Nipah virus (NiV) is a zoonotic pathogen that causes high case-fatality rates (CFRs) in humans. Two NiV strains have caused outbreaks: the Malaysia strain (NiV_M), discovered in 1998–1999 in Malaysia and Singapore (≈40% CFR); and the Bangladesh strain (NiV_B), discovered in Bangladesh and India in 2001 (≈80% CFR). Recently, NiV_B in African green monkeys resulted in a more severe and lethal disease than NiV_M. No NiV vaccines or treatments are licensed for human use. We assessed replication-restricted single-injection recombinant vesicular stomatitis vaccine NiV vaccine vectors expressing the NiV glycoproteins against NiV_B challenge in African green monkeys. All vaccinated animals survived to the study endpoint without signs of NiV disease; all showed development of NiV_F Ig, NiV_G IgG, or both, as well as neutralizing antibody titers. These data show protective efficacy against a stringent and relevant NiV_B model of human infection.

Nipah virus (NiV) and Hendra virus (HeV) are highly pathogenic zoonotic agents in the paramyxovirus genus Henipavirus. Human case-fatality rates (CFRs) for these viruses historically have ranged from 40% to >90% (1). NiV is categorized as a Biosafety Level 4 (BSL-4) pathogen because of the substantial illness and death it causes and the lack of approved vaccines and therapeutic drugs for human use. In 2015, the World Health Organization listed NiV as a priority pathogen because it is likely to cause severe outbreaks and, in early 2018, placed NiV on the Blueprint list of priority diseases (https://www.who.int/blueprint/priority-diseases). This WHO designation was bolstered because of a deadly NiV outbreak (CFR 89%) during spring 2018 in southwestern India, where NiV had not previously been reported (2).

Bats of the genus Pteropus are the primary reservoir in nature for NiV (3), but several other mammal species can be infected by NiV (4–7). Analysis of NiV genomes has identified 2 NiV strains responsible for outbreaks: Malaysia strain NiV_M and Bangladesh strain (NiV_B). NiV_M caused the first identified outbreak of NiV during 1998–1999 in Malaysia and Singapore (≈270 persons infected; CFR ≈40%) (8,9) and perhaps was responsible for a 2014 outbreak in the Philippines (CFR ≈52%); however, this speculation is based on short genomic reads, so the NiV strain that caused this outbreak is not known (10). NiV_B has caused repeated outbreaks in Bangladesh and northeastern India; outbreaks occurred almost every year during 2001–2015 (11–15). These NiV_B outbreaks had higher CFRs, averaging ≈80% (14), and showed documented human-to-human transmission (11,16).

Eight experimental preventive candidate vaccines against henipaviruses have been evaluated in NiV_M animal models: 1) canarypox and 2) vaccinia viruses encoding the NiV_M fusion protein (F) or the NiV_M attachment protein (G) that have shown protection against NiV_M in hamsters and pigs (17,18); 3) a recombinant adeno-associated virus expressing the NiV_M G protein that completely protected hamsters against homologous NiV_M challenge (19); 4) recombinant vesicular stomatitis viruses (rVSVs) expressing the NiV_M F protein or the NiV_M G protein that had 100% efficacy in hamsters against NiV_M (20); 5) rVSVs expressing the NiV_B F protein or the NiV_B G protein that completely protected ferrets from NiV_M disease (21); 6) an rSV expressing the Zaire ebolavirus (EBOV) glycoprotein (GP) and the NiV_M G protein (rSVV-EBOV-GP-NiVG) that demonstrated efficacy in NiV_M hamster (22) and African green monkey (Chlorocebus aethiops) (23) models; 7) a recombinant measles virus vector expressing the NiV_M G

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protein that had efficacy in the NiV<sub>B</sub> African green monkey model (24); and 8) a recombinant subunit vaccine based on the HeV G protein (sG<sub>HeV</sub>) that completely protected small animals against lethal HeV and NiV<sub>M</sub> infections (25–27) and was efficacious in the robust African green monkey model of HeV (28) and NiV<sub>M</sub> infection (29). Of 8 vaccines, the sG<sub>HeV</sub> vaccine is furthest along in evaluation; it has received licensure as a veterinary vaccine for HeV in horses (Equivac HeV, Zoetis, https://www.zoetis.com) in Australia and is being considered as a human vaccine against NiV. When tested against NiV, these 8 vaccine vectors have been tested only against NiV<sub>M</sub> infection in animal models, and although the antigenicity of these vaccines should not be a concern given that HeV G is an immunogen against NiV<sub>M</sub> infection, there are new data on the NiV<sub>B</sub> African green monkey model to consider as far as dose/regimen of vaccines.

