuvant in humans and nonhuman primates (39, 40). As demonstrated here and previously, AGMs vaccinated with sG and CpG (ODN 2006) mount robust immune responses and are protected from lethal HeV or NiV challenge. Interestingly, all sG vaccinated animals were protected from lethal HeV challenge in the current study, but the animals that received sG with CpG had significantly higher antibody titers.

Importantly, all specifically vaccinated animals were protected from HeV disease (Fig. 1B, Table 1). In addition, there was no evidence of clinical illness or infectious HeV in any vaccinated animal across all of these efficacy studies. The efficacy of the sGHeV vaccine against HeV infection described in this report provides further critical evidence that this vaccine should be moved toward further development as a human use vaccine against HeV and NiV using either alum alone or alum and CpG as adjuvant choices based on the neutralizing antibody observations in this study.

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Health and Human Services (Washington, DC) and the Henry M. Jackson Foundation for the Advancement of Military Medicine Inc. (Bethesda, MD). All other authors declare that they have no competing interests. Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by UTMB or the Department of Defense.
Figure Legends

**FIG. 1.** (A) Diagram of the sGHeV vaccination schedule and sampling days post HeV challenge in AGMs. Black arrow; prime vaccination with sGHeV (100 μg) + adjuvant or adjuvant alone. Grey arrow; boost vaccination with sGHeV + adjuvant or adjuvant alone. * depicts the day of i.t. HeV challenge with 5 x 10^5 pfu. (B) Kaplan-Meier survival curve for the control group (black, n= 4; 2 non-vaccinated, 1 alum only, and 1 alum + CpG only) and the vaccinated groups; sGHeV + alum (teal, n= 4) and sGHeV + alum + CpG (orange, n= 4).

**FIG. 2.** Viral load from AGM control cohort (all vaccinated negative, not shown) as detected by genome equivalents/ml by qRT-PCR from nasal swabs (A), oral swabs (B), rectal swabs (C), and blood (D). R335 and R372 (green and red respectively) non-vaccinated controls, O7498 (white) alum only control, and O7521 (black) alum + CpG only control. Error bars are s.d.

**FIG. 3.** Viral load from AGM control cohort (all vaccinated negative, not shown) as detected by genome equivalents/mg by qRT-PCR from tissues. R335 and R372 (green and red respectively) non-vaccinated controls, O7498 (white) alum only control and O7521 (black) alum + CpG only control. Right upper (R.U.), right middle (R.M.), right lower (R.L.), left upper (L.U.), left middle (L.M.), left lower (L.L.), lymph node (LN). Error bars are s.d.

**FIG. 4.** Lack of HeV antigen in representative sGHeV vaccinated tissues and localization of HeV antigen in representative control tissues by immunohistochemical staining. Lung, spleen, kidney, and brain stem were labeled with an N protein-specific polyclonal rabbit antibody and
images taken (lung 20x, spleen 20x, kidney 40x, brain stem 60x). sG + CpG + Alum: all tissues
O7515. sG + Alum: all tissues O7500. Control: lung O7521, spleen O7521, kidney R335, brain
stem R335.

FIG. 5. Detection of HeV G specific antibodies from sGHeV + adjuvant vaccinated AGMs.
Mean fluorescence intensities (MFI) are shown on the y-axis and represent binding of specific Ig
(IgG, and IgM) to sGHeV. Error bars represent the s.d. of fluorescence intensity across 100 beads
for each sample.
Table 1. Clinical description and outcome of Hendra virus challenged AGMs

