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Recombinant Nipah Virus Vaccines Protect Pigs against Challenge

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Abstract

Nipah virus (NiV), of the family *Paramyxoviridae*, was isolated in 1999 in Malaysia from a human fatality in an outbreak of severe human encephalitis, when human infections were linked to transmission of the virus from pigs. Consequently, a swine vaccine able to abolish virus shedding is of veterinary and human health interest. Canarypox virus-based vaccine vectors carrying the gene for NiV glycoprotein (ALVAC-G) or the fusion protein (ALVAC-F) were used to intramuscularly immunize four pigs per group, either with 10^8 PFU each or in combination. Pigs were boosted 14 days postvaccination and challenged with 2.5×10^5 PFU of NiV two weeks later. The combined ALVAC-F/G vaccine induced the highest levels of neutralization antibodies (2,560); despite the low neutralizing antibody levels in the F vaccinees (160), all vaccinated animals appeared to be protected against challenge. Virus was not isolated from the tissues of any of the vaccinated pigs postchallenge, and a real-time reverse transcription (RT)-PCR assay detected only small amounts of viral RNA in several samples. In challenge control pigs, virus was isolated from a number of tissues ($10^{4.4}$ PFU/g) or detected by real-time RT-PCR. Vaccination of the ALVAC-F/G vaccinees appeared to stimulate both type 1 and type 2 cytokine responses. Histopathological findings indicated that there was no enhancement of lesions in the vaccinees. No virus shedding was detected in vaccinated animals, in contrast to challenge control pigs, from which virus was isolated from the throat and nose ($10^{2.9}$ PFU/ml). Based on the data presented, the combined ALVAC-F/G vaccine appears to be a very promising vaccine candidate for swine.

Disciplines

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Recombinant Nipah Virus Vaccines Protect Pigs against Challenge

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Nipah virus (NiV), of the family *Paramyxoviridae*, was isolated in 1999 in Malaysia from a human fatality in an outbreak of severe human encephalitis, when human infections were linked to transmission of the virus from pigs. Consequently, a swine vaccine able to abolish virus shedding is of veterinary and human health interest. Canarypox virus-based vaccine vectors carrying the gene for NiV glycoprotein (ALVAC-G) or the fusion protein (ALVAC-F) were used to intramuscularly immunize four pigs per group, either with 10^8 PFU each or in combination. Pigs were boosted 14 days postvaccination and challenged with 2.5×10^5 PFU of NiV two weeks later. The combined ALVAC-F/G vaccine induced the highest levels of neutralization antibodies (2,560); despite the low neutralizing antibody levels in the F vaccinees (160), all vaccinated animals appeared to be protected against challenge. Virus was not isolated from the tissues of any of the vaccinated pigs postchallenge, and a real-time reverse transcription (RT)-PCR assay detected only small amounts of viral RNA in several samples. In challenge control pigs, virus was isolated from a number of tissues ($10^{4.4}$ PFU/g) or detected by real-time RT-PCR. Vaccination of the ALVAC-F/G vaccinees appeared to stimulate both type 1 and type 2 cytokine responses. Histopathological findings indicated that there was no enhancement of lesions in the vaccinees. No virus shedding was detected in vaccinated animals, in contrast to challenge control pigs, from which virus was isolated from the throat and nose ($10^{2.9}$ PFU/ml). Based on the data presented, the combined ALVAC-F/G vaccine appears to be a very promising vaccine candidate for swine.

Nipah virus (NiV), a member of the family *Paramyxoviridae*, genus *Henipavirus*, emerged in Malaysia in 1998 as an etiological agent in an outbreak of severe febrile encephalitis in humans, with a clinical case mortality of 40%. The virus was isolated in 1999 from the cerebrospinal fluid of a human fatality. Although *Pteropus* bats are considered to be a reservoir of the virus (7), human infections in Malaysia were considered to be due to transmission of the virus from pigs (1). In the field, the infection in pigs may go unnoticed or cause respiratory disease and, rarely, encephalitis (porcine respiratory and encephalitis syndrome) (26). Retrospective investigations suggested that NiV could have been introduced into the swine population as early as 1996 or 1997 (10), but it was not recognized due to the nonspecific clinical signs, relatively low morbidity, and low mortality. The virus is on the list of agents that could be used in biological terrorism; at this time it is classified as a biosafety level 4 (BSL4) agent due to the unknown route of transmission to humans, high virulence in humans, and absence of any vaccine or treatment. NiV is closely related to Hendra virus (HeV), a second member and the type species of the *Henipavirus* genus (32).

Canarypox virus (ALVAC) vaccine vectors induce antibody and cytotoxic T-cell responses, critical in the immune defense against viruses, to vectored viral antigens in a range of mammalian species (12, 14, 25, 29). Replication of canarypox virus vectors is abortive in mammalian cells, eliminating the safety concerns that exist for vaccinia virus vectors. The canarypox virus infects mammalian cells and produces viral proteins, with the replication block occurring at the time of viral DNA synthesis (32). Licensed vaccines for dogs, cats, and horses are commercially available (2), and an ALVAC-vectored human immunodeficiency virus vaccine is entering phase III clinical trials (3, 10).

The NiV envelope proteins F (fusion) and G (glycoprotein) were chosen for vaccine development, based on work by Guillaume et al. (17) with a vaccinia virus-based recombinant vaccine expressing the NiV F and G proteins in golden hamsters and on knowledge of immunity to other paramyxoviruses. For example, antibodies against the measles virus F protein contribute to virus neutralization, likely by preventing fusion of the virus with the cell membrane at the time of virus entry (23). Antibodies against measles virus hemagglutinin (H), the attachment protein of the virus analogous to the Nipah virus G protein, are the most important neutralizing antibodies (11, 15). In addition, the F and G proteins may be involved in inducing the $CD8^+$ cytotoxic T-cell response to NiV, analogous to the role of measles virus proteins H and F (16). A previously developed NiV early-infection model in pigs (34) was used in the challenge part of the work.

The purpose of this study was to obtain preliminary data on the efficacy of a veterinary vaccine against NiV in target species by using for the first time in pigs the canarypox virus vector, which is proven and approved for commercial use in domestic animals. The aim of the vaccine was not only to prevent disease in animals but most importantly to prevent virus shedding, in order to protect the human population by breaking the chain

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of transmission and to stop virus spread in swine herds, especially in areas of endemicity. On the other hand, in areas where the virus is not endemic, where it may be introduced intentionally or by accident, the vaccination may be used in outbreak control, with emphasis on the rapid establishment of protective immunity in swine herds following vaccination. To meet the specific requirements, different optimal vaccination doses/regimens may need to be designed.

MATERIALS AND METHODS

Cells. African green monkey epithelial kidney cells (Vero 76), human kidney epithelial cells expressing the simian virus 40 large T antigen (293T), baby hamster kidney cells (BHK-21), and porcine turbinate cells (PT-K75) obtained from ATCC were maintained according to instructions.

Porcine peripheral blood mononuclear cells (PBMC) were obtained from the experimental animals at different time points during vaccination by blood collection from the right cranial vena cava into cell preparation tubes (Becton Dickinson and Co., Franklin Lakes, NJ), with processing according to the manufacturer's instructions. The cells were maintained in RPMI–10% fetal bovine serum–100 IU penicillin–100 μg/ml streptomycin (Pen/Strep)–4 mM L-glutamine (WISENT Inc., St. Bruno, Quebec, Canada).

