Newcastle disease virus-vectored rabies vaccine is safe, highly immunogenic, and provides long-lasting protection in dogs and cats

Jinying Ge1§, Xijun Wang1§, Lihong Tao1, Zhiyuan Wen1, Na Feng2, Songtao Yang2, Xianzhu Xia2, Chinglai Yang3, Hualan Chen1, and Zhigao Bu1*

1State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences1, People's Republic of China;
2The 11th Institute, Academy of Military Medical Sciences, Changchun, People's Republic of China;
3Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, USA.

§ J.G. and X.W. equally contributed to this paper.
* Corresponding author. Mailing address: Harbin Veterinary Research Institute, CAAS, 427 Maduan Street, Harbin 150001, People’s Republic of China. Phone: 86-451-85935062. Fax: 86-451-82733132. E-mail: zgb@yahoo.com

Keywords: Recombinant Newcastle disease virus, glycoprotein, rabies, vaccine

Running title: Newcastle disease virus-vectored rabies vaccine
Abstract

Effective, safe, and affordable rabies vaccines are still being sought. Newcastle disease virus (NDV), an avian paramyxovirus, has shown promise as a vectored vaccine for mammals. Here, we generated the recombinant avirulent NDV LaSota strain expressing the rabies virus glycoprotein (RVG) and evaluated its potential to serve as a vaccine against rabies. The recombinant virus, rL-RVG, retained its high-growth property in chicken eggs, with titers of up to $10^{9.8}$ EID$_{50}$/ml of allantoic fluid. RVG expression enabled rL-RVG to spread from cell to cell in a rabies virus-like manner, and RVG was incorporated on the surface of the rL-RVG viral particle. RVG incorporation did not alter the trypsin-dependent infectivity of the NDV vector in mammalian cells. The rL-RVG and LaSota NDV showed similar sensitivity to a neutralization antibody against NDV and similar resistance to a neutralization antibody against rabies virus. Animal studies demonstrated that rL-RVG is safe in several species, including cats and dogs, which was administered as multiple high doses of recombinant vaccine. Intramuscular vaccination with rL-RVG induced a substantial rabies virus neutralization antibody response and provided complete protection from challenge with circulating rabies virus strains. Most importantly, rL-RVG induced strong and long-lasting protective neutralization antibody responses to rabies virus in dogs and cats. A low vaccine dose of $10^{8.3}$EID$_{50}$ completely protected dogs from challenge with a circulating strain of rabies virus for more than a year. This is the first study to demonstrate that immunization with an NDV-vectored vaccine can induce long-lasting, systemic protective immunity against rabies.
Introduction

Rabies virus (RV), which belongs to the genus Lyssavirus of the family Rhabdoviridae, causes a fatal neurologic disease in humans and animals (15). More than 55,000 people die of rabies each year, with about 95% of those deaths occurring in Asia and Africa (3). The number of human deaths attributed to rabies worldwide is greater than that from avian influenza, polio, meningococcal meningitis, Japanese encephalitis, yellow fever, and severe acute respiratory syndrome combined (62).

Rabid dogs remain the main source of human exposure (3, 24). The most cost-effective strategy for preventing rabies in humans is by eliminating rabies in dogs through animal vaccination (39). However, the vaccination rate of dogs in many developing countries is low, especially in rural areas, mainly due to low economic development and the high costs of vaccine (35). A live attenuated RV (SAG-2) and a recombinant vaccinia virus expressing the rabies virus glycoprotein (RVG) have been licensed and shown to be effective oral vaccines (29, 60). However, the recombinant vaccinia virus expressing RVG can cause intense skin inflammation and systemic vaccinia infection in humans (1, 45), and SAG-2 induces a low level of virus-neutralizing antibody (VNA) responses, which complicates evaluation of its efficiency (3, 24). Therefore, effective, safe, and low-cost rabies vaccines are still being sought (21, 42, 49, 63, 67).

Newcastle disease virus (NDV) is a member of the genus Avulavirus of the family Paramyxoviridae. NDV strains are classified as nonvirulent (lentogenic), moderately virulent (mesogenic) or highly virulent (velogenic) for poultry (4). This virulence is mainly determined by the amino acid sequence of the protease cleavage site of the fusion (F) protein.
precursor (53). Lentogenic strains contain fewer basic amino acids at this site and can only be
cleaved by trypsin-like extracellular proteases that are largely confined to the respiratory tract,
whereas highly virulent strains are cleaved by ubiquitous intracellular proteases, potentially
resulting in systemic infection(50). Currently, lentogenic strains, such as the La Sota strain,
are used as live attenuated vaccines against NDV in poultry (5).