NiV<sub>B</sub> infection in African green monkeys is more pathogenic than NiV<sub>M</sub> infection (30). This difference resulted in significantly reduced efficacy of antibody therapy because of temporal differences in viral load. Specifically, the human monoclonal antibody m102.4 that had been shown to completely protect African green monkeys against lethal NiV<sub>M</sub> disease when treatment was delayed until day 5 after virus exposure provided no protection when African green monkeys were challenged with NiV<sub>B</sub> and treated beginning at day 5 after virus challenge (30,31). Considering these new data, the current vaccines against NiV need to be evaluated for possible differences in dose/regimen against the more pathogenic NiV<sub>B</sub> infection in the robust African green monkey model. To assess single-dose vaccine efficacy, we evaluated the rVSV vaccine vectors expressing either the NiV<sub>B</sub> F or NiV<sub>B</sub> G proteins 28 days after a single-dose vaccination in the NiV<sub>B</sub> African green monkey model, which most faithfully recapitulates human disease (5,30).

Methods

rVSV Vaccine Vectors and NiV<sub>B</sub> Challenge Stock
We recovered the rVSV NiV<sub>B</sub> vaccines (rVSV-ΔG-NiV<sub>B</sub>/F-GFP and rVSV-ΔG-NiV<sub>B</sub>/G-GFP) and rVSV-ΔG-GFP using methods as previously described (21,32). The isolate of NiV<sub>B</sub> used in this vaccine study was obtained from a fatal human case (200401066) described previously (30).

Statistical Analyses
Animal studies in BSL-4 and nonhuman primate work generally restrict the number of animals used, the volume of biological samples that can be obtained, and the ability to repeat assays independently and thus limit statistical analysis. Consequently, we present these data as the mean calculated from replicate samples, not replicate assays, and error bars represent SD across replicates (Figure 1, panels B, C, and D).

Animal Ethics Considerations and Experiments
Healthy adult African green monkeys were handled in the animal BSL-4 containment space at the Galveston National Laboratory (Galveston, TX, USA). Research was approved under animal protocol 1310040 by the University of Texas Medical Branch Institutional Animal Care and Use Committee (Appendix, https://wwwnc.cdc.gov/EID/article/25/6/16-1820-App1.pdf).

We used 10 adult African green monkeys weighing 3.5–6.0 kg in this study. One animal served as control (received G<sub>M</sub>* rVSV-ΔG-GFP), and 3 animals per vaccine group received G<sub>*</sub> rVSV-ΔG-NiV<sub>B</sub>/F-GFP, G<sub>*</sub> rVSV-ΔG-NiV<sub>B</sub>/G-GFP, or rVSVΔG-NiV<sub>B</sub>/F/G. For vaccination, animals were anesthetized with ketamine and vaccinated with ≈10<sup>7</sup> PFU by intramuscular injection (day −28). Twenty-eight days after vaccination, the animals were exposed to ≈5×10<sup>4</sup> PFU of NiV<sub>B</sub>; the dose was equally divided between the intratracheal and the intranasal routes for each animal. Animals were monitored for clinical signs of illness (i.e., temperature, respiration quality, blood count, and clinical pathologic findings) at 0, 3, 6, 8, 10, 15, 21, and 28 days postchallenge (dpc).

NiV<sub>B</sub> Serum Neutralization Assays
We determined neutralization titers against NiV<sub>B</sub> using a conventional serum neutralization assay. In brief, we serially diluted serum 5-fold or 2-fold depending on magnitude of neutralization titers and incubated with ≈100 PFU of NiV<sub>B</sub>; the dose was equally divided between the intratracheal and the intranasal routes for each animal. Animals were monitored for clinical signs of illness (i.e., temperature, respiration quality, blood count, and clinical pathologic findings) at 0, 3, 6, 8, 10, 15, 21, and 28 days postchallenge (dpc).

