Nipah virus (NiV) and Hendra virus (HeV) are closely related, recently emerged paramyxoviruses that are capable of causing considerable morbidity and mortality in several mammalian species, including humans. Henipavirus-specific vaccines are still commercially unavailable, and development of novel antiviral strategies to prevent lethal infections due to henipaviruses is highly desirable. Here we describe the development of adeno-associated virus (AAV) vaccines expressing the NiV G protein. Characterization of these vaccines in mice demonstrated that a single intramuscular AAV injection was sufficient to induce a potent and long-lasting antibody response. Translational studies in hamsters further demonstrated that all vaccinated animals were protected against lethal challenge with NiV. In addition, this vaccine induced a cross-protective immune response that was able to protect 50% of the animals against a challenge by HeV. This study presents a new efficient vaccination strategy against henipaviruses and opens novel perspectives on the use of AAV vectors as vaccines against emergent diseases.

**Keywords.** Henipavirus; Vaccine; Humoral Immunity; AAV vectors.
induce a protective immune response [11–15]. This form of vaccination requires several injections of recombinant proteins coupled to adjuvants to achieve a significant immune response. Few other vaccination strategies have used recombinant viral vectors to directly express the G and/or F glycoproteins of NiV or HeV in vivo. In particular, recombiant vectors derived from vaccinia virus or canarypox virus were shown to induce a humoral response against the NiV G protein, which could protect hamsters and pigs, respectively, against a challenge with wild-type NiV [16, 17]. However, these vectors also encode for several other viral proteins that may cause undesired immune response in humans.

This study presents a new vaccination strategy that is based on the use of recombinant vectors derived from adeno-associated virus (AAV). AAV vectors are currently considered to be powerful tools for in vivo gene transfer and gene therapy. Indeed, these vectors are able to transduce a wide variety of tissues in vivo, leading to a strong and sustained expression of the transgene [18]. However, the use of AAV vectors for vaccination purposes has only recently been investigated in detail [19–25].

We have evaluated the ability of AAV vectors expressing the NiV G protein to induce a protective immune response. The comparison of 3 different AAV serotypes and 2 vaccination routes indicated that a single injection of an AAV8 vector expressing NiV G (AAV8-Niv.G) in mice induced a potent and long-lasting antibody (Ab) response, which was only modestly enhanced by a boost injection with an alternative AAV serotype. Translational studies in the hamster model, which is highly susceptible to Henipavirus infection [26], indicated that a single injection of the AAV8-Niv.G vector was sufficient to protect the hamsters against a lethal challenge with NiV. In addition, 50% of the animals also survived a challenge with HeV, thus indicating the induction of cross-protecting immune responses. These findings open new perspectives for the development of AAV-vector vaccines against henipaviruses.

**MATERIALS AND METHODS**

**Cell Lines and Viruses**

Human epithelial cells (HeLa and HEK-293), and African green monkey fibroblasts (Vero) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 1% penicillin-streptomycin (5000 U/mL). NiV (isolate UMMC1; GenBank accession number AY029767) and HeV, obtained from Porton Down Laboratory (Porton Down, United Kingdom), were prepared at the INSERM Jean Mérieux biosafety level 4 (BSL4) laboratory (Lyon, France) by infecting Vero cells.

**Plasmids**

The pAAVhCMV-mSEAP and the pAAV-GFP vector plasmids encode for the mouse-secreted embryonic alkaline phosphatase (mSEAP) and the enhanced green fluorescent protein (eGFP) complementary DNAs, respectively, driven by the cytomegalovirus (CMV) promoter. The pAAVhCMV-NiV.G vector plasmid was obtained by inserting the NiV.G expression cassette derived from the phCMV-NiV.G plasmid into the SSV9 plasmid. The phCMV-NiV.Gs plasmid encodes for a soluble NiV G (Gs) protein.

**AAV Vector Production**

Stocks of AAV vector particles were generated by calcium phosphate transfection of HEK-293 cells as described previously [27]. The helper plasmids used for production were pXX6 [28], pDF1 [29], pDG8 (a kind gift from P. Moullier, INSERM U649, France), and pAAVrh32.33 (a kind gift from J. Wilson and the University of Pennsylvania Vector core) [22]. The vector particles were purified on cesium chloride gradients, and the number of genome particles per milliliter was titrated by quantitative reverse transcription polymerase chain reaction (qRT-PCR).