Viruses. Human isolates of NiV and HeV were kindly provided by Thomas Ksiazek and Pierre Rollin, CDC, Atlanta, GA. NiV stocks for the animal experiments were prepared in porcine turbinate PT-K75 cell monolayers in T75 flasks (Corning Costar Corp., Corning, NY); the monolayers were infected with NiV at a multiplicity of infection (MOI) of 0.1 and incubated at 33°C for 72 h, or until 80% cytopathic effect was reached. Virus was harvested by cell freezing and thawing, including the supernatant, and clarified at 2,000 × *g* (4°C, 20 min). The titer of the original virus stock was $10^{7.1}$ PFU/ml on Vero 76 cells. An HeV original stock was prepared in Vero 76 cells, with a titer of $10^{7.5}$ PFU/ml.

Subsequent virus stocks for both viruses yielded titers in the range of $10^{5.5}$ to 10^{6} PFU/ml.

Virus plaque assay. The virus plaque assay was performed in 12-well plates (Corning Costar Corp.) with Vero 76 confluent monolayers. The virus inoculum (400 μl/well) was incubated on cells for 1 h at 33°C, 5% CO_2, and then replaced with 2 ml of 2% carboxymethylcellulose sodium salt (medium viscosity)–Dulbecco modified Eagle medium (Sigma Chemical Co., St. Louis, MO)–2% fetal bovine serum overlay and incubated at 33°C, 5% CO_2. The cells were fixed after 5 days with 4% formaldehyde and stained with 0.5% crystal violet–80% methanol–phosphate-buffered saline (PBS).

Vaccine vectors. The canarypox virus-based recombinant vaccine vectors (ALVAC) vCP2199, carrying the NiV glycoprotein (G) gene, and vCP2208, carrying the NiV fusion protein (F) gene, were developed and supplied by Sanofi Pasteur and Merial. For practical reasons in further work, the vaccine vectors were designated ALVAC-G and ALVAC-F, respectively. NiV RNA for development of the vaccine was kindly provided to Merial by Paul Rota, CDC, Atlanta, GA. Expression of the NiV F and G proteins was verified by fusion in cells coinfected with ALVAC-G and ALVAC-F and by Western blot assay of the infected cell lysates.

Coinfection of cells with two vaccine vectors carrying the NiV F and G proteins. Twenty four-well plates were seeded with Vero 76, PT-K75, or BHK-21 cells and (i) mock inoculated, (ii) inoculated with ALVAC-F, (iii) inoculated with ALVAC-F and ALVAC-G, or (iv) inoculated with ALVAC-G recombinant virus at an MOI of 10. The formation of syncytia was monitored starting at 24 h postinoculation.

Western blotting. BHK-21 cells in six-well plates were inoculated with either ALVAC-F or ALVAC-G at an MOI of 10 and incubated for 6, 24, 27, 30, and 48 h at 37°C, 5% CO_2. The supernatant was removed and assayed for lack of infectivity and, by electron microscopy, for the absence of virus particles. Washed cells were lysed with 200 μl/well of sodium dodecyl sulfate (SDS) gel loading buffer, and the lysate was sonicated and boiled for 5 min at 95°C. Following electrophoresis on a 10% resolving gel (SDS-polyacrylamide gel electrophoresis), the samples were transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA) by the semidry transfer system. The membranes were blocked in PBS–0.1% Tween 20–5% skim milk powder overnight at 4°C and then incubated with guinea pig anti-NiV serum (prepared as described previously [34] against live virus) and diluted 1:1,000 in PBS–0.1% Tween 20–5% skim milk powder for 1 h at room temperature. Immunoreactive bands were detected by enhanced chemiluminescence (ECL Plus Western blotting kit; GE Healthcare Biosciences Corp., Piscataway, NJ) with secondary rabbit anti-guinea pig horseradish peroxidase-conjugated antibodies (Sigma) diluted 1:10,000 in PBS–0.1% Tween 20–5% skim milk and developed with Lumigen PS-3 Acridan as a substrate. The resulting light was detected on autoradiography film (HyperfilmTM ECL; GE Healthcare Biosciences Corp.).

Animals. Twelve four-week-old crossbred Landrace female pigs were obtained from a high-health-status herd (free of *Mycoplasma hyopneumoniae* and porcine respiratory and reproductive syndrome virus; Sunnyside Colony LTD, Manitoba, Canada) and acclimatized for 1 week prior to the first vaccination. Four pigs for challenge control (pigs 39, 40, 41, and 42) were received at the age of 7 weeks and challenged following 1 week of acclimatization. Negative-control pigs (pigs C1, C2, and C3) were received at 5 to 7 weeks of age and euthanized shortly after arrival. Animal housing met BSL3 (vaccinated pigs) and BSL4 (challenged pigs) conditions, and all animal manipulations were approved by the Animal Care Committee of the Canadian Science Centre for Human and Animal Health and met Canadian Council on Animal Care guidelines. Sampling, vaccination, and inoculation of animals were done under inhalation anesthesia with Isoflurane.

Experimental design. (i) Vaccination and prechallenge sample collection schedule. Four pigs (pigs 35, 36, 37, and 38) were intramuscularly vaccinated with 1 ml of 10^{8} PFU of ALVAC-G per pig (group G), four (pigs 31, 32, 33, and 34) were intramuscularly vaccinated with 1 ml of 10^{8} PFU of ALVAC-F per pig (group F), and four (pigs 43, 44, 45, and 46) were intramuscularly vaccinated with 1 ml of 10^{8} PFU of ALVAC-G and 1 ml of 10^{8} PFU of ALVAC-F per pig (group F/G). The pigs were boosted 14 days postvaccination (dpv) with the same vaccine dose and route. Serum was collected before vaccination and at 7, 14, 21, and 28 dpv. Nasal washes and pharyngeal swabs were collected before vaccination and on dpv 28. The vaccination part of the experiment was done under BSL3 conditions, and the pigs were transferred prior to challenge to the BSL4 cubicle.

(ii) Challenge and sample collection schedule. The virus dose used for the challenge was based on work by Middleton et al. (24) and Weingartl et al. (34). Since the maximum virus titer obtained from the nasal or pharyngeal swabs in our infection studies was 10^{4} PFU/ml, the challenge dose was estimated to be within the range of a dose during natural exposure to the virus.

Vaccinated pigs were challenged intranasally by slowly injecting 1.5 ml of the inoculum into each nostril, with a total of 2.5×10^{5} PFU per pig at dpv 28. A total volume of 3 ml was administered in order to cover well the mucosal surface and with anesthetized pigs in a dorsal position to minimize the loss of inoculum.

The challenge control pigs were inoculated intranasally with the same dose of virus following a week of acclimatization. Nasal washes, pharyngeal swabs, and serum were collected prior to challenge (sampling on dpv 28 for vaccinated pigs), on sampling days, and on the day of euthanasia. Two pigs from each group were sampled at 1 and 3 days postinoculation (dpi), and two were sampled at 2 and 4 dpi to decrease the stress from anesthesia. Two pigs from each group were euthanized at 6 dpi, and two were euthanized at 7 dpi by exsanguination under inhalation anesthesia with Isoflurane.

The animals were observed daily for clinical signs by measuring their body temperature and assessing their alertness, willingness to stand up and move, food uptake, feces production, respiratory difficulties, gait, and body posture.