RNA Isolation from NiV<sub>B</sub>-Infected African Green Monkeys
We isolated RNA from NiV<sub>B</sub>-infected animals as described previously (30). For viremia, we added 100 µL of blood to 600 µL of AVL viral lysis buffer (QIAGEN, https://www.qiagen.com) for RNA extraction. For virus load in tissue, we stored ≈100 mg in 1 mL RNAlater (QIAGEN) for 7 d to stabilize RNA, removed the RNA later completely, and homogenized tissues in 600 µL RLT buffer (QIAGEN) in a 2-mL cryovial using a tissue lyser (QIAGEN) and ceramic beads.

Detection of NiV<sub>B</sub> Load
We isolated RNA from blood or tissues and assessed it using primers and probe targeting the N gene and the intergenic region between N and P genes of NiV<sub>B</sub> for quantitative reverse transcription PCR (qRT-PCR). The probe used was 6FAM-5′CGT CAC ACA TCA GCT CTG ACA -30 T called TAMRA (Life Technologies, https://www.thermo.com) in the infected African green monkey model to consider as far as dose/regimen of vaccines.

Hematology and Serum Biochemistry
We assessed clinical pathology of NiV<sub>B</sub>-infected African green monkeys by hematology and serum biochemistry
analysis as described previously (30). We performed the hematology assays using a laser-based hematologic analyzer (Beckman Coulter, https://www.beckmancoulter.com) and serum biochemistry analysis using a Piccolo point-of-care analyzer and Biochemistry Panel Plus analyzer discs (Abaxis, https://www.abaxis.com).

**Histopathology and Immunohistochemistry**

We performed necropsies on all animals and collected tissue samples of all major organs. We performed histopathologic and immunohistochemical examination and analyses as described previously (30).

**Results**

**Immunization of African Green Monkeys and Measuring the Humoral Immune Response**

Previously, single-injection, single-round replication rVSV vaccine vectors expressing the NiV F or NiV G proteins were described, characterized, and shown to be efficacious against NiV challenge in ferrets (21). To assess the efficacy of these vectors in the NiV African green monkey model, 4 groups of African green monkeys received a single intramuscular vaccination of rVSV vectors on day –28 (Figure 2). To analyze the antibody response to rVSV-ΔG-NiV vaccinations, we assessed circulating antibodies for neutralization activity against NiV before and after vaccination by using a 50% plaque-reduction neutralization titer (PRNT<sub>50</sub>) assay. All 4 groups had no detectable neutralizing antibody titers before vaccination (Table 1, day –28). On the day of challenge, the control animal (C-1) did not have detectable neutralizing antibody titers against NiV, whereas all animals from the specific NiV protein vaccination groups (F, G, and F/G) had detectable neutralizing antibodies against NiV (Table 1, day 0). Overall, the detectable neutralizing antibody response against NiV reached a 1:640 dilution titer in the G and F/G groups and from 1:160 to 1:640 in the F group.

**NiV Challenge and Viral Load of Vaccinated African Green Monkeys**

To determine the efficacy of the rVSV-ΔG-NiV vectors against NiV disease in African green monkeys, we challenged these animals by combined intratracheal and intranasal routes with a lethal challenge dose of NiV on day 0 (Figure 1). All African green monkeys were closely monitored for up to 28 dpc for clinical signs of illness. The NiV antigen vaccinated animals in the F (F-1–3), G (G-1–3), and F/G (F/G-1–3) groups showed no signs of clinical illness (Table 2) and were 100% protected against NiV challenge (Figure 1, panel A), whereas the animal in the nonspecific vaccinated control group (C-1) exhibited clinical signs of disease (Table 2). In addition, the control animal was the only NiV-infected animal to have lymphopenia and serosanguinous nasal discharge during the course of disease (Table 2).

