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

Chad E. Mire, a,b Joan B. Geisbert, a,b Krystle N. Agans, a,b Yan-Ru Feng, c Karla A. Fenton, a,b Katharine N. Bossart, d Lianying Yan, c Yee-Peng Chan, e Christopher C. Broder, f Thomas W. Geisbert a,b

Galveston National Laboratory a and Departments of Microbiology and Immunology, b University of Texas Medical Branch, Galveston, Texas, USA; Department of Microbiology and Immunology, Uniformed Services University, Bethesda, Maryland, USA c; Integrated Research Associates, LLC, San Francisco, California, USA d

ABSTRACT

Hendra virus (HeV) is a zoonotic emerging virus belonging to the family Paramyxoviridae. HeVs cause 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 the human disease. Here, we assessed the utility of a newly developed recombinant subunit vaccine based on the HeV attachment (G) glycoprotein 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 the 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.

IMPORTANT

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

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 4 (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 pteropod 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 nearly annual basis since the initial outbreak, with all episodes involving infection of horses. In total, >80 horses have succumbed to HeV infection, with a case fatality rate of approximately 75%. There have been seven human HeV infections recorded, most recently in 2009, of which four have been fatal (57%) (8). All patients initially presented with influenza-like symptoms after an incubation period of 7 to 16 days. While two of the patients recovered from the influenza-like illness, one developed pneumonia and succumbed to multiorgan 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 approved vaccines or antiviral drugs for combating human HeV or NiV infection. Regarding treatment options for henipavirus infection, an open-label ribavirin trial was performed with 140 patients during the initial outbreak of NiV in Malaysia in 1998; however, the results of that study remain controversial (10). In addition, three of the seven recorded human HeV cases were treated with ribavirin and one of the patients survived (8). The utility of ribavirin as a countermeasure against 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 postexposure treatment for henipavirus infection appears to be an experimental human monoclonal antibody (MAb). This MAb, m102.4, targets the ephrin-B2 and -B3 receptor binding domain of the henipavirus envelope attachment (G) glycoprotein (12–16). m102.4 is a potent cross-reactive neutralizing antibody in vitro (17, 18) and had been shown to protect ferrets from a lethal NiV challenge (19) and AGMs from a lethal HeV challenge (20). Importantly, in 2010,
m102.4 was administered to two individuals in Australia who had a significant risk of exposure to HeV under a compassionate-use protocol. It was also used in 2012 to treat an individual with possible exposure to HeV in Australia and in 2013 to treat an individual with possible laboratory exposure in the United States. To date, all four of these individuals have no evidence of henipavirus infection.

In addition to the postexposure treatments, two experimental preventive vaccines against henipaviruses have been evaluated in animal models. A recombinant adeno-associated virus vaccine expressing the NiV G protein completely protected hamsters against a homologous NiV challenge and protected 50% of the animals against a heterologous HeV infection (21). In addition, a recombinant subunit vaccine based on the HeV G protein (sGHeV) completely protected small animals against lethal HeV and NiV infections (22–25) and more recently was shown to be efficacious in the robust AGM model of NiV infection (26). sGHeV (amino acids 73 to 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 the performance of this vaccine in a nonhuman primate model of HeV infection, which is a necessary prerequisite for the licensure of such a vaccine for use in humans. Here, we report for the first time the prophylactic efficacy of the sGHeV vaccine in a lethal HeV AGM model that faithfully recapitulates human HeV infection.

MATERIALS AND METHODS

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

Viruses and cells. HeV was kindly provided by Tom Ksiazek and was obtained from a patient who was part of the 1994 outbreak in Australia. The virus was propagated on Vero E6 cells in Eagle’s minimal essential medium supplemented with 10% fetal calf serum. The titer of the HeV stock used was \(1 \times 10^7\) PFU/ml. The HeV challenge virus stock was assessed for the presence of endotoxin with the Endosafe-PTS portable test system (Charles River, Wilmington, MA). Virus preparations were diluted 1:10 in Limusia amebocyte lysate (LAL) reagent water in accordance with the 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 the limit of detection, while positive controls showed that the tests were valid.