Sample collection. Blood from the right cranial vena cava was collected into serum separator tubes (Becton Dickinson Co., Sparks, MD) and processed, according to the manufacturer's instructions, for antibody and virus detection. Nasal washes (10 ml of PBS–0.5% bovine serum albumin (BSA)–Pen/Strep) and pharyngeal swabs (polyester fiber swabs; Becton Dickinson Co., Sparks, MD), immersed in 2 ml of PBS supplemented with Pen/Strep, were collected for virus isolation and real-time reverse transcription-PCR (rRT-PCR). After euthanasia, the following tissues were collected for virus isolation and rRT-PCR: brain, trigeminal ganglion, olfactory bulb, cerebrospinal fluid, spleen, nasal turbinates, trachea, lung, and submandibular and bronchial lymph nodes. Samples collected for pathology are listed in "Histology," below.

Virus isolation. Virus isolation was attempted from samples positive by rRT-PCR for the presence of viral RNA in a plaque assay format with Vero 76 cells in 12-well plates (Corning Costar Corp., Corning, NY), as described above, with samples prepared as follows. Tissue homogenates (10%, wt/vol) were prepared in cold, Ca^{++}- and Mg^{++}-free Dulbecco PBS (Sigma Chemical Co., St. Louis, MO) supplemented with 1% (vol/vol) Pen/Strep by homogenization either in a closed plastic bag in a Bagmixer "MiniMix" blender (Interscience Laboratories Inc., Weymouth, MA) for 30 s at 9 strokes/s or in a MixerMill stainless steel homogenizer (Retsch Inc., Newtown, PA) for 1 min at 30 Hz. The homogenized tissues were clarified by centrifugation (2,000 × *g*, 20 min). Prior to virus isolation from swabs, 500 μg/ml (final concentration) of gentamicin was added to the diluent following removal of the swab. The sample was then incubated at room temperature for 1 h and clarified at 1,550 × *g* for 10 min.

rRT-PCR. rRT-PCR was performed according to the method of Guillaume and others (18) with modifications (34) by using a SmartCycler (Cepheid; Fisher

Scientific). The probe and primers targeted the nucleocapsid (N) gene of NiV. All samples were run minimally in duplicate.

Neutralizing antibody titers. Neutralizing antibody titers against NiV in heat-inactivated sera (1 h, 56°C) were determined by the microtiter plaque reduction neutralization assay, as previously described (35), with Vero V-76 cells, 500 PFU/well, and a 1% carboxymethylcellulose overlay. Wells with 90% plaque reduction were considered positive for presence of NiV neutralizing antibodies. The sera were also tested for cross-neutralization of HeV.

Indirect NiV ELISA. Binary ethylenimine-inactivated, safety-tested, and sucrose gradient-purified NiV was used as an antigen in an enzyme-linked immunosorbent assay (ELISA) (4). Plates were coated with 0.42 μg of antigen per well, with PBS as a coating buffer. Swine antisera were serially diluted from 1:100 up to 1:51,200 in blocking buffer (5% skim milk–0.1% Tween in PBS). The bound antibody was detected with goat anti-swine immunoglobulin G (IgG)/IgM horseradish peroxidase-conjugated antibodies (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD) and 3% hydrogen peroxide solution with ABTS [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid)] as a substrate. Values for the optical density at 405 nm (OD_{405}) of higher than 0.200 were considered positive for the presence of anti-NiV antibodies, based on cutoff values established with sera from pigs not infected with NiV.

Immunofluorescence detection of anti-F and anti-G antibodies. 293T cells in 96-well plates coated with poly-D-lysine were transfected at 80% confluence with 0.25 μg of plasmid DNA carrying the gene for the NiV F or G protein and the gene for green fluorescent protein (GFP) (either pCZCFG5-NivG or pCZCFG5-NivF, or pCZCFG5 as a control) by using Lipofectamine 2000 (Invitrogen Corp.) according to the manufacturer's instructions. The efficacy of transfection was determined 24 h later by fluorescence microscopy detection of the GFP. Cells were fixed with 10% buffered formalin (Fisher Scientific) containing 0.6% Triton X-100 for 30 min at 37°C, blocked for 30 min with PBS–1% BSA–0.5% Tween 20 at 37°C, and probed with serial dilutions of swine sera in blocking buffer starting at 1:20 for 1 h at room temperature. The bound antibodies were detected with biotin-streptavidin-conjugated goat anti-swine IgG (H+L) (Jackson ImmunoResearch Inc., West Grove, PA), diluted 1:2,000 in blocking buffer, by incubation for 1 h at room temperature and an additional 1 h with the streptavidin-phycoerythrin conjugate (Calbiochem, EMD Biosciences Inc., San Diego, CA), also diluted 1:2,000 in blocking buffer. Cells were washed three times with PBS–1% BSA–0.5% Tween 20 between all steps and prior to examination under the fluorescent microscope.

Cytokine detection. Interleukin 10 (IL-10), gamma interferon (IFN-γ), and tumor necrosis factor alpha (TNF-α) ELISAs were performed with commercially available kits from Biosource International Inc., Camarillo, CA, according to the manufacturer's instructions, by using cell culture supernatants from PBMC cultivated for 48 h under the conditions described below. The amounts of the cytokines were determined based on a standard curve.

PBMC were harvested, as described in "Cells," above, prior to immunization (nonstimulated and stimulated cells) and 14 days postimmunization prior to the boost (restimulated cells). Cells (5×10^5/well) (in duplicates or triplicates per treatment, depending on the success of the harvest from individual animals) in microtiter plates were treated as follows.

Cells harvested prior to vaccination were (i) cultivated with no treatment to determine baseline cytokine production in the animals and (ii) stimulated (cultivated) with 2.5×10^5 PFU of NiV per well to determine the stimulation of cytokine production due to NiV infection.

Cells harvested 14 dpv were cultivated with 2.5×10^5 PFU of NiV per well. These cells were primarily exposed in vivo to the NiV F and/or G antigens in the canarypox virus context and restimulated in vitro for these antigens in the NiV context. They were thus considered restimulated for the respective specific antigens F and/or G.

Control stimulation with 5 μg/ml (final concentration) of concanavalin A (Sigma Chemical Co.) at 0 and 14 dpv resulted in no difference in cytokine production between different vaccine groups. Following the stimulation, mean (from all vaccinees) TNF-α levels were 125 pg/ml at 0 dpv and 223 pg/ml at 14 dpv; mean IFN-γ levels were 25.5 and 32 pg/ml, respectively; and mean IL-10 levels were 71 and 90 pg/ml, respectively. The control stimulation with concanavalin A confirmed cell viability and stimulation.

Control stimulation with 2.5×10^5 PFU of the respective canarypox virus vectors for each vaccine group was done for all vaccinees at 0 dpv. In addition, cells from ALVAC-F-vaccinated animals were restimulated at 14 dpv with the ALVAC-G vector and vice versa, to determine immune cell priming and memory by the canarypox virus vector itself.

Histology. Tissues fixed in 10% formalin for 5 days were trimmed inside the BSL4 cubicle and fixed for an additional 24 h before being removed from containment. Histologic sections stained with hematoxylin and eosin were prepared according to standard protocols. The histologic sections were evaluated without knowledge of vaccination status, with the exception of the tissues from the three uninfected pigs, for which the treatment status was known. The following tissues from each pig were examined: brain (olfactory bulb, cerebral cortex, basal nuclei, thalamus, midbrain, cerebellum, medulla with pons, and obex), optic nerve, lacrimal gland, lymph nodes (submandibular, bronchial, mesenteric, and inguinal), palatine tonsil, spleen, tongue, nasal turbinate, ethmoid turbinate, trachea, lung, kidney, and bone marrow.