To determine the level of NiV replication in animals after challenge, we assessed viral load by qRT-PCR on
nasal and oral swab samples and whole blood samples (Figure 1, panels B–D). We detected NiV\(_{\beta}\) genome equivalents (GEq) from nasal swab samples (Figure 1, panel B) in the control, F, G, and F/G groups. The following animals were positive for viral RNA: C-1 at 6 dpc; F-1 at 3 dpc; F-2 at 3, 6, and 10 dpc; G-1 at 3 dpc; G-3 at 6 dpc; F/G-1 at 3, 6, and 8 dpc; and F/G-3 at 3 dpc. At 6 dpc, when C-1 was positive for NiV RNA in nasal swab samples, the levels were >1 log higher than they were for the NiV-antigen vaccinated groups F, G, and F/G. Oral swab samples were negative for NiV RNA in all animals in the G-vaccinated group (Figure 1, panel C), and NiV\(_{\beta}\) GEq were detected from oral swab samples in the control, F, and F/G groups. The following animals were positive for viral RNA: C-1 at 3 and 6 dpc, F-1 at 3 dpc, and F/G-1 at 3 and 6 dpc. Within these oral swab sample results, C-1 had NiV RNA levels up to 100-fold higher than the F and F/G animals that had positive oral swab samples (Figure 1, panel C). Unlike the results for swab samples, which represent tissues initially exposed to NiV, systemic and circulating detection of NiV\(_{\beta}\) GEq were not detected in whole blood from animals in the F, G, and F/G groups, whereas the control animal was positive in the blood sample from 6 dpc (Figure 1, panel D). The lack of systemic and circulating detection of NiV\(_{\beta}\) RNA correlated with survival (Table 2; Figure 1, panel A).

### Gross Pathologic, Histopathologic, and Immunohistochemical Analyses of NiV\(_{\beta}\)-Infected African Green Monkeys

In the F, G, and F/G groups, we observed no gross pathologic findings at study endpoint. However, in the control animal that died of NiV\(_{\beta}\) infection, gross pathologic findings included serosanguinous pleural effusion, failure of all lung lobes to collapse with severe pulmonary hemorrhage and congestion, and multifocal to coalescing hemorrhage of the mucosal surface of the urinary bladder.

Lung sections examined from the control animal had moderate lymphoplasmacytic interstitial pneumonia characterized by a diffuse thickening of alveolar septae by moderate numbers of lymphocytes, plasma cells, polymerized fibrin, and edema fluid. The alveolar spaces were flooded by edema fluid, polymerized fibrin, foamy alveolar macrophages, and cellular debris. Endothelial syncytial cells were most apparent in medium- to small-caliber vessels (Figure 3, panel A). The animals in the F, G, and F/G groups had no major histologic findings in the lung sections (Figure 3, panels C, E, G). Immunohistochemical analysis revealed strong NiV antigen immunoreactivity within scattered alveolar macrophages and the endothelium of the alveolar septae and syncytial cells within medium- to small-caliber vessels in up to ≈75% of the examined pulmonary tissues (Figure 3, panel B). The lung sections of the F, G, and F/G groups were devoid of detectable NiV antigen (Figure 3, panels D, F, H).

Spleen sections from the control animal were depleted of lymphocytes in the multifocal follicular germinal centers within the splenic white pulp and were effaced by hemorrhage, fibrin, syncytial cell formation (Figure 4, panel A). Spleens from the F, G, and F/G groups had no major histologic findings (Figure 4, panels C, E, G). Immunohistochemical analysis of the spleen from the