The development of a reverse genetics system has provided a method to generate
recombinant NDV-based live virus-vectored vaccines (12, 48). NDV has been actively
developed (27, 28, 30, 52, 59) and evaluated for its potential as a vaccine vector for the
control of human and animal diseases (9, 12). NDV does not usually infect mammals because
of host-range restriction and it is antigenically distinct from mammalian paramyxoviruses,
suggesting that mammals would not be susceptible to NDV. After intranasal or intratracheal
inoculation with a mesogenic or lentogenic strain of NDV, African green and rhesus monkeys
experienced only a low level, asymptomatic infection of the respiratory tract with little or no
virus shedding (19). Studies with mice have shown that NDV expressing the protective
antigens of H1 influenza virus, H5 highly pathogenic avian influenza virus, and human
respiratory syncytial virus were immunogenic and provided protection from challenges (27,
43, 46, 47). Moreover, recombinant NDV expressing SARS coronavirus S or H5 avian
influenza virus hemagglutinin were safe and immunogenic in non-human primates and also
provided protection from challenges (10, 17, 18, 20). These results strongly indicate that
NDV is a promising vaccine vector for humans and other mammals. Here, we generated a
recombinant NDV expressing RVG, the major antigen for protective immune responses
against RV (15). The feasibility of this recombinant NDV to serve as live virus-vectored rabies vaccine for mammalian animals was evaluated.
Materials and Methods

Viruses and cells

HEp-2 cells, BHK-21 cells, Vero cells, A549 cells and neuroblastoma cells (NA) of A/J mouse origin were grown in Eagle's minimum essential medium (DMEM) containing 10% fetal bovine serum (FBS). The Vero-adapted RV Evelyn-Rokitnicki-Abelseth (ERA) strain and the RV CVS-11 strain came from the China Veterinary Culture Collection. The NDV vector virus rL was rescued from the genomic cDNA of the NDV LaSota vaccine strain (27). Recombinant NDV strains were grown and titrated in 9-day-old specific-pathogen-free (SPF) embryonated chicken eggs by inoculation of the allantoic cavity. Recombinant NDV was also grown and titrated in BHK-21 or NA cells in six-well or 96-well plates in fresh Opti-MEM (Invitrogen Corp., Carlsbad, CA, USA) with or without 1µg/ml of TPCK trypsin (Sigma, St. Louis, MO, USA). The infection of NDV in BHK-21 and NA cells was detected by using an indirect immunofluorescence assay (IFA) and observing the cells under an immunofluorescence microscope. The RV ERA and CVS-11 strains were propagated and titrated in Vero cells and BHK-21 cells, respectively. The modified vaccinia strain Ankara expressing the T7 RNA polymerase (64) was grown and titrated in primary chicken embryo fibroblasts. The RV street virus GX/09, isolated from the brain of a dog that died of rabies in the Guangxi Province of China in 2009, was propagated and titrated in the brains of adult mice, as previously described (58). The recombinant vesicular stomatitis virus (VSV) expressing GFP, rVSV-EGFP, was generated and prepared as previously described (40). Viral titration results were calculated by using the method of Reed and Muench (54). All viruses were stored at -70°C before use.
**Plasmid construction and virus rescue**

To construct the full-length recombinant NDV genomic cDNA, the cDNA that represents the open reading frame (ORF) of the G gene of the RV ERA virus was amplified from the genome RNA by using the following primer pair:

5′-GACTGTTAAAACTTAGAAAAATACGGGTAGA AGTGCCACCATGGTTCCTAGCTCTCCTG-3′, and

5′-GACTGTTAAAACCTACAGTCTGGTCCTACCCCCACTC-3′, in which the gene end and gene start sequences of NDV (underlined), the optimal Kozak sequence (italic) and the Pme I restriction sites (bold) were included. The G gene of ERA was introduced into the NDV genome of pLa through the introduction of a Pme I site in the P-M intergenic region at nucleotide position 3165 of the NDV genome, as described previously (27). The resultant plasmid was designated as pLa-RVG and used for virus rescue by using reverse genetics as described previously (27). The presence of rescued virus and RVG expression in recombinant NDV were confirmed by IFA. The recovered viruses were also confirmed by sequencing of the entire viral genome. The resultant recombinant virus was designated as rL-RVG.

**Immunofluorescence**

NDV infection of cultured cells was detected by IFA by using mouse or chicken serum against NDV as previously described (27). For confocal assays, BHK-21 cells were grown in 24-well plates or plated on cover slips in 35-mm-diameter dishes and were infected with NDV rL or rL-RVG. At 24 h post-infection, cells were fixed in ice-cold 3% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature, and then washed with PBS three times. Cells were blocked in PBS containing 1% (wt/vol) bovine serum albumin (BSA)
at 4°C for 1 h. Cells were then incubated with mouse serum against RV or chicken serum against NDV for 30 min at room temperature. Cells were then washed three times with PBS containing 0.05% Tween 20 and stained with a TRITC-conjugated goat anti-mouse antibody (Sigma) or an FITC-conjugated rabbit anti-chicken antibody (Sigma) for 30 min. Cells were washed three times with PBS and their nuclei stained with DAPI. Cells were analyzed with a fluorescence microscope or confocal laser microscope. Images were acquired with a Zeiss (Thornwood, N.Y.) Axioscop microscope equipped for epifluorescence with a Sensys charge-coupled device camera (Photometrics, Tucson, Ariz.) by using IPLab software (Scanalytics, Vienna, Va.).

**Western blotting**

BHK-21 cells were infected with rL and rL-RVG at a multiplicity of infection (MOI) of 10. At 36 h post-infection, lysates were prepared for the analysis of cell-associated proteins. For the analysis of virion-associated proteins, virus particles were isolated as previously described (46) from the allantoic fluid of SPF chicken eggs infected with rL or rL-RVG. Briefly, the allantoic fluid was clarified by low-speed centrifugation, layered onto a 40% to 60% (wt/vol) sucrose gradient, and centrifuged at 90,000 g for 90 min (Beckman Coulter, Fullerton, CA). The resulting band of virus particles was isolated and resuspended in PBS. As a control, allantoic fluid from mock-infected eggs was processed in parallel. Proteins from the lysates of infected cells or from purified virus particle preparations were separated by using SDS-10% PAGE under denaturing conditions for Western blot analyses with chicken serum against NDV or mouse serum against RV. Chicken or mouse serum binding was detected with horseradish peroxidase (HRP)-conjugated rabbit-anti-chicken IgG or goat-anti-mouse IgG.
respectively (Sigma, St. Louis, MO, USA).