Each histologic section was graded from 0 to 4 as follows: 0, no lesions; 1, equivocal lesions; 2, mild lesions; 3, moderate lesions; 4, severe lesions. Mean histologic scores were calculated for each pig. The mean histologic score for brain samples was based on lesions of lymphocytic meningitis and encephalitis for each of the nine areas of the brain evaluated. The mean histologic score for

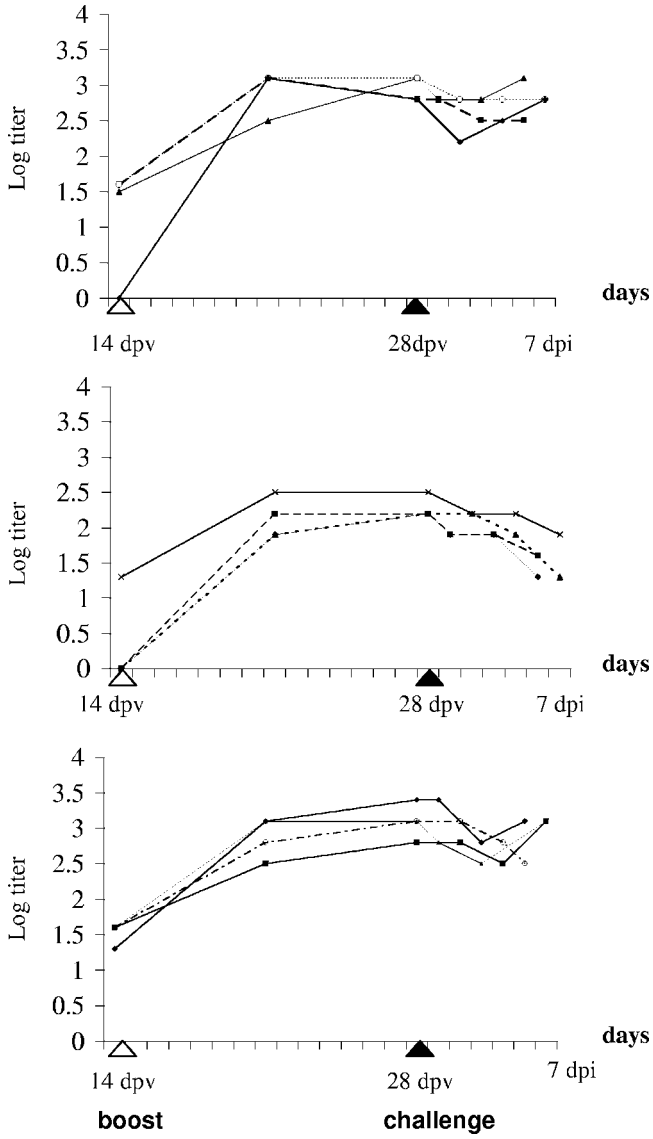

FIG. 1. Neutralizing antibody response following vaccination and challenge. Antibody titers in sera collected by serial bleeding of individual animals are plotted separately against the day of serum collection. (Top) Logarithms of neutralizing antibody titers for pigs vaccinated with ALVAC-G, starting at dpv 14. (Middle) Logarithms of neutralizing antibody titers for pigs vaccinated with ALVAC-F, starting at dpv 14. (Bottom) Logarithms of neutralizing antibody titers for pigs vaccinated with a combination of ALVAC-G and ALVAC-F, starting at dpv 14. Statistical analysis was not attempted due to the small numbers of animals per group.

TABLE 1. Comparison of neutralization activities of anti-NiV G and anti-NiV F antibodies against HeV and titers of anti-NiV F and anti-NiV G antibodies as detected by IFA on transfected cells expressing the respective genes

Time point	Target	Titer of antibodies elicited by the indicated vaccine in the indicated pig											
		ALVAC-F				ALVAC-G				ALVAC-F/G			
		31	32	33	34	35	36	37	38	43	44	45	46
Neutralization													
28 dpv	NiV	200	200	200	400	640	1,280	1,280	1,280	1,280	1,280	640	1,280
28 dpv	HeV	0	0	50	0	0	20	0	0	80	80	80	80
Immunofluorescence													
28 dpv	Anti-F	2,560	2,560	2,560	2,560	0	0	0	0	2,560	2,560	1,280	2,560
6/7 dpi	Anti-F	640	640	640	1,280	0	0	0	0	1,280	1,280	320	640
28 dpv	Anti-G	0	0	0	0	1,280	2,560	2,560	2,560	2,560	2,560	1,280	2,560
6/7 dpi	Anti-G	0	0	0	0	640	1,280	1,280	1,280	1,280	1,280	640	1,280

lymphoid necrosis included scores for submandibular, bronchial, mesenteric, and inguinal lymph nodes, tonsil, and spleen. The mean histologic score for respiratory tissues was based on nasal and tracheal necrosis and lung lymphohistiocytic infiltrates. Mean histologic scores were weighted by dividing the mean score for each pig by the average of the mean scores for all pigs, so that each tissue contributed equally to the calculated total histologic score. The total histologic score was calculated for each pig as the sum of the weighted mean scores for brain, lymphoid, and respiratory tissues. Statistical analysis was performed with GraphPad Prism (GraphPad Software Inc., San Diego, CA). The histologic scoring data were analyzed by one-way analysis of variance (ANOVA), and Tukey's multiple-comparison test was used to evaluate differences between vaccine groups.

Immunohistochemistry for porcine reproductive and respiratory syndrome virus, porcine circovirus, and swine influenza virus antigens was performed on lung tissues from three pigs by the Animal Health Laboratory, University of Guelph.

RESULTS

Expression of NiV glycoproteins in canarypox (ALVAC) virus vectors was verified prior to immunization by Western blotting and by syncytium formation in cells. Simultaneous coinfection of cells with the ALVAC-G and ALVAC-F vectors at an MOI of 10 resulted in syncytium formation, while single infections with either ALVAC-F or ALVAC-G recombinant viruses did not result in syncytium formation even after 5 dpi. All three cell lines tested, Vero 76, PT-K75, and BHK-21, developed syncytia within 72 h postinfection.

Western blots confirmed the expression of specific NiV F and G proteins. The guinea pig serum reacted strongly with the uncleaved F_0 protein, at an apparent molecular size of approximately 60 kDa; the F_1 fragment, at approximately 50 kDa; and the G protein, at approximately 70 to 75 kDa, corresponding to the published sizes for these proteins (data not shown) (5).

Development of neutralizing antibodies. The first neutralizing antibodies against NiV were detected at 14 dpv, and following the boost at that time, all vaccinated pigs developed neutralizing antibodies with titers depending on the immunizing antigen. Neutralizing antibodies in sera from pigs vaccinated either by ALVAC-G only or by ALVAC-F/G reached titers of around 1,280 (Fig. 1, top and bottom, respectively), while the NiV F protein vaccinees had lower titers of neutralizing antibodies (around 160) (Fig. 1, middle). The antibody titers reached peaks at 21 to 28 dpv (1 day prior to challenge). A slight drop in antibody titers was observed immediately after the challenge in pigs vaccinated with the G or F/G proteins (1 and 2 dpi), with recovery to the higher titers (maximum, 1,280) within 1 week (Fig. 1, top and bottom), while the anti-F antibodies appeared to have a decreasing trend after challenge (Fig. 1, middle). The challenge control pigs (pigs 39 to 42) did not develop detectable neutralizing antibodies during the experiment.