### Table 2. Clinical findings and outcome of Nipah virus Bangladesh strain–challenged African green monkeys*

<table>
<thead>
<tr>
<th>Animal no.</th>
<th>Sex</th>
<th>Group</th>
<th>Clinical Illness</th>
<th>Clinical and gross pathology findings†</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-1</td>
<td>F</td>
<td>Control ΔG vaccine</td>
<td>Loss of appetite (d 6–8); labored breathing (d 6–8). Died on d 8.</td>
<td>Lymphopenia (d 6); serosanguinous nasal and oral discharge (d 8); serosanguinous pleural fluid, severely inflamed, enlarged lungs with severe congestion and hemorrhage of all lobes, multifocal to coalescing hemorrhage of the mucosal surface of the urinary bladder.</td>
</tr>
<tr>
<td>F-1</td>
<td>F</td>
<td>F vaccine</td>
<td>None</td>
<td>Thrombocytopenia (d 15); &gt;3 fold increase in ALT (d 6), &gt;3 fold increase in AST</td>
</tr>
<tr>
<td>F-2</td>
<td>M</td>
<td>F vaccine</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>F-3</td>
<td>M</td>
<td>F vaccine</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>G-1</td>
<td>F</td>
<td>G vaccine</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>G-2</td>
<td>M</td>
<td>G vaccine</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>G-3</td>
<td>M</td>
<td>G vaccine</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>F/G-1</td>
<td>F</td>
<td>F + G vaccine</td>
<td>None</td>
<td>Increase in CRP (d 8)</td>
</tr>
<tr>
<td>F/G-2</td>
<td>M</td>
<td>F + G vaccine</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>F/G-3</td>
<td>M</td>
<td>F + G vaccine</td>
<td>None</td>
<td>Thrombocytopenia (d 8)</td>
</tr>
</tbody>
</table>

*ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRP, C-reactive protein.
†Lymphopenia is defined as a ≥30% decrease in number of lymphocytes; thrombocytopenia is defined as a ≥30% decrease in number of platelets.
control animal revealed strong immunoreactivity for NiV antigen within the endothelium, syncytial cells, and scattered mononuclear cells in up to ≈50% of the examined splenic tissue (Figure 4, panel B), whereas the spleen sections of groups F, G, and F/G were devoid of detectable NiV antigen (Figure 4, panels D, F, H).

Discussion

An important step in the preclinical development of a vaccine is efficacy testing in standards of animal models of disease. For NiV, the standard is the African green monkey model. Although the initial studies on the NiV<sub>M</sub> model in African green monkeys were reported as near uniformly lethal, data from several groups have revealed the model is not 100% lethal, depending on dose and route of infection (5,24,29–31,33,34). Combining the control animals from these studies, in which African green monkeys were challenged with various combinations of routes (e.g., intratracheal, intranasal, intraperitoneal, oral, small particle aerosol) at various doses, revealed that 18 (72%) of 25 animals died; however, most of the control animals were positive for circulating NiV RNA and had signs of clinical disease to varying degrees. Historically, our previous studies with the NiV<sub>M</sub> model has resulted in the deaths of all 14 control African green monkeys; the mean time to death was 7.14 days (Figure 1, panel A). We recently compared the pathogenesis of NiV<sub>M</sub> and NiV<sub>B</sub> strains in African green monkeys and observed that NiV<sub>B</sub> caused more pulmonary and splenic pathologic findings (30). We also observed the efficacy of time to treatment post-NiV challenge with a human monoclonal antibody m102.4 was shorter for NiV<sub>B</sub>-infected animals than for NiV<sub>M</sub>-infected animals (30). With these animal data in mind and the fact that NiV<sub>B</sub> has been responsible for most NiV outbreaks since 2002, we wanted to test our rVSV NiV vaccine vectors expressing NiV<sub>B</sub> F and G proteins as immunogens, which had 100% efficacy against NiV<sub>M</sub> challenge in ferrets (21), against NiV<sub>B</sub> challenge in African green monkeys.

In this study, we vaccinated 1 control African green monkey with a nonglycoprotein rVSV vector control, G<sub>Ind</sub>*/ rVSV-ΔG-GFP, and 3 groups of 3 African green monkeys with NiV antigen vectors: G<sub>Ind</sub>*/ rVSV-ΔG-NiV<sub>B</sub>/F-GFP, G<sub>Ind</sub>*/ rVSV-ΔG-NiV<sub>B</sub>/G-GFP, or G<sub>Ind</sub>*/ rVSV-ΔG-NiV<sub>B</sub>/F/G-GFP. The control animal, C-1, did not develop NiV<sub>B</sub> neutralizing antibodies by the day of challenge; had detectable circulating NiV RNA at 6 dpc; had clinical signs of NiV-mediated disease; and ultimately died of infection, showing typical NiV gross pathology and histopathologic findings. Conversely, the 3 rVSV NiV vaccine groups had animals in which detectable circulating NiV F, G, or F and G IgG developed, and circulating neutralizing antibody titers developed in all 3 groups by 28 days postvaccination. Each vaccine cohort had detectable NiV<sub>B</sub> RNA in nasal swab samples and only the F and F/G groups in oral swab samples, but none of the cohorts had any detectable circulating NiV<sub>B</sub> RNA throughout the course of the study. Consistent with the vaccine response from each cohort and the control of systemic spread of NiV<sub>B</sub> infection and control of NiV-mediated disease, all of the specifically vaccinated African green monkeys survived NiV<sub>B</sub> challenge.