**Animal Immunization and Challenge**

All animals were handled in strict accordance with good animal practice, as defined by the French national charter on the ethics of animal experimentation. BALB/c male mice were injected with AAV vectors either intramuscularly (2.10^{10} genome particles) or intradermally (1.10^{10} genome particles). Golden hamsters were immunized intramuscularly (6.10^{11} genome particles). For challenge with NiV or HeV, animals were housed in ventilated containers in the BSL4 laboratory and injected intraperitoneally with 10^4 particle-forming units of NiV or HeV. Animals were observed daily for the appearance of clinical signs (body weight, dyspnea, tremor, and limb paralysis) and immediately euthanized in case of symptoms. Animals that survived were euthanized 29 days after challenge.

**Enzyme-Linked Immunosorbent Assay (ELISA)**

Stocks of soluble NiV G protein were produced by transfection of HEK-293 cells with the phCMV-NiV.Gs plasmid. For the ELISA, Ni-NTA plates (Qiagen) were coated overnight at 4°C with either soluble NiV G protein or control supernatant. Dilutions of sera were added for 2 hours at room temperature, followed by incubation with the secondary antibody for 1 hour at room temperature. Plates were then incubated with the 3′,3′,5,5′-tetramethylbenzidine substrate (Sigma-Aldrich), and the optical density was measured at 450 nm (OD_{450 nm}). The secondary antibodies (Southern Biotech) used were horse-radish peroxidase (HRP)–goat anti-mouse immunoglobulin G (IgG; 1/10 000), HRP-goat anti-mouse IgG1 (1/10 000), HRP-goat anti-mouse IgG2a (1/10 000), HRP-goat anti-hamster IgG (1/5000), HRP-mouse anti-hamster IgG1 (1/1 000), and HRP-mouse anti-hamster IgG2 (1/5000).
**Seroneutralization Assays**

**Neutralization Assay With NiV Virus-Like Particles (VLPs)**

NiV VLPs were produced as previously described by Szecsi et al [30]. Briefly, NiV VLPs were produced in 293T cells by transient transfection of plasmids pTG5349, pTG13077, phCMV-NiV.GΔ20, and phCMV-NiV.FA24 [31]. A plasmid coding for the vesicular stomatitis virus G protein was used to generate control VLPs. VLPs containing supernatants were harvested 48 hours after transfection, concentrated, and titered. For the neutralization assay, serial dilutions of sera were mixed with $1 \times 10^5$ VLPs, incubated for 1 hour at 37°C, and then added on HeLa cells. The transduction efficiency was determined 72 hours later by measuring the percentage of GFP-positive cells, using fluorescence-activated cell sorting analysis.

**Neutralization Assay With NiV and HeV in the BSL4 Laboratory**

NiV and HeV neutralizing Ab (NAb) were assayed using 2-fold dilutions of serum, as described previously [32]. The cytopathic effect was revealed by staining cells with crystal violet, and titers were defined as the reciprocal of the last dilution that completely inhibited the cytopathic effect.

**qRT-PCR Analyses**

RNAs were purified from tissues in accordance with the manufacturer’s instructions (RNeasy Mini kit, Qiagen), treated with Turbo DNaseI (Ambion, Life Technologies), and reverse transcribed using the qScript complementary DNA Super Mix (Quanta Biosciences). Total genomic DNA was extracted from tissue, using the Wizard SV Genomic DNA Purification System (Promega). The qRT-PCR reaction was conducted on 10 ng of complementary DNA or genomic DNA, using the FastStart Universal SYBR Green Master reagent (Roche Diagnostics). qRT-PCR was run on the Step One Plus Real Time PCR system (Applied Biosystems). The sequence of the primers is available on request. All samples were run in duplicate, and results were analyzed using ABI StepOne software v2.1.

**Statistical Analyses**

Data are expressed as mean ± standard error of the mean. Statistical analyses were performed using the 2-tailed Student $t$ test or 1-way analysis of variance with the GraphPad software. Differences were considered statistically significant when $P < .05$.

**RESULTS**

**Characterization of AAV-NiV.G–Induced Humoral Immune Responses in Mice**

The first part of this study focused on the characterization of the AAV-induced anti-NiV G immune responses in mice to compare several vaccination conditions. The AAV serotypes chosen were AAV1, for its ability to efficiently transduce the muscle [33]; AAV8, for its broad tropism [34]; and AAVrh32.33, a recently isolated variant that demonstrated a superior capacity to induce cellular immune responses to the transgene product [22, 35]. For both intramuscular and intradermal injection routes, anti-NiV G Ab were detected 4–6 weeks after the priming injection (Figure 1A and 1B); levels peaked between 8 and 12 weeks but then did not significantly change until 16 weeks after priming, except for AAV1 and AAV8 injected intramuscularly and intradermally, respectively. In general, humoral responses generated after intramuscular injection were significantly lower than those observed after intramuscular injection, and only minor differences were observed among the 3 AAV serotypes. In contrast, AAV8 injected by the intramuscular route was superior to the 2 other serotypes in terms of kinetics and Ab level (Figure 1A). Both IgG1 and IgG2a isotypes were detected for each AAV serotype independently of the injection route (Figure 1C and 1D).