**Immuno-electron microscopy**

Purified virus particles were bound to 200-mesh Formvar/carbon-coated nickel grids (Electron Microscopy Sciences, Hatfield, PA). For immuno-labeling, grids were blocked in PBS containing 2% globulin-free BSA (Sigma-Aldrich, St. Louis, MO) and incubated with mouse anti-NDV polyclonal IgG or mouse anti-RV polyclonal IgG. Grids were then washed in blocking solution and incubated in goat anti-mouse IgG conjugated to 10-nm gold beads (Sigma). The grids received a final wash, followed by negative staining with 1% phosphotungstic acid. They were examined under a model H7500 transmission electron microscope (Hitachi High Technologies, Schaumburg, IL) at 80 kV. All images were obtained by using an XR100 digital camera system (Advanced Microscopy Techniques, Danvers, MA).

**Quantification of interferon induction and inhibition of viral replication**

A549 cells were incubated with rL or rL-RVG at an MOI of 5 for 1 h at 37 °C; the viruses were then removed by washing five times with PBS. After the cells were incubated for an additional 24 h, cell supernatants were harvested and UV-treated for 2 h, and then mixed with mouse serum against NDV at a dilution of 1:20 and incubated for 1 h at 37 °C. The complete neutralization of residual infectious NDV in the supernatant-serum mixtures was confirmed by inoculation of chicken eggs. The anti-viral activity of the cell supernatants was then detected in A549 cells and quantified as described previously (40). The anti-viral activity was quantified in terms of an inhibition unit (InU), where one InU was defined as the final dilution at which 50% inhibition of rVSV-EGFP infection occurred. For the interferon inhibition test, type I interferon was generated in A549 cells by treating them with poly I:C as...
described previously (32), and the anti-viral activity in the supernatants was quantified in InUs in A549 cells. Serial dilutions of the generated type I interferon were used to treat A549 cells for 24 h. The pre-treated A549 cells were then infected with 100 TCID$_{50}$ of rL or rL-RVG in the presence of trypsin. Virus replication in A549 cells was detected at 48 h post-inoculation by using IFA with chicken serum against NDV.

**Pathogenicity in poultry and mice**

To determine the pathogenicity of rL-RVG in poultry, the mean death time (MDT), the intracerebral pathogenicity index (ICPI) and the intravenous pathogenicity index (IVPI) were determined in embryonated SPF chicken eggs or in SPF chickens following the OIE Manual (2). To assess the pathogenicity of recombinant viruses in mice, groups of 19 three-week-old BALB/c mice were inoculated intramuscularly (i.m.) and intracerebrally (i.c) respectively, with rL and rL-RVG. For each inoculation method, mice were inoculated i.m. at thigh of rear leg with $2 \times 10^{8} \text{EID}_{50}$ of the viruses in a volume of 0.03 ml. Three mice from each group were killed on days 3, 5, and 7 post-inoculation, and organs including brain, lung, liver, spleen, kidney, and heart were collected and homogenated in 0.5 ml PBS for virus titration in embryonated SPF chicken eggs by inoculation of the allantoic cavity (5). The remaining 10 mice were observed daily for signs of disease, weight loss, or death for three weeks.

**Immunization studies**

For mouse immunization studies, four-week-old female BALB/c mice were divided into four groups of 10 animals each. Three groups were inoculated i.m. in the gastrocnemius muscle with 100 µl of $10^{8.3} \text{EID}_{50}$, $10^{7.3} \text{EID}_{50}$, and $10^{6.3} \text{EID}_{50}$ of rL-RVG. The fourth group was inoculated i.m. with 100 µl of $10^{8.3} \text{EID}_{50}$ of rL. All mice were bled from the retro-orbital
sinus under isoflurane inhalation anesthesia for the serologic assay. For cat immunization assays, outbred cats (0.5–2-years old) were divided into two groups of five animals each. One group was inoculated i.m. in the quadriceps muscle with 1 ml of $10^{9.8}$ EID$_{50}$ of rL-RVG and other group was inoculated with 1 ml of $10^{6}$ focus forming units (FFU) of ERA. For dog immunization, three-month-old Beagle dogs were arranged in four groups of ten animals each. Three groups were inoculated i.m. in the quadriceps muscle with 1 ml of $10^{9.8}$ EID$_{50}$, $10^{9.3}$ EID$_{50}$, and $10^{8.3}$ EID$_{50}$ of rL-RVG, respectively. The fourth group was inoculated with 1 ml of $10^{6}$ FFU of ERA by the same route. At three and 60 weeks post initial vaccination, the cats and dogs received second and third vaccine doses, respectively. They were bled from the vein of the front leg prior to vaccination and at different times post-vaccination for serological assessment. All cats and dogs used in this study had no record of prior rabies vaccination.

**Challenge test**

The mouse challenge was carried out at three weeks post-immunization. Mice were i.m. injected at the thigh muscle of hind legs with 50 MLD$_{50}$ of RV (strain GX/09, 100 µl). Mice were observed for four weeks for clinical signs of rabies. Any mouse that showed definitive clinical signs of rabies, such as paralysis, tremors, or spasms was euthanized by CO$_2$ intoxication. Survival rates obtained with the different vaccination groups were compared.