Vaccine formulation. Production and purification of sGHeV were 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-modified backbone (sGHeV-alum-CpG). The vaccine without CpG was formulated with 100 µg of sGHeV and 2.5 mg of aluminum ion (sGHeV-alum) per vaccinated animal. The vaccine with CpG was formulated with 100 µg of sGHeV, 2.5 mg of aluminum ion, and 150 µg of ODN 2006 per vaccinated animal. For the vaccine containing both Alhydrogel and CpG, the Alhydrogel and sGHeV were mixed before ODN 2006 was added. Each vaccine dose was adjusted to 1 ml with phosphate-buffered saline (PBS), and mixtures were incubated on a rotating wheel at room temperature for at least 2 to 3 h before injection. Each AGM received the same dose of 1 ml for prime and boost vaccinations, and all vaccine doses were given via intramuscular (i.m.) injection. The 100-µg dose used in this study was based on the protective efficacy against NiV and the prolonged level of IgG in AGMs vaccinated with this dose rather than 10 or 50 µg (26).

Animals. Animal studies were performed in BSL-4 biocontainment at the Galveston National Laboratory 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 adhered to the principles stated in reference 29. 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 to 6 kg (PreLabs, Hines, IL) were used in this study. AGMs were anesthetized by i.m. injection with ketamine and vaccinated with sGHeV by i.m. injection on day −42 (prime vaccination) and day −21 (boost vaccination) (Fig. 1, black and gray arrows, respectively). The vaccine formulations used are described above. Four AGMs received two doses of vaccine containing sGHeV and Alhydrogel; four animals received two doses of vaccine containing sGHeV, 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 \(5 \times 10^7\) 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 mucosae on days 0, 3, 5, 7, 10, 15, and 30 postchallenge. AGMs in the vaccine cohorts were euthanized on day 30 postchallenge, whereas three of the four control AGMs had to be euthanized according to approved humane end-points on day 8 postchallenge. All of the other AGMs survived until the end of the study.

Measurement of serum or plasma HeV G-specific antibodies. AGM serum samples collected at the time points indicated were tested for immunoglobulin (Ig) antibodies against HeV G with previously developed multiplexed microsphere assays (11). In brief, 96-well filter plates were primed with PBS. Test serum samples were diluted in PBS at 1:10 at prevaccination time points and at 1:10,000 at postvaccination time points. Biotinylated goat anti-human IgM/IgG and streptavidin-phycocerythrin (strept-PE) were also diluted in PBS. Coupled microspheres (sG-HeV) were prepared by sonication for 1 min, followed by vortex mixing for 1 min each, and then diluted into PBS. Priming liquid was removed from the plates with a Bio-Plex Pro II Wash Station (Bio-Rad Laboratories, Hercules, CA), and 100 µl containing 1,500 coupled microspheres was added to each well. The microsphere mixture was removed by vacuum, 100 µl of diluted test serum was added to appropriate wells, and the mixture was incubated at room temperature for 30 min while shaking in the dark. Diluted test samples were removed by vacuum, 100 µl of diluted biotinylated goat anti-human antibody (1:500; Pierce Protein Biology Products, Thermofisher Scientific, Waltham, MA) was added to each well, and the mixture was incubated as described above. Liquid was removed by vacuum, 100 µl of strep-PE (1:1,000; Qiagen Inc., Valencia, CA) was added to each well, and the mixture was incubated for 30 min. All of the liquid was removed from the plates with a vacuum manifold and washed twice with 300 µl of PBS, and the liquid was removed between wash steps. Finally, 125 µl of PBS was added to each well and incubated for 2 min 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 with Bio-Plex Manager Software (v 6.1) (Bio-Rad). MFI and the standard deviation (SD) 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, serum samples were serially diluted 2-fold and incubated with ~100 PFU of HeV for 1 h at 37°C. Virus and antibodies were then added to individual wells of six-well plates of Vero cells. Plates were stained with neutral red 2 days after infection, and plaques were counted 24 h after staining. The 50% neutralization titer was determined as the serum dilution at which there was a 50% reduction in plaque counts versus the control well.