All of the prechallenge sera (28 dpv) were also tested for cross-neutralizing activity against HeV. A titer of 50 was detected in serum from pig 33 (ALVAC-F), and a titer of 20 was detected in serum from pig 36 (ALVAC-G). Pigs vaccinated with both antigens (ALVAC-F/G) developed moderate neutralizing titers (around 80) against HeV (Table 1).

Development of antibodies detected by ELISA and immunofluorescence. The whole NiV indirect ELISA was first able to detect antibody (titers in the range of 200 to 400) in F and F/G vaccinees 7 days after primary immunization (Fig. 2B and C). Unfortunately, there were no later samples available for the G group at 7 dpi to determine whether the same would be true for development of anti-G antibodies. (Originally, gamma-irradiated cell lysate from NiV-infected cells was used as an antigen in the indirect ELISA. However, we were unable to detect anti-F antibodies, despite the presence of neutralizing activity in the sera of the ALVAC-F vaccinees.)

As with the neutralizing antibody titers, the highest antibody titers were detected at 21 dpv with a decreasing trend, even after challenge (Fig. 2). The highest antibody titers were detected for the F/G group (titers around 25,600), followed by the G group. The F vaccinees had somewhat lower antibody titers, reaching a maximum of 12,800; however, the titers among the three groups were comparable.

Low antibody titers were detected in the challenge control pigs at 6 and 7 dpi, when the experiment was terminated. Pigs 39 and 40 had a 6-dpi titer of 100, and pigs 41 and 42 had 7-dpi titers of 200 and 400, respectively.

The specificities of the antibodies produced by the different groups of vaccinees were confirmed by immunofluorescence assay (IFA) for sera collected at 28 dpv and 6 or 7 dpi with transfected cells expressing the specific antigen. The ALVAC-F and ALVAC-F/G groups reached the highest titers, around 2,560, as detected on 293T cells transfected with the pCZCFG5-NivF plasmid. The ALVAC-G and ALVAC-F/G groups reached the highest titers, around 2,560, as detected on 293T cells transfected with pCZCFG5-NivG. The ratio of anti-F and anti-G antibodies in the ALVAC-F/G vaccinees was 1:1 prior

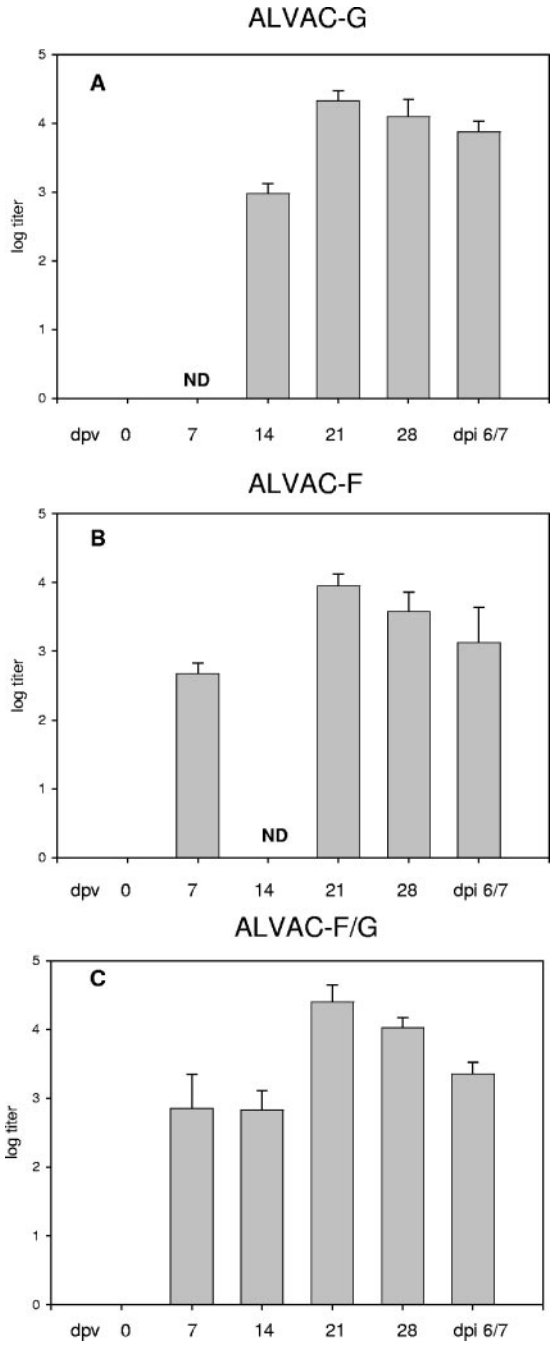

FIG. 2. Antibody response measured by indirect ELISA using binary ethylenimine-inactivated, sucrose gradient-purified NiV as an antigen. Mean antibody titers for each group of vaccinees are plotted against the day of serum collection. (A) Logarithm of mean antibody titers in sera from pigs vaccinated with ALVAC-G. (B) Logarithm of mean antibody titers in sera from pigs vaccinated with ALVAC-F. (C) Logarithm of mean antibody titers in sera from pigs vaccinated with ALVAC-F/G. Statistical analysis was not attempted due to the small numbers of animals per group.

to challenge (Table 1). Following challenge, no anti-F antibodies were detected in the G vaccinees, and vice versa. No anti-F or anti-G antibodies were detected by IFA at 6 or 7 dpi in the challenge control animals.

Cytokine production by PBMC detected by indirect ELISAs indicated upregulation of cellular immune responses. The canarypox virus vector stimulation induced the same levels of cytokines in the PMBC from all vaccine groups harvested at 0 dpv (mean levels were 11 pg/ml for IL-10, 25 pg/ml for IFN-γ, and 125 pg/ml for TNF-α). No significant increase in production of cytokines was observed upon restimulation of cells from ALVAC-F-vaccinated animals with the ALVAC-G vector, and vice versa, at 14 dpv, and no significant difference was observed between the two vaccine groups. The mean values for the eight animals were 22.5 pg/ml for IL-10, 49 pg/ml for IFN-γ, and 95 pg/ml for TNF-α.

The cytokine levels in supernatants of in vitro-cultivated PBMC from the vaccinated pigs upon stimulation with NiV are summarized in Fig. 3, grouped according to the type of cytokine. Figure 3A summarizes the IL-10 levels in unstimulated cells, NiV-stimulated cells, and cells restimulated for NiV F and/or G antigens (primary exposure in vivo to NiV F and/or G antigens in the canarypox virus vector context and restimulation in vitro for the respective antigens in the NiV context). Figure 3B summarizes the TNF-α levels, and Fig. 3C represents IFN-γ. As we did not determine the cytokine-producing cells, we describe the type of cytokine response as type 1 or type 2 rather than Th1- or Th2-type.