To examine the possibility of enhancing the humoral response by a second AAV injection, animals primed with either AAV1 or AAV8 received a second injection of AAVrh32.33 at week 16. Interestingly, only a moderate increase of IgG or NAb titers was observed 4 weeks after the boost injection (Figure 1E and 1F). Also, the boost did not alter the dominant IgG2a response. Production of NAb against NiV followed the same kinetics, with the strongest and most rapid response obtained after injection of AAV8-NiV.G by the intramuscular route (data not shown).

**Analysis of Vector Persistence Over Time**

Persistence of expression of the AAV-encoded transgene may be important for the induction of a long-lasting immune response. Because generation of transgene-specific immunity may result in elimination of AAV-transduced cells, we analyzed the persistence of an AAV8-NiV.G vector over time and compared it to an AAV8 vector encoding GFP that was reported to persistently express the transgene over time [35]. The measurement of AAV-GFP DNA copies in the injected muscles indicated that, after an initial drop 2–6 weeks after injection, the number of AAV-GFP copies remained stable for at least 16 weeks (Figure 2A). As expected, follow-up evaluation of AAV-NiV.G DNA indicated increased elimination of vector DNA 6 weeks after injection, compared with AAV-GFP samples. However, interestingly, AAV-NiV.G DNA levels remained stable thereafter, and quantification of NiV G RNA 16 weeks after injection indicated that the transgene was still...
expressed at a significant level (Figure 2B). These results confirmed that AAV vector DNA was still present and continued to express the transgene 16 weeks after injection.

**Protection Against Henipavirus Infection in the Golden Hamster Model**

We next evaluated AAV vaccination in golden hamsters, an animal model currently used to study henipavirus pathogenesis [26, 32]. Animals were injected with the AAV8 NiV.G vector via the intramuscular route. The follow up of the anti-NiV G IgG response indicated a different kinetics than that observed in mice (Figure 3A), with an earlier onset of Ab production at 2 weeks, which peaked 4–6 weeks after injection, progressively declined until week 10, but remained stable thereafter. Moreover, as observed in mice, a dominant IgG2 response was induced (data not shown). The NAb levels before the challenge followed the same kinetics as the IgG levels. However the NAb levels remained stable until at least 12 weeks after injection, further suggesting the persistent induction of high-affinity NAb (Figure 3B).

To verify that the level of Ab responses achieved with the AAV vaccine was sufficient to protect the animals against
challenge with NiV, vaccinated animals were injected intraperitoneally with a lethal dose of wild-type NiV at 5 weeks after vaccination. All control animals, which were injected with AAV8-GFP, developed clinical signs of infection on day 5 after challenge. In contrast, 100% of vaccinated animals survived until the end of the experiment, without appearance of clinical signs (Table 1). Immunohistochemistry analysis confirmed the absence of detectable NiV antigens in the organs of vaccinated animals (Supplementary Figure 3).

Some studies have shown that in vivo delivery of a soluble HeV G protein can result in cross-protection against NiV [13–15]. In contrast, the NiV G protein is generally considered unable to induce cross-protective immunity against HeV [17, 32]. To evaluate this, animals vaccinated with AAV8-NiV.G were challenged with HeV. Follow up of the animals indicated that 3 of 6 survived the challenge (Figure 3D). As expected, none of the vaccinated animals had detectable anti-HeV cross-neutralizing NAb before challenge (Table 2). After challenge, only 1 of the 3 surviving animals (animal NiV.G-9) developed anti-HeV NAb. However, HeV RNA was not detected in the organs of any of the animals surviving the challenge (Table 2 and Figure 4B). Similarly, HeV antigens were not detected by immunohistochemistry analysis (Supplementary Figure S3). Together, these results strongly suggest that a single injection of the AAV8-NiV.G vector induced sterilizing immunity against NiV infection, as well as cross-protective immunity sufficient to protect 50% of the animals against a lethal challenge with HeV.