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 DMEM (Sigma-Aldrich) and vortexed for 30 s. Immediately following sampling, 100 μl of blood or 100 μl of DMEM from individual swab samples was added to 600 μl of AVL viral lysis buffer (Qiagen) for RNA extraction. For tissues, approximately 100 mg was stored in 1 ml of RNAlater (Qiagen) for 7 days to stabilize the RNA. RNAlater was completely removed, and tissues were homogenized in 600 μl of RLT buffer (Qiagen) in a 2-ml cryovial with a TissueLyser (Qiagen) and stainless steel beads. The tissues sampled included conjunctiva, tonsil, oro- and nasopharynx, nasal mucosa, trachea, right bronchus, left bronchus, 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 with the QIAamp viral RNA kit (Qiagen) and from tissues by using the RNeasy minikit (Qiagen) according to the manufacturer’s instructions supplied with each kit.

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

Histopathology and immunohistochemistry analyses. Necropsy was performed on all AGMs. Tissue samples of all major organs were collected for histopathologic and immunohistochemical examinations and immersion fixed in 10% neutral buffered formalin for at least 21 days at BSL-4. Subsequently, the formalin was changed; specimens were removed from BSL-4, processed at BSL-2 by conventional methods, embedded in paraffin, and sectioned at a 5-μm thickness. For immunohistochemistry analysis, specific anti-HeV immunoreactivity was detected with an anti-HeV N protein rabbit primary antibody at a 1:5,000 dilution for 30 min. The tissue sections were processed for immunohistochemistry analysis with the Dako Autostainer (Dako, Carpinteria, CA). The secondary antibody used was biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) at 1:200 for 30 min, followed by Dako LSAB2 streptavidin-horseradish peroxidase (Dako) for 15 min. Slides were developed with Dako 3,3′-diaminobenzidine chromogen (Dako) for 5 min and counterstained with hematoxylin for 1 min. Nonimmune rabbit IgG was used as a negative staining control.

Detection of HeV loads. RNA was isolated from nasal, oral, and rectal swabs; blood; or tissues and analyzed with primers/probe targeting the N gene of HeV for quantitative real-time PCR (qRT-PCR) (11), with the probe used here being 6-carboxyfluorescein–5′-TCCGTAGCTCGCCTGAGGGCGGT-3′ (Life Technologies, Carlsbad, CA). HeV RNA was detected with the CFX96 detection system (Bio-Rad) in One-Step Probe qRT-PCR kits (Qiagen) under the following cycling conditions: 50°C for 10 min, 95°C for 10 s, and 40 cycles of 95°C for 10 s and 59°C for 30 s. Threshold cycle values representing HeV genome equivalents (GEq) were calculated by 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 the 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 of 8 to 10 days. The purpose of the present
study was to determine whether vaccination with sG<sub>HeV</sub> could prevent HeV infection and illness in AGMs. Additionally, we tested whether protection from HeV infection requires vaccination with sG<sub>HeV</sub> plus two adjuvants (sG<sub>HeV</sub>-alum-CpG) or a single adjuvant (sG<sub>HeV</sub>-alum). A time line of the vaccination regimen, HeV challenge, and specimen collection is shown in Fig. 1A. All four control AGMs 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, and one also developed 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, it began to recover on postchallenge 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 loads.** To determine if there was HeV replication in animals postchallenge, virus shedding was assessed by qRT-PCR of nasal, oral, and rectal swabs (Fig. 2A, B, and C, respectively) and viremia was also screened for by qRT-PCR of whole blood samples (Fig. 2D). HeV GEq were observed in all swab and blood samples from two of the control animals (R335 and O7521, Fig. 2), while we detected HeV GEq in oral swabs from another control animal (R372) on day 7 postchallenge (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 detected only in the respiratory tissues, the axillary LN, and femoral bone marrow of 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 each animal’s outcome and gross pathology (Fig. 1B and Table 1).