The challenge in dogs was carried out 60 weeks after the second dose of vaccination by injecting 2×0.5 ml containing $10^{5.8}$ MLD$_{50}$ of GX/09 into the bilateral masseter muscle, 0.5 ml per side. The dogs were observed for 12 weeks and any animal showing definitive rabid signs was euthanized with a barbiturate solution administered intravenously. All dogs that survived the challenge at the end of the observation period were also euthanized and their
brain tissue checked for rabies virus antigen by using a direct immunofluorescence (IF) test (41).

Serologic tests

Animal sera were tested for RV neutralizing antibodies (VNA) by using the rapid fluorescent focus inhibition test (RFFIT) as described elsewhere (57). Rabies virus neutralizing antibodies (VNA) were assayed by using RFFIT, and titers were expressed in international units per milliliter of serum (IU/ml) with a WHO standard as a reference.

Hemagglutination inhibition (HI) antibody to NDV was tested as previously described (27).

Laboratory facility

All experiments related to the rabies virus GX/09 were conducted in a BSL-3 facility at the Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences. All animal studies were approved by the Review Board of Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences.
Results

Generation of recombinant NDV viruses expressing the RVG gene

To generate recombinant NDV expressing RVG, an infectious recombinant genomic cDNA clone of NDV LaSota was constructed by inserting the RVG gene between the P and M genes (Fig 1A). The resultant recombinant virus, rL-RVG, was rescued from the full-length genomic cDNA clone as described in the Materials and Methods, and the presence of the RVG gene was confirmed by using RT-PCR. Expression of the G protein by rL-RVG was detected by immunostaining infected BHK-21 cells 36 h post-infection. As expected, cells infected with rL were not stained by mouse serum against RV, but were stained by chicken serum against NDV (Fig 1B). By contrast, cells infected with rL-RVG were stained by both the mouse antiserum to RV and the chicken antiserum to NDV (Fig 1B). RVG expression by the recombinant viruses was also confirmed by western blot analysis with polyclonal mouse antiserum to RV (Fig 1C).

The growth properties of rL and rL-RVG in eggs were examined. As shown in Fig 1D, rL-RVG and rL grew to similar levels and reached peak titers of 9.8logEID<sub>50</sub>/ml at 72 h post-inoculation. This titer was approximately one-fifth of a log lower than that of rL. The stability of the RVG gene within rL-RVG was assessed by serially passaging the virus in SPF chicken eggs for 10 passages. After 10 passages, the RVG gene was assessed by using RT-PCR and immunofluorescence and found to be stably maintained and expressed (data not shown).

RVG expression enables the NDV vector to spread from cell to cell and alters its replication in cell culture in the absence of trypsin
Progeny rabies virions that bud from infected cells are spread from cell to cell in cell culture, presumably as they do in animals. Rabies viruses can spread to contiguous or to non-contiguous cells, which are surrounded by the interstitial space (16). To investigate whether RVG expression alters the cell spread of NDV vector, BHK-21 and NA cells were infected with rL or rL-RVG each at an MOI of 0.05. The infected cells were detected at different times post-inoculation by using indirect IFA with chicken serum against NDV. As a low pathogenicity NDV strain, rL infected individual cells but did not spread to adjacent BHK-21 or NA cells in the absence of TPCK trypsin (1mg/ml). Therefore, the percentage of infected cells at 96 h post-infection was almost identical to that at 24 h post-infection (Fig 2A, B). However, rL-RVG acquired the ability to spread from cell to cell in the absence of TPCK trypsin and spread from the initial infected cell to adjacent cells, forming larger and larger plaques. These plaques gradually fused together and all of the cells in observation field were infected at 96 h post-inoculation (Fig 2A, B). To further confirm that the cell-to-cell spread was specifically caused by the RVG that was displayed on the cell surface, we added diluted anti-NDV mouse serum (at 12.5× neutralization titer), anti-RV mouse serum (at 22.5×neutralization titer) or 10×-diluted naïve mouse serum to the culture media at 1 h post-inoculation with rL-RVG at an MOI of 0.05. The spread of the infection from cell to cell in BHK-21 (Fig 2C I ) and NA cells (Fig 2C II ) was completely blocked by the presence of the anti-RV mouse serum. However, the anti-NDV mouse and naïve mouse sera had no effect on infection spread. These results suggest that the RVG expression dramatically altered the cell-to-cell spread in vitro.