The results of this study are similar to what we observed with these rVSV NiV constructs in the ferret model, which showed 100% protection regardless of the vaccine construct (21). Differences were that we found higher PRNT<sub>50</sub> results for neutralizing antibody titers on day of challenge in this study and detected no circulating NiV RNA in the African green monkeys but did have detectable viral RNA at 6 dpc in the ferret study. Although we
did not detect circulating viral RNA in the African green
monkeys, the increase of neutralizing antibody titers at
the study endpoint suggests sterilizing immunity was not
achieved, and dosing or regimen will require further test-
ing to reach sterilizing immunity with this single-round
replication vaccine vector.

The single-round replication rVSV NiV vectors in
this study and the replication-competent rVSV-EOBV-
GP-NiVG (23) are the only vaccine vectors to show
100% single-dose vaccine efficacy against NiV in the Af-
rican green monkey model. Although both studies used
this model, they differed in several ways. Our study used
NiV\textsubscript{B} and challenged through the intratracheal and intra-
nasal routes, whereas the other study used NiV\textsubscript{M} by the in-
tratracheal route only (intratracheal challenge route used
in initial model [5]). Here, we report detectable levels of
NiV RNA in nasal swab samples at early times postchal-
lenge, whereas the rVSV-EOBV-GP-NiVG study did not
report any detectable NiV RNA in nasal swab samples.
Whether these differences resulted from use of the intra-
nasal route as part of the challenge cannot be determined
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report any detectable NiV RNA in nasal swab samples.
In summary, we found that single-round replication rVSV vectors against NiV provided 100% efficacy against NiV challenge using a single-dose regimen. The rVSV vaccine platform has received attention recently because the replication-competent rVSV-ZEBOV GP vaccine vector against EBOV has now been given to >16,000 humans in clinical trials ranging from phase 1 to phase 3 and has been safe and efficacious (36); however, data for pregnant women and immunocompromised persons are not yet available. A single-round replication rVSV vaccine vector that is immunogenic and efficacious would have an attractive safety profile. Whether these single-round replication rVSV NiV vaccine vectors are as safe as the recombinant subunit sG\textsubscript{HeV} vaccine has yet to be determined, and the subunit vaccine has yet to be tested with a single-dose vaccine regimen. Although multidose vaccine regimens would be a potential strategy for laboratory and healthcare workers and for first responders in stable settings with defined risk for an NiV outbreak, an outbreak setting or a case of deliberate release of NiV would require rapid protection with a single administration of vaccine. The single-dose strategy was successfully enacted using a close-contact ring vaccination strategy with the rVSV-ZEBOV-GP vaccine at the end of the 2013–2016 EBOV epidemic (37–39). The strategy was so successful that it became the World Health Organization recommendation for future EBOV outbreaks and has recently been set into motion in the ongoing outbreak in the Democratic Republic of the Congo (40). Recent studies also suggest that the ring vaccination strategy for viruses such as EBOV (depending on transmissibility) that are endemic to countries that might not be able to afford a mass herd-immunity vaccination strategy might be more effective than mass vaccinations at controlling outbreaks (41). Further studies should examine the time to immunity of the G\textsubscript{Ind}\textsuperscript{*} rVSV-ΔG-NiV\textsubscript{B}/G in the NiV\textsubscript{B} African green monkey model because these data will be instrumental in providing information about whether this vaccine vector could be implemented in a ring vaccination strategy during future NiV outbreaks, such as the current one in India (2).