**Histopathological and immunohistochemical analysis of HeV-infected AGMs.** Histopathologic analysis of samples from the three control animals that succumbed to a HeV challenge was mostly consistent with previous findings on henipavirus-infected AGMs (11, 20, 26, 30), and lesions from control animals R335 and O7521 were more prominent than those from 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 to HeV antigen

**TABLE 1 Clinical descriptions and outcomes of HeV-challenged AGMs**

<table>
<thead>
<tr>
<th>AGM</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 (day 7), depression (day 8), lethargy (day 8), loss of appetite (days 7, 8), labored breathing (days 5–8), dehydration (days 5, 7), death (day 8)</td>
<td>Thrombocytopenia (day 8), &gt;2-fold increase in WBC count, &gt;3-fold increase in BUN (day 8), &gt;2-fold increase in CRE (day 8), &gt;4-fold increase in AST (day 8), &gt;10-fold increase in CRP (days 7, 8), excess blood-tinged pleural fluid, severely inflated enlarged lungs with severe congestion and hemorrhage of all lobes, darkened liver</td>
</tr>
<tr>
<td>R372</td>
<td>Female</td>
<td>Control</td>
<td>Fever (days 3, 5), Depression (days 10–13), lethargy (days 10–13), loss of appetite (days 4–8), labored breathing (days 7, 8), dehydration (days 7, 8), chills (day 8), hind limb paresis (day 8), euthanasia (day 8)</td>
<td>Moderately inflated enlarged lungs with multifocal areas of congestion</td>
</tr>
<tr>
<td>O7498</td>
<td>Male</td>
<td>Control</td>
<td>Depression (day 8), lethargy (day 8), loss of appetite (days 8–15), labored breathing (days 7–13), dehydration (day 10), splenomegaly (day 7) survival</td>
<td>None</td>
</tr>
<tr>
<td>O7521</td>
<td>Male</td>
<td>Control</td>
<td>Fever (day 7), depression (day 8), loss of appetite (days 7, 8), labored breathing (days 5–8), death (day 8)</td>
<td>Thrombocytopenia (day 7), &gt;2-fold increase in WBC count (day 8), &gt;2-fold increase in BUN (day 8), &gt;5-fold increase in CRE (day 8), &gt;2-fold increase in AST (day 8), &gt;10-fold increase in CRP (day 8), excess blood-tinged pleural fluid, inflated enlarged lungs with multifocal areas of congestion and 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>
<td>None</td>
</tr>
<tr>
<td>O7494</td>
<td>Male</td>
<td>sG-Alum-CpG</td>
<td>None</td>
<td>None</td>
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<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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was present within the alveolar capillary endothelium and alveolar macrophages and also within scattered mononuclear cells in the subcapsular and medullary sinuses of various LNs (Fig. 4). Strong immunoreactivity to HeV antigen was also present in the kidneys within segmental regions of the endothelium of the glomerular tufts (Fig. 4). Neuropathology was not as prominent in the control animals as in HeV-infected AGMs in a previous study (11). Nonetheless, HeV antigen was a notable finding within the endothelium

**FIG 2** Viral loads of the AGM control cohort (all vaccinated; negative data not shown) expressed in GEq/ml and determined by qRT-PCR assay of nasal swabs (A), oral swabs (B), rectal swabs (C), and blood (D). R335 and R372 (green and red, respectively), nonvaccinated controls; O7498 (white), alum-only control; O7521 (black), alum-CpG-only control. Error bars are SDs.

**FIG 3** Viral loads of the AGM control cohort (all vaccinated; negative data not shown) as detected by GEq/mg by qRT-PCR assay of tissues. R335 and R372 (green and red, respectively), nonvaccinated controls; O7498 (white), alum-only control; O7521 (black), alum-CpG only control. R.U., right upper; R.M., right middle; R.L., right lower; L.U., left upper; L.M., left middle; L.L., left lower. Error bars are SDs.
of the brain stem (Fig. 4). Representative tissue sections from the control animals are shown in Fig. 4. Examination of tissue sections from vaccinated AGMs revealed only normal tissue architecture. Importantly, HeV antigen was not detected in any tissue of any sGHeV-vaccinated AGM by 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 used in this study were vaccinated with sGHeV with adjuvant; therefore, we were interested in measuring the humoral immune responses to HeV G and serum neutralizing titers induced in all of the 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 serum samples before prime vaccination (day −42) but we were able to detect Ig directed against HeV G in serum samples from the sGHeV-vaccinated cohorts on the day of boost vaccination (day −21) along with an increase in titer after the boost vaccination (day 0), as well as detectable HeV G-specific Ig after the challenge (Fig. 5, teal and orange). This pattern was also seen when we tested the circulating neutralizing antibody titers in serum samples against HeV, with both specifically vaccinated groups producing good neutralizing antibody titers with higher levels detected after the boost vaccination (Table 2).