To investigate whether cell-to-cell spread alters the replication of the NDV vector in cell
culture, one-step and multiple-step growth kinetics of rL and rL-RVG in BHK-21 cells were compared in the presence and absence of TPCK (1mg/ml). In the multi-step growth assay, BHK-21 cells were infected with egg propagated rL or rL-RVG at an MOI of 0.01. In the presence of TPCK trypsin, the growth kinetics of rL-RVG was almost identical to those of rL in BHK-21 cells. The titers of rL and rL-RVG in the cell supernatant gradually increased and peaked at $10^{7.5}$ EID$_{50}$/ml and $10^{7.2}$ EID$_{50}$/ml, respectively, at 96 h post-inoculation (Fig. 3A). In the absence of TPCK trypsin, rL and rL-RVG showed different growth kinetics in cell culture. As expected, the titers of rL showed slowly increasing during the 5-day observation period after inoculation. However, the titers of rL-RVG gradually increased after inoculation, reaching a peak titer of $10^{7.6}$ EID$_{50}$/ml at 96 h post-inoculation (Fig. 3B). In the one-step growth assay, BHK-21 cells were infected with rL or L-RVG at an MOI of 5. The viruses in the cell supernatant were collected at different times after inoculation and titrated in chicken eggs. The mean titers of rL and rL-RVG gradually increased and reached peak titers ($10^{7.0}$ - $10^{7.3}$ EID$_{50}$/ml) at 72 h post-inoculation (Fig. 3C, 3D). No significant differences were found in peak titers between rL and rL-RVG in the presence (Fig. 3C) or absence of (Fig. 3D) TPCK trypsin. These results show that the insertion and expression of the RVG gene significantly affected the growth of the NDV vector in cell culture in the absence of trypsin. RVG is incorporated into the surface of NDV particles but does not alter trypsin-dependent infectivity in mammalian cell culture

To investigate whether RVG was incorporated into recombinant NDV particles, virions were purified from the allantoic fluid of SPF chicken embryos inoculated with rL or rL-RVG and subjected to SDS-PAGE and Western blotting. SDS-PAGE clearly separated the purified
virions of rL and rL-RVG into the major NDV structural proteins, including HN, N, M, and F1 (Fig. 4A Ⅰ). The RVG proteins were probed with the anti-RV mouse serum in the purified virions of rL-RVG, but not in those of rL (Fig. 4A Ⅱ). Partially purified virions of rL and rL-RVG were also observed by electron microscopy by using negative staining. Both virions showed typical paramyxovirus morphology with densely arrayed spikes on their envelopes. Immunogold staining revealed that the polyclonal antibody to NDV bound to whole viral particles of rL and rL-RVG (Fig. 4B), whereas the polyclonal antibody to RV bound only to the viral particles of rL-RVG (Fig. 4B). These results indicate that the RVG that was incorporated into the surface of the recombinant NDV particles did not change the morphology of NDV.

To further investigate whether RVG incorporation influences the trypsin-dependent infectivity of the NDV vector in mammalian cells, rL and rL-RVG were propagated in BHK-21 cells in the presence or absence of trypsin. Cell supernatants were collected 24 h post-inoculation and then used to infect BHK-21 and NA cells in the absence of trypsin. The infections were detected by using an IFA with chicken serum against NDV. Both the rL and rL-RVG that were produced in BHK-21 cells in the presence of trypsin were able to infect BHK-21 (Fig. 5A) and NA cells (Fig. 5B). By contrast, the rL and rL-RVG that were generated in BHK-21 cells in the absence of trypsin could not infect BHK-21 (Fig. 5A) or NA cells (Fig. 5B), indicating that the RVG incorporation into viral particles did not alter the trypsin-dependent infectivity of the NDV vector in mammalian cells.

To examine whether RVG incorporation altered the neutralization and infectivity characteristics of the recombinant NDV, 10^4 EID\textsubscript{50} of rL and rL-RVG, propagated in chicken...
eggs, was mixed with 2-fold serially diluted mouse serum against NDV, mouse serum against RV, or naïve mouse serum. After a 1 h-incubation at 37°C, the residual infectious viruses in the virus-serum mixtures were evaluated by inoculation into chicken eggs. The incubation with the anti-NDV serum completely prevented the infection of rL and rL-RVG. The mean of neutralization titers for rL and rL-RVG were 896 and 768, respectively (Fig. 6). However, the anti-RV and naïve mouse sera showed no significant inhibition of the infection of rL and rL-RVG (Fig. 6). These results indicate that the recombinant rL-RVG has the same sensitivity to the NDV neutralization antibody as that of the parent vector virus. The incorporation of RVG into the viral envelope did not change the sensitivity of the NDV vector to the NDV or RV neutralization antibody.

**RVG expression does not significantly influence the effect of the NDV vector on the innate immune responses of mammalian cells**

To investigate whether RVG expression alters the ability of the NDV vector to stimulate type I interferon responses in mammalian cells, human A549 cells were infected with rL and rL-RVG at an M.O.I. of 5. At 24 h post-infection, the antiviral activity in the supernatants was quantified by using a replication inhibition assay as described in the materials and methods. The interferon activity stimulated by rL and rL-RVG was 1228.8 InU and 1433.6 InU, respectively. By contrast, there was no detectable antiviral activity in the supernatant of mock infection A549 cells. The results show that rL-RVG and rL similarly induce type I interferon responses in mammalian cells (Fig. 7A). To further compare the sensitivity of rL and rL-RVG to the interferon of mammalian cells, A549 cells were pretreated with type I interferon that was generated in A549 cells. The minimal amount of interferon that completely inhibit the
replication of rL and rL-RVG was 1.6 InU and 1.4 InU respectively; rL and rL-RVG thus showed similar sensitivity to mammalian type I interferon (Fig. 7B). These results suggest that RVG expression does not significantly influence the effect of the NDV vector on the innate immune responses of mammalian cells.