In the ALVAC-F group restimulation of PBMC with live NiV caused a very moderate increase in IL-10 production compared to the ALVAC-F/G group and almost no increase in IFN-γ production. The cytokine profile in the PBMC from the ALVAC-G vaccinees, with a large amount of IFN-γ being produced and almost no increase in production of IL-10, indicated an up-regulation of the type 1 cytokine response. TNF-α, an indicator of monocyte/macrophage activation, was not upregulated in the PBMC from ALVAC-F-vaccinated animals and was only very moderately increased in the PBMC from the ALVAC-G vaccinees.

The cytokine upregulation in the ALVAC-F/G group seemed to elicit a balanced immune response by increased production of IL-10, TNF-α, up-regulation, and a moderate increase in IFN-γ expression.

Vaccination prevented nasal and pharyngeal shedding of the virus following challenge with NiV. Pharyngeal swabs and nasal washes from all challenge control pigs tested positive for the presence of viral RNA, and the virus was isolated at 3 dpi, with titers between 10 and 740 PFU/ml in the individual samples (Table 2). In contrast, virus was not detected by rRT-PCR or by isolation in any pigs vaccinated with ALVAC-G or ALVAC-F/G at any time point. rRT-PCR detected viral RNA in only one pig, vaccinated with ALVAC-F (pig 32), at 1 dpi in nasal wash and at 3 dpi in both nasal wash and pharyngeal swabs. We were not able to isolate NiV from pharyngeal swabs or nasal washes of any of the ALVAC-F-vaccinated animals.

Virus and NiV RNA were not detected following NiV challenge in organs of pigs vaccinated with the combination of F/G proteins. Low levels of viral RNA were detected by rRT-PCR in three pigs vaccinated with ALVAC-F and three pigs vaccinated with ALVAC-G in the olfactory bulb, trigeminal ganglion, and trachea. Viral RNA was detected in eight organs/samples from the challenge control (unvaccinated) pigs selected for analysis, with the exception of lung and spleen (Table 3).

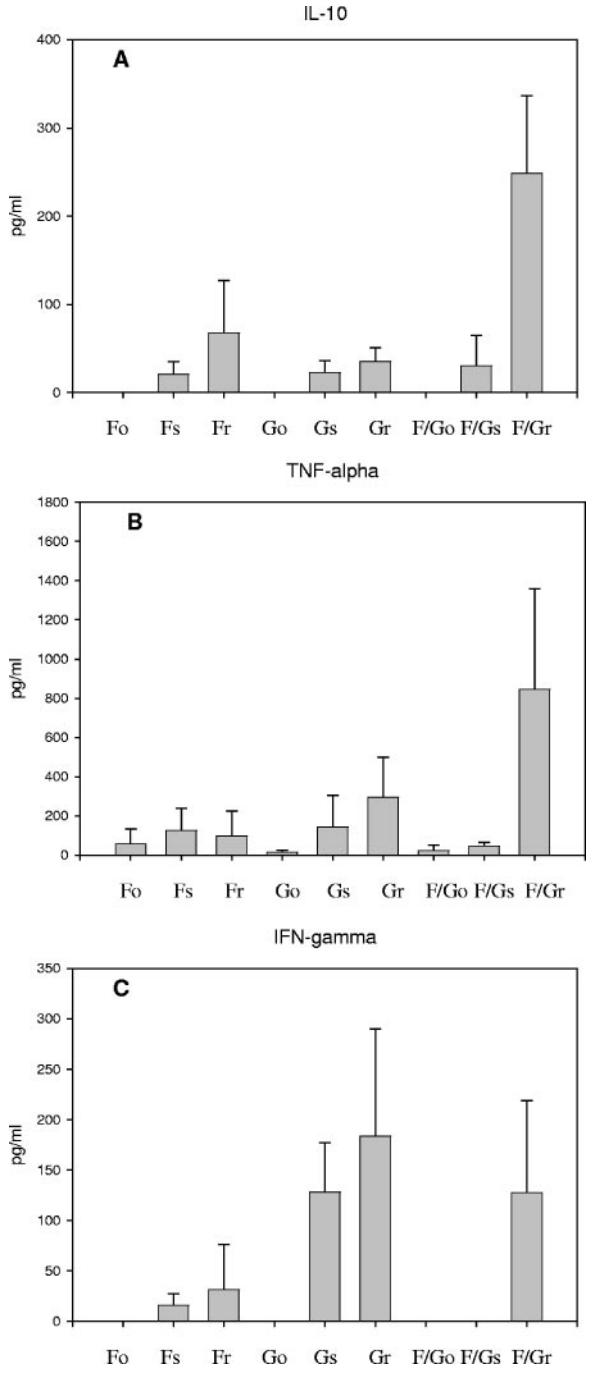

FIG. 3. Cells harvested prior to vaccination were divided into several aliquots. Supernatants from untreated (nonstimulated) cells were assayed for baseline levels of cytokines (F_0, G_0, and F/G_0). Cytokines from cells treated with live NiV (stimulated) cells (F_s, G_s, and F/G_s) were assayed for cytokine levels induced by NiV. Cells collected 14 days post-primary vaccination were treated with live NiV. These cells were considered restimulated for the respective specific antigens: F and/or G (F_r, G_r, and F/G_r). They were primarily exposed in vivo to the NiV F and/or G antigens in the canarypox virus context and restimulated in vitro for these antigens in the NiV context. F represents ALVAC-F vaccinees, G represents ALVAC-G vaccinees, and F/G represents ALVAC-F/G vaccinees. (A) Mean IL-10 levels produced by PBMC. IL-10 levels for F_0, G_0, and F/G_0 (below an OD of 0.08) were considered negative and are noted on the graph as zero. (B) Mean TNF-α levels produced by PBMC. (C) Mean IFN-γ levels produced by PBMC. IFN-γ quantities for F_0, G_0, F/G_0, and F/G_s were considered below the detectable levels and are noted on the graph as zero. Statistical analysis was not considered due to the small numbers of animals per group.

Virus was isolated from the challenge control pigs only in the range of 5 to 2,750 PFU per gram of tissue (Table 4).

No clinical disease was observed in any of the vaccinated animals. Clinical signs developed in two challenge control pigs (pigs 41 and 42) at 7 dpi. The pigs had increased temperature (pig 41, 41.2°C; pig 42, 40.2°C) and were lethargic, and pig 42 was unwilling to stand and had labored breathing with cough. However, no gross pathological changes linked to NiV infection were observed in any of the animals.

Histopathological findings indicated that there was no enhancement of lesions in the vaccinees. Severe brain lesions were present only in the challenge control group, although mild meningitis and/or encephalitis in the form of lymphocytic aggregates was noted in several pigs in all vaccine groups but not in uninfected pigs. Since the lesions were very mild, the absence of signs of neurological disease was not unexpected.

Epithelial necrosis in the nasal mucosa and trachea were present only in the challenge control group and in one pig (pig 32) in the ALVAC-F vaccine group. Lymphoid necrosis was noted only in the challenge group. Significant differences were identified between the unvaccinated challenge control group and all three vaccinated groups for lymphoid necrosis ($P < 0.05$) and total histologic score ($P < 0.01$) (one-way ANOVA with Tukey's multiple-comparison test) (Fig. 4).