**DISCUSSION**

A substantial increase in the number of independent HeV spill-over events between bats and horses occurred in Australia in 2011 (31). Notably, the first case of HeV seroconversion in a dog in Australia was also reported during these episodes (32). These last findings, which have since repeated, have had a major impact on equine veterinary practices (33). 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 sGHeV vaccine Equivac HeV for use in horses (28, 33). 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 implemented the Animal Efficacy Rule in 2002. This rule specifically applies to vaccines or therapeutics with data generated from studies performed in animal models that faithfully recapitulate human disease.

**TABLE 2** Serum HeV neutralizing antibody titers of vaccinated AGMs

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>AGM</th>
<th>Day −42&lt;sup&gt;ab&lt;/sup&gt;</th>
<th>Day −21&lt;sup&gt;ab&lt;/sup&gt;</th>
<th>Day 0&lt;sup&gt;ab&lt;/sup&gt;</th>
<th>Day 30&lt;sup&gt;ab&lt;/sup&gt;</th>
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<tr>
<td>sG-Alum</td>
<td>O7500</td>
<td>20</td>
<td>160</td>
<td>1,280</td>
<td>640</td>
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<tr>
<td>O7503</td>
<td>&lt;20</td>
<td>80</td>
<td>640</td>
<td>320</td>
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<tr>
<td>O7499</td>
<td>&lt;20</td>
<td>160</td>
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<td>160</td>
<td>1,280</td>
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<tr>
<td>sG-Alum-CpG</td>
<td>O7515</td>
<td>&lt;20</td>
<td>160</td>
<td>1,280</td>
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<tr>
<td>O7494</td>
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<td>320</td>
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<td>&lt;20</td>
<td>640</td>
<td>1,280</td>
<td>640</td>
<td></td>
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<tr>
<td>Alum only</td>
<td>O7498</td>
<td>&lt;20</td>
<td>&lt;20</td>
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<td>40</td>
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<td>CpG only</td>
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<sup>a</sup> Reciprocal serum dilution at which 50% of virus was neutralized. Boldface values highlight moderate to good antibody titers.

<sup>b</sup> Day after HeV challenge.

<sup>c</sup> NS, not sampled.
ease. At this time, the ferret and AGM appear to be the models that most accurately reflect human HeV infection. The protective efficacy of sGHeV 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 sGHeV vaccine has been tested in ferrets 1 year postvaccination against a 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 the challenge (Table 2) which were comparable to the levels of antigen-specific Ig and neutralizing antibody against HeV seen in a previous study (26). While both of the vaccine formulations in the present study resulted in HeV G-specific Ig and significant titers of neutralizing antibody against HeV, the cohort of AGMs receiving the sGHeV-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 sGHeV-alum alone is also capable of providing complete protection from a 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 the immunization route used (34–36), the most recent sG vaccine studies have used 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 used previously in humans (37–39) and cats. Mucosal IgA was detected in vaccinated and protected animals (23), suggesting that 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 (40, 41). As demonstrated here and previously, AGMs vaccinated with sG and CpG (ODN 2006) mounted robust immune responses and are protected from a lethal HeV or NiV challenge. Interestingly, all sG-vaccinated animals were protected from a lethal HeV challenge in the present 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 with alum alone and alum plus CpG as adjuvant choices based on the neutralizing antibody observations in this study.

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K.N.B. and C.C.B. are coinventors on pending U.S. patents and Australian patent 2005327194, pertaining to soluble forms of Hendra and Nipah G glycoproteins; assignees are the United States of America as represented by the Department of Health and Human Services (Washington, DC) and the Henry M. Jackson Foundation for the Advancement of Military Medicine Inc. (Bethesda, MD). The rest of us have no competing interests. All of the opinions, interpretations, conclusions, and recommendations presented here are ours and are not necessarily endorsed by UTMB or the Department of Defense.

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Hendra virus Glycoprotein Subunit Vaccine


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