**Expression of the RVG gene does not increase the virulence of the NDV vector in poultry or mice**

To investigate whether expression of the RVG gene alters the pathogenicity of the NDV vector, we compared the biological character and pathogenicity of rL-RVG with its vector in poultry and mice. The MDT, ICPI and IVPI tests are internationally accepted methods for assessing the pathogenicity of NDV strains in poultry (24). NDV strains are categorized into three groups on the basis of their MDTs: velogenic (< 60 h), mesogenic (60-90 h), and lentogenic (> 90 h) (24). The MDT values for rL and rL-RVG were both greater than 120 h (Fig. 8A), indicating that these two viruses are lentogenic. After receiving rL and rL-RVG, all chickens remained healthy during the observation period. The ICPI values for rL and rL-RVG were 0.4 and zero, respectively (Fig. 8A); the IVPI values for rL and rL-RVG were both zero (Fig. 8A). These data show that rL-RVG and rL are of low pathogenicity to SPF chickens and embryos, suggesting that the insertion of the RVG genes did not increase the virulence of the NDV vector.

To investigate the replication and pathogenicity of the recombinant virus in mammalian animal, mice were inoculated *i.c.* and *i.m.* with rL and rL-RVG, respectively. All of the mice survived after inoculation. There were no differences in body weight changes between rL-infection and rL-RVG-infection groups, after intracerebral (Fig. 8B) or intramuscular
(Fig. 8B Ⅱ) administration. Lung, heart, spleen, kidney, and brain tissues, collected at 3, 5, and 7 days after inoculation, showed no evidence of virus after inoculation in eggs. These data suggest that the expression of RVG does not change the pathogenicity of vector virus in mice, and both rL and rL-RVG have limited replication in specific major organs of mammalian animals.

**Recombinant virus induces a dose-dependent immune response and protective efficacy against rabies in mice**

Three groups of 10 mice each were intramuscularly inoculated at thigh muscle of hind legs with $10^{8.3}\text{EID}_{50}$, $10^{7.3}\text{EID}_{50}$, and $10^{6.3}\text{EID}_{50}$ of rL-RV, respectively; a fourth group of 10 mice was inoculated with $10^{8.3}\text{EID}_{50}$ of rL as a control. Three weeks after inoculation, sera were collected for NDV HI antibody and RV VNA assays. Meanwhile, the mice were challenged with 50 MLD$_{50}$ of RV GX/09. Significant NDV HI antibody was detected in all mice (Fig. 9AⅠ), but RV VNA was detected only in the blood of mice inoculated with rL-RV, not rL (Fig. 9AⅡ). Both the NDV HI antibody and RV VNA showed significant dose-dependent responses. The mean titers of RV VNA for the $10^{8.3}\text{EID}_{50}$, $10^{7.3}\text{EID}_{50}$, and $10^{6.3}\text{EID}_{50}$ dose groups were 12 IU, 6 IU, and 1.3 IU, respectively. The $10^{8.3}\text{EID}_{50}$ and $10^{7.3}\text{EID}_{50}$ doses elicited considerably higher levels of NDV HI antibody (Fig. 9AⅠ) and RV VNA (Fig. 9AⅡ) than did the $10^{7.3}\text{EID}_{50}$ dose. As shown in Fig. 9B, all mice inoculated with the $10^{8.3}\text{EID}_{50}$ or $10^{7.3}\text{EID}_{50}$ dose of rL-RVG survived from challenge of RV GX/09, and showed no signs of rabies. In the $10^{6.3}\text{EID}_{50}$ dose group, 6 of the 10 mice survived the challenge; 4 mice died within 12 days of exposure to RV GX/09. All mice in the rL inoculated group died within 12 days of exposure to RV GX/09. These mice showed severe neurologic signs, including...
hunching and hinder leg paralysis. Rabies virus antigens were detected in the brains of all of
the mice that died during the challenge. These results demonstrate that rL-RVG is highly
immunogenic and efficacious against rabies in mice.

Antibody responses induced by the recombinant NDV in dogs and cats

Dogs and cats represent two sizeable animal populations that need to be vaccinated for
rabies control. We, therefore, further evaluated the immunogenicity of rL-RVG in these
animals by using the immunization schedules detailed in the methods section. Three weeks
after the first dose, RV VNA was detected in all four groups of dogs (Fig. 10A). In the ERA
vaccinated dogs and the dogs vaccinated with $10^{9.8} \text{EID}_{50}$, $10^{9.3} \text{EID}_{50}$ and $10^{8.3} \text{EID}_{50}$ of rL-RV,
the mean titers of RV VNA were 20.1 IU (6/6 $\geq$ 1 IU), 30.7 IU (6/6 $\geq$ 1 IU), 21.6 IU (10/10 $\geq$ 1
IU), and 16.8 IU (10/10 $\geq$ 1 IU) respectively. Three weeks after the second dose, the mean
titers of RV VNA for the above four groups increased to 101.3 IU, 96 IU, 67.2 IU, and 70.4
IU, respectively. After that, the RV VNA gradually declined. At 22 weeks post-vaccination,
the mean titers of RV VNA for the four groups were 85.3 IU, 80.4 IU, 46.2 IU, and 40.6 IU,
respectively. There was no significant difference in RV VNA titers among all four vaccinated
groups at 22 weeks post-vaccination. From this point, the VNA to RV in all four groups of
dogs remained relatively stable. At 60 weeks post-vaccination, the mean titers of RV VNA in
the four groups were 5.8 IU (5/6 $\geq$ 1 IU), 9.8 IU (6/6 $\geq$ 1 IU), 12.4 IU (10/10 $\geq$ 1 IU), and 5.5
IU (10/10 $\geq$ 1 IU), respectively. After receiving the third dose at 60 weeks post-vaccination, all
four groups of dogs showed substantial re-boost responses to RV VNA. The titers of RV VNA
in all four groups rose sharply to 98.6 IU, 101.8 IU, 88.6 IU, and 82.8 IU, respectively, at 3
weeks post the third dose. In cats, all animals seroconverted to RV VNA following vaccination (Fig. 10C). The mean titers of RV VNA for the rL-RVG group at 3, 6, and 60 weeks post-vaccination were 30.8 IU (5/5 ≥ 1 IU), 65.2 IU, and 2.4 IU (5/5 ≥ 1 IU), respectively. For ERA-vaccinated cats, the mean titers of RV VNA at 3, 6, and 60 weeks post-vaccination were 15.8 IU (5/5 ≥ 1 IU), 54.8 IU, and 3.7 IU (5/5 ≥ 1 IU), respectively. After receiving the third dose, all cats experienced sizeable re-boost responses to RV VNA. The RV VNA titers in the rL-RVG and ERA groups rose sharply to 67 IU and 72 IU, respectively. No significant difference was seen in RV VNA titer between the rL-RVG and ERA groups.