In the challenge control group, three out of four pigs (pigs 40, 41, and 42) had lymphocytic meningoencephalitis, which was most severe and consistent in the olfactory bulb, in addition to the cerebrum and midbrain. Brain lesions included lymphocytic meningitis with vasculitis and rare endothelial syncytia, as well as lymphocytic encephalitis with focal gliosis and variable numbers of neutrophils and eosinophils (Fig. 5A). Respiratory lesions included epithelial necrosis and lymphocytic inflammation affecting the nasal, tracheobronchial, and bronchiolar mucosa (pigs 40, 41, and 42) (Fig. 5B) and lymphohistiocytic interstitial pneumonia with lymphocytic arteritis (pigs 39, 40, and 42). Pigs 39, 41, and 42 had necrosis in the submandibular or bronchial lymph nodes, characterized by multiple foci of necrosis or depletion of lymphocytes with increased numbers of histiocytic/dendritic cells. Syncytial cells and intracytoplasmic inclusion bodies were rare (Fig. 5C).

Immunohistochemical staining for other porcine pathogens (porcine reproductive and respiratory syndrome virus, porcine circovirus, and swine influenza virus) performed on lung tissue was negative.

DISCUSSION

The present study indicates that the tested recombinant canarypox (ALVAC-vectored) NiV vaccine candidates have the potential to protect pigs from disease and to restrict virus replication and nasal and pharyngeal shedding, thereby strictly limiting the chance for spread of the virus to uninfected animals/individuals. Interestingly, unlike in several other species

TABLE 2. rRT-PCR of pharyngeal swabs and nasal washes[a]

Group and pig no.[b]	rRT-PCR cycle threshold/100 μl of sample[c]											
	1 dpi		2 dpi		3 dpi		4 dpi		6 dpi		7 dpi	
	N	P	N	P	N	P	N	P	N	P	N	P
ALVAC-F												
31	—	—			—	—			—	—		
32	25.4	—			26	282			—	—		
	25.5					8						
vi	Tox				Tox	0						
33			—	—			—	—			—	—
34			—	—			—	—			—	—
Challenge control												
39	—	—			27	31.3			23.4	25.3		
						31.8			23.4	27.3		
vi					Tox	0			415	740		
40	—	—			27.9	29.4			22.8	28.5		
					29.6	27.7			19.2	28.3		
vi					10	10			60	10		
41			—	—			21.8	—			25.3	27.3
							22.2				26.8	24.8
vi							90				10	45
42			—	—			24	—			25.4	24.6
											25.4	25.8
vi							40				0	0

[a] No NiV was isolated from samples of vaccinated pigs, and viral RNA was not detected by rRT-PCR in samples from pigs vaccinated with ALVAC-G or ALVAC-F/G. Since the volume of collected nasal washes differed for individual pigs, the amount of recovered virus may not indicate the actual virus load.
[b] "vi" indicates that values are PFU/ml of swab/wash material.
[c] N, nasal washes; P, pharyngeal swabs; Tox, virus isolation attempt was unsuccessful due to toxicity; —, negative.

(cats, bats, and hamsters), NiV has not been detected in urine (or urinary tract) or feces of infected pigs (24, 34). In contrast to the challenge control animals, NiV was not isolated from tissues or swabs of any of the vaccinated pigs postchallenge. Clinical disease was not observed in any of the vaccinated animals, while the challenge control (unvaccinated) animals showed increased body temperature and lethargy at 7 dpi. Vaccinated animals did not show enhancement of histological lesions compared to the infected, nonvaccinated animals.

High levels of antibodies were induced by all vaccines, with ALVAC-G and ALVAC-F/G inducing higher neutralizing titers than the ALVAC-F vaccine. Previous work by Guillaume et al. (17, 19) indicated that antibodies against the G and F proteins have a crucial role in protection against NiV. The previously published vaccine study with golden hamsters using vaccinia virus vector reported an increase in antibody titers postchallenge, as detected by virus neutralization at 11 dpi (by ELISA at 5 dpi), and a decline at 18 dpi (17). In our experiment, an increase in antibody titers after infection was not observed. Although it cannot be excluded that the experiment was terminated before the challenge "boost" could take effect (termination was at 7 dpi), the lack of an anamnestic response in vaccinated animals following virus challenge may be real and consistent with other paramyxoviruses, e.g., canine distemper or measles virus (9, 30).

In swine, significant levels of IgG antibodies are present in the nasal mucosa as a result of transudation from serum, and it has been shown that mucosal immunity can be obtained by parental vaccination (8, 21). We speculate that the observed mild drop in serum anti-NiV IgG levels shortly postchallenge may have been due to the formation of virus-antibody complexes shortly after challenge on the nasal mucosa, as indi-

TABLE 3. rRT-PCR of tissue homogenates

Tissue type	rRT-PCR cycle threshold/100 μl of 10% tissue homogenate for indicated pig[a]											
	ALVAC-F group				ALVAC-G group				Challenge control group			
	31	32	33	34	35	36	37	38	39	40	41	42
Brain	–	–	–	–	–	–	–	–	29.5	24.8	24	–
Cerebrospinal fluid	–	–	–	–	–	–	–	–	ND	–	29.5	28.9
Olfactory bulb	32	–	–	–	30	–	–	–	25	19	23.8	ND
Trigeminal ganglion	–	–	28.5	30	32	–	–	–	26	18	25.1	18.8
Turbinate	–	–	–	–	–	–	–	–	19	18	27.5	25.6
Trachea	–	–	–	29	–	–	31	31	18.5	19	28.2	–
Submandibular lymph node	–	–	–	–	–	–	–	–	21.5	23	ND	–
Bronchiolar lymph node	–	–	–	–	–	–	–	–	22.5	24	–	–

[a] ND, not done; –, negative.

TABLE 4. Virus isolation in tissues positive by rRT-PCR[a]

Tissue type	PFU/100 μl of 10% tissue homogenate for indicated pig[b]			
	39	40	41	42
Brain	–	0.5	1.25	–
Olfactory bulb	0.125	ND	–	ND
Trigeminal ganglion	0.125	71.3	–	262.5
Nasal turbinate	102.5	12	0.25	4.5
Trachea	42	8	1.6	–
Submandibular lymph node	20	1.5	ND	–
Bronchial lymph node	8.4	1.25	–	–

[a] The only positive tissues were those from nonvaccinated challenged pigs; no virus was isolated from any of the vaccinees.
[b] ND, not done; –, negative.

cated, e.g., for influenza virus (31), and subsequent recruitment of more antibodies from the sera. The presumed formation of NiV antibody complexes could be supported by the lack of an anamnestic response (neutralizing of virus early in infection and decreasing antigen presentation to the immune system below levels sufficient to elicit the "boost" effect).

The lack of virus detection and the lack of an anamnestic response in the vaccinated animals after challenge, along with the presence of high neutralizing antibody levels in the sera of the animals at the time of challenge, suggest that only very limited virus replication took place. It cannot be excluded that in case of the ALVAC-F/G group, where even the viral RNA was not detected, the vaccination led to sterile immunity. The presence of preexisting high levels of neutralizing antibodies at the time of challenge can, in case of NiV, lead to sterile immunity, as demonstrated by Guillaume and others (19).