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Figure 4. Results of testing for Nipah virus (NiV) in spleen tissue from representative vaccinated African green monkeys (Chlorocebus aethiops). A, C, E, G) Hematoxylin and eosin staining; B, D, F, H) immunohistochemistry of tissues labeled with NiV N protein–specific polyclonal rabbit antibody. In stained tissue from the control animal (A), moderate necrosis and drop out of the white pulp (*), with hemorrhage, and fibrin within germinal centers are seen; stained sections examined from the NiV F (C), NiV G (E), and NiV F/G (G) groups were devoid of any significant lesions compared with sections from the control animal. In antibody-labeled tissues from the control animal (B), strong immunolabeling for NiV antigen with scattered mononuclear cells (white arrow) and syncytial cells within germinal centers were found, and the endothelium of small caliber vessels had strong cytoplasmic immunolabeling for NiV antigen; no immunolabeling for NiV antigen was identified from the NiV F (D), NiV G (F), and NiV F/G (H) groups. Original magnification ×20.
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Appendix

Additional Methods

rVSV Vaccine Vectors and NiV* Challenge Stock

The rVSV NiV* vaccines (rVSV-ΔG-NiV*F-GFP and rVSV-ΔG-NiV*G-GFP) and rVSV-ΔG-GFP were recovered using methods as previously described (1,2). These rVSVΔG viruses were propagated on BHK-21 cells transiently expressing the VSV glycoprotein (G\textsubscript{Ind}) and were titered as previously described (2). Viruses complemented with VSV G\textsubscript{Ind} are denoted as G\textsubscript{Ind}* rVSV-ΔG-GFP, G*rVSV-ΔG-NiV*B-F-GFP, and G* rVSV-ΔG-NiV*B-G-GFP. To make the vaccine in the F/G group, as previously described Vero 76 cells were co-infected with G*rVSV-ΔG-NiV*B-F-GFP and G* rVSV-ΔG-NiV*B/G-GFP at MOI 5 for each virus (1). Supernatants were collected 24 h post infection (h.p.i.) and titered on BHK-21 cells transiently expressing VSV G\textsubscript{Ind}.

The isolate of NiV*B used in this vaccine study was an isolate obtained from a fatal human case, 200401066 (kindly provided by Dr. Thomas G Ksiazek), as described previously (3).

Statistics

Animal studies in BSL-4 and NHP work generally restricts the number of animal subjects, the volume of biologic samples that can be obtained, and the ability to repeat assays independently and thus limit statistical analysis. Consequently, data are presented as the mean calculated from replicate samples, not replicate assays, and error bars represent the SD across replicates.
Animal Ethics and Experiments

Healthy, adult African green monkeys (AGMs) were handled in the animal BSL-4 containment space at the Galveston National Laboratory (GNL), Galveston, Texas. Research was approved under animal protocol 1310040 by the University of Texas Medical Branch (UTMB) Institutional Animal Care and Use Committee (IACUC). Animal research at UTMB was conducted in compliance with the Animal Welfare Act and adhered to the guidelines in the eighth edition of the Guide for the Care and Use of Laboratory Animals (4). This facility is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All steps were taken to ameliorate the welfare and to avoid the suffering of the animals in accordance with the “Weatherall report for the use of nonhuman primates” recommendations (royalsociety.org/-/media/Royal_Society_Content/policy/publications/2006/Weatherall-Report.pdf). Animals were housed in adjoining individual primate cages allowing social interactions, under controlled conditions of humidity, temperature, and light (12-h light/12-h dark cycles). Food and water were available ad libitum. Animals were monitored (pre- and post-infection) and fed commercial monkey chow, treats, and fruit twice daily by trained personnel. Environmental enrichment consisted of commercial toys. All procedures were conducted by trained personnel under the oversight of an attending veterinarian, and all invasive clinical procedures were performed while animals were anesthetized using ketamine or telazol. Endpoint criteria was specified and approved by the UTMB IACUC. Animals were euthanized using a pentobarbital-based euthanasia solution.