**DISCUSSION**

Strategies against henipavirus infection can be either preventive or therapeutic [36]. For viruses that cause sporadic outbreaks, therapeutic treatment after virus contact seems to be the most suitable strategy. Accordingly, recent efforts have focused on the development of a monoclonal antibody capable of recognizing the G glycoprotein of both NiV and HeV [37–39]. However, because henipaviruses can rapidly replicate and spread after entry, this strategy has a very narrow therapeutic window. Consequently, preventive vaccination remains a more suitable alternative, particularly to protect people who may be in contact with infected animals and susceptible livestock.

Vaccination strategies against henipavirus infection have mostly been based on repeated injections of high doses of HeV G protein mixed with adjuvants to induce a protective humoral response [13–15]. Alternative strategies based on viral vectors expressing henipavirus antigens have also been
reported, but except for canarypox virus, these vectors are not currently used in humans [16, 17, 40, 41]. Several arguments favor the use of AAV vectors as vaccines against henipaviruses. First, AAV vectors are derived from a nonpathogenic virus, and their lack of toxicity has been documented in several preclinical and clinical studies [18, 42]. Second, the wide tropism of AAV and the existence of several AAV serotypes allow efficient gene transfer via different

### Table 1. Neutralizing Antibody (NAb) Titers and Nipah Virus (NiV) Detection in Hamsters Challenged With NiV

| Animal | Time of Death, d | Titer Before Challenge | Titer After Challenge | NiV RNA qRT-PCR Result, by Organ |
|--------|-----------------|------------------------|-----------------------|----------------------------------|
|        |                 | 0 d                    | 5 d                  | 12 d                    | 29 d                  | Spleen | Lung | Brain |
| NiV.G-1| 29              | 1:40                   | 1:80                 | 1:80                   | 1:60                  | −      | −    | −     |
| NiV.G-2| 29              | 1:160                  | 1:160                | 1:120                  | 1:160                 | −      | −    | −     |
| NiV.G-3| 29              | <1:10                  | 1:10                 | 1:10                   | 1:160                 | −      | −    | −     |
| NiV.G-4| 29              | 1:120                  | 1:80                 | 1:80                   | 1:160                 | −      | −    | −     |
| NiV.G-5| 29              | <1:10                  | <1:10                | 1:40                   | 1:240                 | −      | −    | −     |
| NiV.G-6| 29              | <1:10                  | <1:10                | 1:20                   | 1:20                  | −      | −    | −     |
| GFP-1  | 5               | <1:10                  | <1:10                | ND                     | ND                    | ND     | +    | +     |
| GFP-2  | 5               | <1:10                  | <1:10                | ND                     | ND                    | ND     | +    | +     |
| GFP-3  | 5<sup>c</sup>   | <1:10                  | ND                   | ND                     | ND                    | ND     | ND   | ND    |
| GFP-4  | 5               | <1:10                  | <1:10                | ND                     | ND                    | ND     | +    | +     |
| GFP-5  | 5               | <1:10                  | <1:10                | ND                     | ND                    | ND     | +    | +     |
| GFP-6  | 5<sup>c</sup>   | <1:10                  | <1:10                | ND                     | ND                    | ND     | ND   | ND    |

Abbreviations: d, days after challenge; ND, not done; qRT-PCR, quantitative reverse transcription polymerase chain reaction; −, negative; +, positive.

<sup>a</sup> Animals were euthanized, unless otherwise indicated.

<sup>b</sup> Anti-NiV NAb titers were quantified in the INSERM Jean Mérieux biosafety level 4 laboratory, using a seroneutralization assay against NiV.

<sup>c</sup> Animal was found dead.
injection routes and in numerous, if not all animal species [43]. Finally, previous studies have suggested that a single injection of AAV vectors can result in a strong and sustained production of Ab against the transgene product [21, 22, 44–46].