Meanwhile, moderate levels of NDV HI antibodies were detected in all dogs (Fig. 10B) and cats (Fig. 10D) vaccinated with rL-RVG or rL at 3 weeks after the first dose, which increased slightly at 3 weeks after the second dose. For dogs, the mean NDV HI antibody titers in the $10^{9.8}\text{EID}_{50}$, $10^{9.3}\text{EID}_{50}$, and $10^{8.3}\text{EID}_{50}$ of rL-RVG vaccinated groups were 4.7, 4.6, and 3.8, respectively, at 3 weeks after the first dose, 24, 9.9, and 11.6, respectively, at 3 weeks after the second dose, and gradually decrease to 2.8, 2.2, and 2.0, respectively, at 60 weeks post-vaccination. After receiving the third dose at 60 weeks post-vaccination, all dogs in the rL-RVG-vaccinated groups experienced re-boost responses to the NDV HI antibody. The mean titers for the three groups increased to 32, 16.8, and 15.2, respectively (Fig. 10B). For cats, the mean NDV HI antibody titers in the rL-RVG vaccinated groups were 3.9, 12.1, and 2.2 at 3, 6, and 60 weeks post-vaccination. After receiving the third dose at 57 weeks after the second dose, all cats in the rL-RV vaccinated groups had re-boost responses to the NDV HI antibody. The mean NDV HI antibody titer increased to 17.7 at 3 weeks post-vaccination (Fig. 10D). Of note, none of the dogs or cats vaccinated with ERA showed any detectable NDV HI
antibody (Fig. 10B and D). These results demonstrate that rL-RVG is an immunogenic candidate rabies vaccine for dogs and cats.

No abnormal clinical signs were observed during the course of the study. These results suggest that the recombinant NDV rL-RVG is safe for dogs and cats, even after repeat inoculation with high dosages.

**Immunization protects dogs from street RV challenge**

To determine the protective efficacy of rL-RVG in dogs against rabies, we conducted challenge tests at 57 weeks after the second immunization doses. Five dogs immunized with $10^{8.3}\text{EID}_{50}$ of rL-RVG, three dogs immunized with ERA, three dogs inoculated with $10^{9.8}\text{EID}_{50}$ of vector NDV rL, and three dogs inoculated with PBS were randomly selected from each group and their individual RV VNA titers determined (Fig. 11A). All dogs were i.m. challenged with $2 \times 10^{5.5}\text{MLD}_{50}$ of RV GX/09 as described in the materials and methods. All animals that were vaccinated with rL-RVG and ERA survived the challenge and showed no clinical signs of rabies during the 12-week observation period. On the other hand, all six dogs inoculated with rL or PBS developed similar clinical signs of rabies 8–12 days post-challenge. Five of these dogs died within three weeks of the challenge and one dog was euthanized at 21 days post-vaccination (Fig. 11B). Rabies virus antigen was detected in the brains of all of the dead or euthanized dogs. All mice, intracerebrally inoculated with brain tissue from each of the dead or euthanized dogs, also developed signs of rabies and died within 12 days of inoculation. Rabies virus antigen was also detected in the brains of these mice. These results demonstrate that rL-RVG provides efficient and long-lasting protection in dogs against rabies.
Discussion

We generated a recombinant NDV that expresses rabies virus glycoprotein, rL-RVG, by using reverse genetics, and evaluated its potential as a novel vectored vaccine against rabies in animals. RVG expression enabled the rL-RVG to acquire the RV-like ability to spread from cell to cell. RVG was incorporated into the surface of the rL-RVG viral particles and did not alter the trypsin-dependent infectivity of the vector in mammalian cells. rL-RVG and rL showed similar sensitivity to VNAs against NDV and similar resistance to VNAs against RV. Animal studies demonstrated that rL-RVG is safe in poultry, mice, cats, and dogs, even at multiple doses as high as $10^{9.8} \text{ EID}_{50}$ in the latter two species. Vaccination with rL-RVG induced significant RV VNA responses and provided complete protection from RV street virus challenge. Most importantly, rL-RVG induced strong and long-lasting protective immune responses to RV in dogs and cats. The vaccinated dogs were completely protected from RV street virus challenge after one year, displaying no signs of disease or death. This is the first study to demonstrate that intramuscular immunization with an NDV-vectored vaccine can induce long-lasting systemic protective immunity against rabies in animals, and this vaccine may also have potential use in high-risk human individuals to control the rabies infections.