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Sex</th>
<th>Group</th>
<th>Clinical illness</th>
<th>Clinical and gross pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>R335</td>
<td>Male</td>
<td>Control</td>
<td>Fever (d7); Depression (d8); lethargy (d8);</td>
<td>Thrombocytopenia (d8); &gt;2-fold increase in WBC; &gt;3-fold increase in BUN (d8); &gt;2-fold increase</td>
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<td></td>
<td></td>
<td></td>
<td>loss of appetite (d7-8); labored breathing</td>
<td>in CRE (d8); &gt;4-fold increase in AST (d8); &gt;10-fold increase in CRP (d7-8); excess blood-tinged</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(d5-8); dehydration (d5,7).</td>
<td>pleural fluid; severely inflamed, enlarged lungs with severe congestion and hemorrhage of all</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Animal expired on d8.</td>
<td>lobes; darkened liver.</td>
</tr>
<tr>
<td>R372</td>
<td>Female</td>
<td>Control</td>
<td>Fever (d3, 5); Depression (d10-13); lethargy</td>
<td>Moderately inflamed, enlarged lungs with multifocal areas of congestion.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(d10-13); loss of appetite (d4-8); labored</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>breathing (d7-8); dehydration (d7-8); chills</td>
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<td></td>
<td></td>
<td>(d8); hind limb paresis (d8). Animal</td>
<td></td>
</tr>
<tr>
<td>O7498</td>
<td>Male</td>
<td>Control</td>
<td>Depression (d8); lethargy (d8); loss of</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>appetite (d8-15); labored breathing (d7-13);</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>dehydration (d10); Splenomegaly (d7); Animal</td>
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</tr>
<tr>
<td>O7521</td>
<td>Male</td>
<td>Control</td>
<td>Fever (d7); Depression (d8); lethargy (d8);</td>
<td>Thrombocytopenia (d7); &gt;2-fold increase in WBC (d8); &gt;2-fold increase in BUN (d8); 5-fold</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>loss of appetite (d7-8); labored breathing</td>
<td>increase in CRE (d8); &gt;2-fold increase in AST (d8); &gt;10-fold increase in CRP (d8); excess</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(d5-8). Animal expired on d8.</td>
<td>blood-tinged pleural fluid; inflated, enlarged lungs with multifocal areas of congestion and</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>hemorrhage particularly of the lower and middle right lobes; darkened liver.</td>
</tr>
<tr>
<td>O7500</td>
<td>Male</td>
<td>sG+Alum</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>O7503</td>
<td>Male</td>
<td>sG+Alum</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>O7499</td>
<td>Male</td>
<td>sG+Alum</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>O7501</td>
<td>Male</td>
<td>sG+Alum</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>O7515</td>
<td>Male</td>
<td>sG+Alum+CpG</td>
<td>None</td>
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</tr>
<tr>
<td>O7494</td>
<td>Male</td>
<td>sG+Alum+CpG</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>O7506</td>
<td>Male</td>
<td>sG+Alum+CpG</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>O7477</td>
<td>Male</td>
<td>sG+Alum+CpG</td>
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</tbody>
</table>
Table 2. HeV Serum Neutralization Titersa in Vaccinated AGMs

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Subject No.</th>
<th>Day -42b</th>
<th>Day -21b</th>
<th>Day 0b</th>
<th>Day 30b</th>
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<tbody>
<tr>
<td>sG + Alum</td>
<td>O7500</td>
<td>&lt;20</td>
<td>160</td>
<td>1280</td>
<td>640</td>
</tr>
<tr>
<td></td>
<td>O7503</td>
<td>&lt;20</td>
<td>80</td>
<td>640</td>
<td>320</td>
</tr>
<tr>
<td></td>
<td>O7499</td>
<td>&lt;20</td>
<td>160</td>
<td>640</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td>O7501</td>
<td>&lt;20</td>
<td>160</td>
<td>1280</td>
<td>320</td>
</tr>
<tr>
<td>sG + Alum + CpG</td>
<td>O7515</td>
<td>&lt;20</td>
<td>160</td>
<td>1280</td>
<td>640</td>
</tr>
<tr>
<td></td>
<td>O7494</td>
<td>&lt;20</td>
<td>320</td>
<td>1280</td>
<td>640</td>
</tr>
<tr>
<td></td>
<td>O7506</td>
<td>&lt;20</td>
<td>320</td>
<td>2560</td>
<td>640</td>
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<tr>
<td></td>
<td>O7477</td>
<td>&lt;20</td>
<td>640</td>
<td>1280</td>
<td>640</td>
</tr>
<tr>
<td>Alum only</td>
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<td>&lt;20</td>
<td>&lt;20</td>
<td>20</td>
<td>40</td>
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<tr>
<td>CpG only</td>
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<td>&lt;20</td>
<td>&lt;20</td>
<td>20</td>
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<tr>
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<td>R335</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>&lt;20</td>
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<td>&lt;20</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>*</td>
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</tbody>
</table>

a reciprocal serum dilution at which 50% of virus was neutralized
b day post-HeV challenge
* not sampled.
References