Ten adult AGMs weighing 3.5–6.0 kg were used in this study with 1 AGM control (received G_ind* rVSV-ΔG-GFP) and 3 AGMs per vaccine group (received either G*/rVSV-ΔG-NiV_B/F-GFP, G*/ rVSV-ΔG-NiV_B/G-GFP, or rVSVΔG-NiV_B/F/G). For vaccination, animals were anesthetized with ketamine and vaccinated with \( \approx 10^7 \) plaque forming units (PFU) by i.m. injection on day –28. Twenty-eight days post-vaccination, AGMs were exposed to \( \approx 5 \times 10^5 \) PFU of NiV_B with the dose being equally divided between the i.t. and the intranasal i.n. routes for each animal. After challenge, animals were monitored for clinical signs of illness, including temperature, respiration quality, blood count, and clinical pathology on days 0, 3, 6, 8, 10, 15, 21, and 28 post-challenge.
**NiV<sub>B</sub> Serum Neutralization Assays**

Neutralization titers against NiV<sub>B</sub> were determined by a conventional serum neutralization assay. Briefly, sera were serially diluted 5-fold or 2-fold, respectively, and incubated with $\approx$100 PFU of NiV<sub>B</sub> for 1 h at 37°C as done previously (3). Virus and antibodies mixtures were then added Vero cells, and after 48 h plates were stained with neutral red and plaque forming units were counted 24 h 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.

**RNA Isolation from NiV<sub>B</sub>-Infected AGMs**

On procedure days, 100 μL of blood was added to 600 μL of AVL viral lysis buffer (Qiagen) for RNA extraction. For tissues, $\approx$100 mg was stored in 1 mL RNAlater (Qiagen) for 7 d to stabilize RNA. RNAlater was completely removed, and tissues were homogenized in 600 μL RLT buffer (Qiagen) in a 2-mL cryovial using a tissue lyser (Qiagen) and ceramic 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, brachial plexus, 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 were inactivated in AVL viral lysis buffer, and tissue samples were homogenized and inactivated in RLT buffer before removal from the BSL-4 laboratory. Subsequently, RNA was isolated from blood and nasal and oral 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.

**Detection of NiV<sub>B</sub> Load**

RNA was isolated from blood or tissues and assessed using primers/probe targeting the N gene and intergenic region between N and P of NiV<sub>B</sub> for reverse transcription quantitative PCR (RT-qPCR) with the probe used here being 6FAM-5’CGT CAC ACA TCA GCT CTG ACA A 3’-6TAMRA (Life Technologies, Carlsbad, CA, USA) as described previously (3). This strategy uses the intergenic region which only allows for genome and anti-genome detection without
detecting contaminating viral mRNA. NiV_B RNA was detected using the CFX96 detection system (Bio-Rad) in One-step probe RT-qPCR kits (Qiagen) with the following cycle conditions: 50°C for 10 min, 95°C for 10 sec, and 40 cycles of 95°C for 10 sec and 59°C for 30 sec. Cycle threshold (Ct) values representing NiV_B genomes were analyzed with CFX Manager Software, and data are presented as GEq. To generate the GEq standard curve, RNA from NiV_B challenge stocks was extracted, and the number of genomes was calculated using Avogadro’s number and the molecular weight of the NiV_B genome.

**Hematology and Serum Biochemistry**

Prior to the study, baseline blood and sera were collected from all 10 AGMs on days −28, 0, 3, 6, 8, 10, 15, 21, and 28 post-challenge for analysis. Hematologic analysis including total leukocyte counts, leukocyte 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). Serum samples were tested for concentrations of albumin, amylase, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), γ-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).

**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 d in BSL-4. After 21 d, the formalin was changed out; specimens were removed from BSL-4, processed in BSL-2 and embedded in paraffin and sectioned at 5 μm thickness. For immunohistochemistry, specific anti-NiV immunoreactivity was detected using an anti-NiV N protein rabbit primary antibody at a 1:5000 dilution for 30 min. The tissue sections were processed for immunohistochemistry using the Dako Autostainer (Dako). Secondary antibody used was biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) at 1:200 for 30 min followed by Dako LSAB2 streptavidin-HRP (Dako) for 15 min. Slides were developed with Dako DAB chromagen (Dako).
for 5 min and counterstained with hematoxylin for 1 min. Non-immune rabbit IgG was used as a negative staining control.

References