Although no virus was isolated from the ALVAC-F-vaccinated pigs postchallenge, viral RNA was detected in several animals (in nasal washes of pig 32 and in pharyngeal swabs and tissues of several pigs). The vaccinated animals developed good antibody titers, as determined by ELISA and IFA, with neutralizing activity about 1 log lower than the G and F/G vaccinees. It needs to be considered that anti-F antibodies are not the primary neutralizing antibodies for paramyxoviruses (23). Based on the limited cytokine production data, it appears

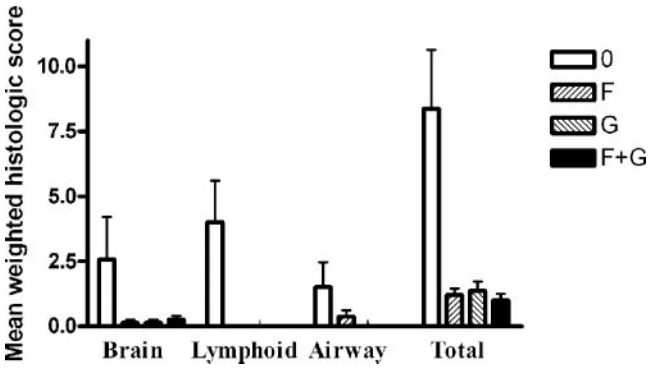

FIG. 4. Histologic scoring data in NiV-infected pigs, which were previously immunized with no vaccine (0) or with vaccine against F, G, or both F and G proteins. Shown are the histologic scores for meningoencephalitis (brain), necrosis of lymphoid organs, necrosis of nasal and tracheal epithelium (airway), and the totals.

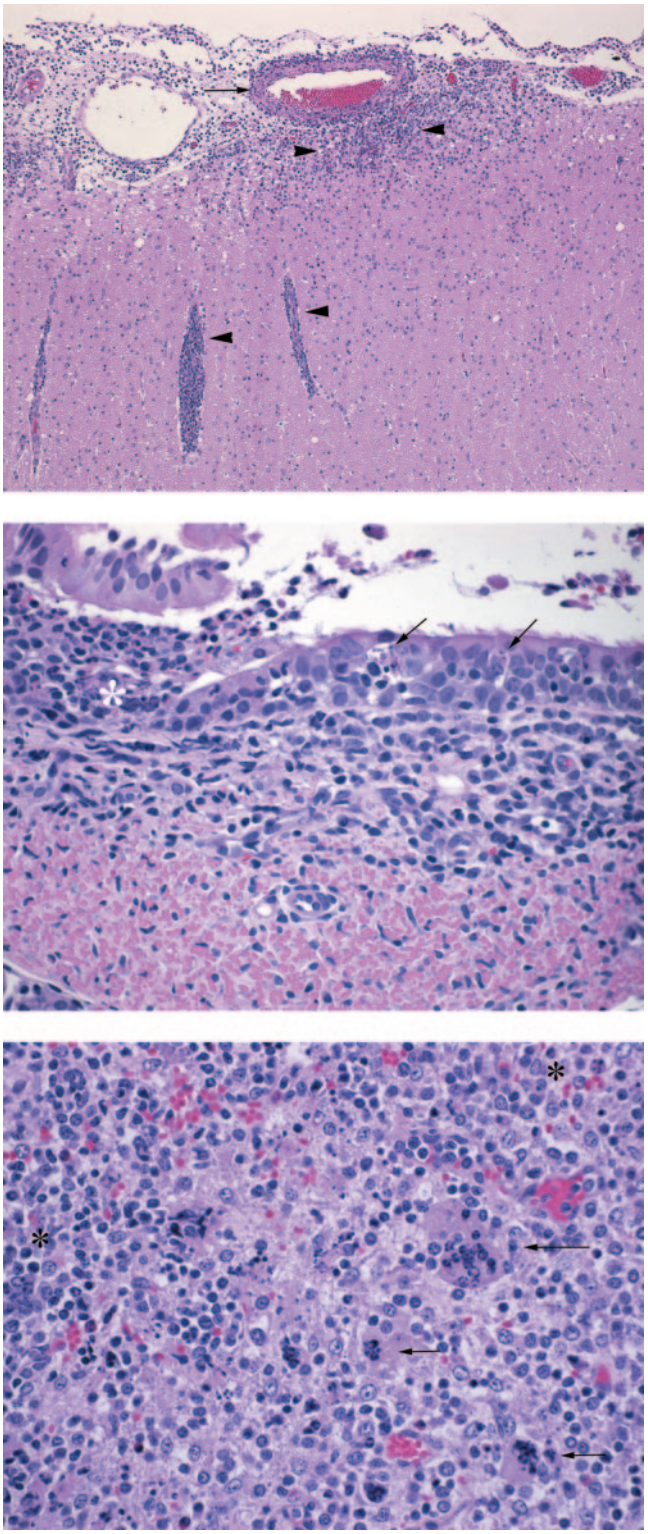

FIG. 5. Histologic lesions in challenge control pigs infected with NiV. (Top) Cerebrum of pig 41, showing lymphocytic meningitis with vasculitis (arrow) and encephalitis (arrowheads). (Middle) Trachea of pig 39, showing epithelial erosion (asterisk) and single-cell necrosis associated with lymphocyte infiltration (arrows). (Bottom) Bronchial lymph node of pig 39, showing syncytial cells (arrows) in a focal area of necrosis, with non-necrotic lymphocytes at the periphery (asterisks).

that the NiV F protein does not induce a type 1 cell response (13) (no increase in IFN-γ and TNF-α levels) and mounts only a small increase in the level of IL-10 compared to that induced by the ALVAC-F/G vaccine.

On the other hand, vaccination with the G protein (ALVAC-G) results in the mounting of high IFN-γ and intermediate TNF-α levels, indicative of a type 1 response. Almost no induction of IL-10 was observed, suggesting low activation of the type 2 cytokine response. Vaccination induced relatively high levels of neutralizing antibodies. Although no viral RNA was detected in pharyngeal swabs and nasal washes, and no virus was isolated from the collected samples, several pigs had viral RNA present in some of the tissues.

No viral RNA or virus was detected in tissues or swabs/washes of the animals vaccinated with the ALVAC-F/G combination. Vaccination led to the development of high antibody titers, including neutralizing antibodies, and appeared to activate both type 1 and type 2 immune responses (induction of TNF-α, IL-10, and IFN-γ). This phenomenon has been seen in natural infections with a number of viruses that induce mixed responses that are predominantly of the Th1 type but have Th2 components (16). Combined vaccination with both the F and G NiV proteins induced the best protection and a balanced type 1/type 2 immune response, which is important not only as an indicator of good immune priming and memory but also because in other paramyxovirus infections, cytokine dysregulation caused by natural infection or by vaccination contributes to the development of disease. For example, in measles virus infection, impaired type 1 immune responses may lead to temporary immunosuppression and increased susceptibility to secondary infections (22, 27), and increased production of Th2 cytokines and reduced production of IFN-γ may contribute to a severe form of respiratory syncytial virus-induced bronchitis (20).

Interestingly, the canarypox virus vaccine vector itself did not really induce immune priming and memory, based on cytokine induction. This unique property (lack of induction of antivector immunity) has been previously observed with this vector in other species and has not been fully explained (2); however, it represents an additional advantage.

Based on the results obtained in this study, vaccination with a combination of NiV F and G proteins in the canarypox virus-based vector may provide better protection than immunization with individual F or G protein. In addition, sera from the ALVAC-F/G-vaccinated animals showed a moderate neutralizing activity against the related HeV. Pursuing these promising results will require gaining more information on the immunogenicity of the vaccine, the vaccination regimen, the duration of the protective effect, and the challenge dose, etc., using not only the swine (or miniature pig) model but also an additional small animal model, e.g., the golden hamster (36). If proven efficacious and safe in animals, vaccine using an ALVAC delivery system may also be considered for human use.

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