The first part of our study was focused on the characterization of the AAV-induced humoral response. We found that the AAV8 vector injected intramuscularly was the most efficient for producing anti-NiV G Ab (Figure 1A and 1C). In both intramuscular and intradermal routes, the kinetics of anti-NiV G Ab was characterized by delayed detection 4–6 weeks after injection and by a peak production 8–12 weeks after injection. This unusual lag phase was previously documented in other studies [23, 24] and likely reflects the time required for the conversion of the single-stranded AAV vector genome into a double-stranded DNA form able to express the transgene [47]. The use of double-stranded AAV vectors, which can circumvent this rate-limiting step and accelerate the synthesis of the antigen, may improve the kinetics of Ab production. Another major characteristic of AAV-induced humoral response in mice was its detection at a high level for an extended period (Figure 1). Many groups have similarly documented a persistent AAV-induced humoral response [21, 45, 46]. Analysis of vector persistence and expression in transduced tissues suggested that the persistent Ab level may be due to continued expression of the transgene (Figure 2). In contrast, the decline in IgG levels 5 weeks after injection in hamsters could be due to reduced vector persistence in this animal species (Figure 3A). Future studies comparing a range of AAV doses in hamsters and mice will indicate whether these differences are linked to the host species or to the vector dose. Finally, we observed that a boost injection with an alternative AAV serotype in mice did not significantly enhance the Ab level (Figure 1E and 1F). In contrast, several studies have documented the possibility of boosting the AAV-induced humoral response by using an adenoviral vector [22–24]. It is possible that a booster effect could be observed only if boosting is performed at a later time, when the humoral response induced by the priming injection has declined. Additional studies should also be performed to determine whether these observations can be transposed to other animal species.

In the second part of our study, we analyzed whether a single AAV injection was sufficient to protect the animals

Figure 4. Detection of henipavirus RNA in tissues from immunized and control animals. Brain, lung, and spleen were collected from animals at the time of euthanasia (Tables 1 and 2). RNA was extracted and analyzed by quantitative reverse transcription polymerase chain reaction, using primers hybridizing either to Nipah virus (NiV; A) or Hendra virus (HeV; B) N gene. Samples were normalized by quantifying the number of CD8 (DNA) or GAPDH (complementary DNA) copies. Results are expressed as the relative number of NiV and HeV RNA copies per microgram of RNA. Numbers below the x-axis correspond to individual animals.

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against a lethal challenge with henipaviruses. All vaccinated animals were protected against a lethal challenge with NiV (Figure 3C). The absence of NiV antigens (Supplementary Figure 3) and RNA (Figure 4A and Table 1) in the tissues, as well as the moderate anamnestic response observed in only 1 of 6 animals (Table 1), strongly suggested that this mode of vaccination induced sterilizing immunity. So far, only few vaccination studies reported the ability to induce sterilizing immunity. In particular, variable results were obtained using a soluble HeV G protein, suggesting that only high, repeated doses of the antigen, coupled with adjuvants, may completely prevent virus replication [12–15]. The induction of sterilizing immunity after a single AAV injection may reflect a unique contribution made by this vaccine approach, which certainly deserves future investigations.

NiV and HeV G proteins are 83% homologous [48, 49]. This level of homology suggests that a cross-neutralizing response can be induced. Accordingly, Ab against the HeV G protein have been reported to cross-neutralize NiV [37, 38, 50]. In contrast, the NiV G protein is considered unable to induce an efficient cross-protective response in vivo. This notion is mostly supported by in vitro neutralization assays showing that anti-NiV G Ab do not efficiently neutralize HeV. However, the potential cross-protective effect of anti-NiV G responses against HeV has not been evaluated so far. In this study, we observed that 50% of the animals vaccinated with AAV-NiV.G survived after a challenge with HeV (Figure 3D). This interesting result suggests that a low level of cross-neutralizing Ab against HeV not detectable by the neutralization assay (Table 2) may be induced after vaccination. Alternatively, it is possible that protection is conferred by the cell-mediated immune response. Even though cellular responses against henipavirus glycoproteins have not been characterized so far, cellular and particularly CD8+ T-cell responses may be induced and play a role in protecting the animals. Accordingly, induction of an adaptive T-helper 1 immune response against NiV G was suggested to occur following injection of canarypox virus vectors encoding NiV G in pigs [17]. Interestingly, intramuscular injection of AAV8-NiV.G induced a dominant IgG2a response in mice and hamsters that was not observed with other AAV serotypes nor via the intradermal route (Figure 1C and 1D). This result suggests that this capsid serotype may induce an anti-NiV G T-helper 1–oriented immune response. A challenge performed at earlier time points or adoptive transfer of T cells should indicate the extent to which cellular responses against NiV G can protect against viral infection. These studies should include the AAVrh32.33 serotype, which was selected for its ability to induce a qualitatively and quantitatively superior cellular response [22].

Together, our results demonstrate that AAV vectors constitute a powerful genetic platform for the in vivo expression of henipavirus antigens that can be used to further explore the immunological basis of anti-henipavirus vaccines and to develop new prophylactic strategies.

### Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The
posted materials are not copublished. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflict of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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