NDV acquires its envelope from the host cell plasma membrane and incorporates foreign glycoproteins at the envelope (10, 17, 20, 46). The ability of foreign proteins to function as new receptor binding proteins has also been demonstrated in other paramyxoviruses (13). RVG mediates both receptor binding and penetration (15, 26), and is an important virulence factor for the virus (22, 58). The introduction of the RVG gene does not increase the virulence of the NDV vector. Recombinant rL-RVG grew to slightly lower titers compared to those of
rL in embryonated eggs and in cell culture in the presence of trypsin. This may be due to
the effects of foreign gene insertion in NDV genome (38, 46). Considering this, it was
remarkable to find that rL-RVG grows to a much higher titer in BHK-21 cells, compared to
rL, in the absence of trypsin. As the virons of rL-RVG generated from BHK-21 cells in the
absence of trypsin lack the ability to re-inf ect BHK-21 cells, and the infectivity of rL-RVG
could be inhibited only by VNAs to NDV but not RV, it is more likely that the change in the
growth property of rL-RVG was the result of RVG expression enabling the vector to spread
from cell to cell. The RVG protein on the cell surface mediates fusion with adjacent cells,
resulting in the transportation of the viral genome and transcription complex of NDV from
one cell to the next. This hypothesis is supported by our finding that the spread of rL-RVG
could be inhibited by the presence of VNAs to RV but not by VNAs to NDV.

Previous study showed that progeny rabies virions that bud from infected cells can
spread to contiguous or to non-contiguous cells, which are surrounded by the interstitial space
(16). In the case of direct spread from cell to cell, the pathogenic RV can spread despite the
presence of rabies VNA, whereas spread of non-pathogenic RV is prevented in the presence
of rabies VNA (16, 23). In our study, rL-NDV was generated with the RVG gene from a
non-pathogenic RV, the ERA strain, which may explain why the cell to cell spread of
rL-RVG could be completely prevented by the presence of VNAs to RV.

Our result that rL-RVG is highly sensitive to VNA to NDV but resistant to rabies VNA
differs from reports with recombinant HPIV3 expressing the GP of Ebola virus (13, 65). In
these studies, HPIV3 VNA completely neutralized the HPIV3 vector alone and weakly
neutralized the infectivity of the recombinant HPIV3 expressing Ebola virus GP on the virion
envelope. The virions containing both the HN/F glycoproteins of HPIV3 and the GP of Ebola virus were more efficiently neutralized by Ebola VNAs than by HPIV3 VNAs despite the greater amount of HPIV3 glycoproteins (13, 65). The reasons for these observations are not fully understood. One possibility is that the Ebola virus GP incorporated on the surface of the HPIV3 particle could mask the neutralization epitopes of the HN and F glycoproteins (55).

NDV is a strong inducer of the interferon response in mammalian cells and is highly sensitive to the interferon induced in such cells (6, 7). The V protein encoded by NDV functions as an alpha interferon antagonist and is usually less efficient in mammalian cells (31, 51). In mammals, the localized release of limited amounts of progeny virus at the primary infection site is not only dependent on trypsin-like proteases, but also on the innate immunity of the host cells (10, 20). In fact, a previous study suggests that the F protein cleavage site does not have the same impact on virulence of NDV in primates as it has in poultry (19). Our results confirm that rL-RVG and rL induce similar levels of, and show similar sensitivity to, interferon in mammalian cells. Therefore, rL-RVG replication should still be limited by the innate immune response in mammalian hosts. This may partially explain why rL and rL-RVG could not be detected in mice two days after infection, even though RVG expression enables the NDV vector to spread from cell to cell in vivo.

Our results also show that RVG expression by NDV is not associated with disease in poultry, mice, cats, or dogs. The MDT, IVPI, and ICPI assays demonstrated that recombinant rL-RVG retained the non-virulent property of rL to poultry; there was no difference in virus replication or tissue distribution in mice between rL and rL-RVG after infection, which further confirms that RVG expression in vivo did not confer RV-specific pathogenicity to the
NDV vector. This is not the first time that a lack of correlation between cell pathologic effect (CPE) in vitro and virulence in vivo has been observed. Previous studies have reported that the increased CPE in vitro was accompanied by a decrease in the replication of recombinant HPIV3 expressing Ebola virus GP, compared to that of HPIV3 vector, which was not associated with increased virulence in guinea pigs or non-human primates (13, 65). For RSV, increasing the expression of the fusion F glycoprotein, by repositioning its gene in the genome, resulted in increased CPE in vitro, but no change in virulence was observed in mice (37). Also, RSV with a deletion in its M-2 regulatory protein showed a considerable increase in CPE in vitro, but was highly attenuated in chimpanzees (8). The precise mechanism for the attenuation of recombinant virus in vivo requires further investigation.

The development of an immune response against one particular vector would probably make that vector ineffective for subsequent vaccination in the same individual. The prevalence of immunity to a vector, such as vaccinia virus or adenovirus type 5 vector virus, would restrict the replication of the recombinant virus vaccine, resulting in a reduced immune response to the expressed foreign antigen (33, 44, 56, 66). This does not seem to be a problem for the NDV vector, as most of the rabies vaccine target population, including cats and dogs, is sero-negative for the NDV vector. Furthermore, the second dose induced sizeable boost immune responses, as has been documented with several other virus-vectored vaccines (27) (11, 14, 25, 61). For HPIV3 and goat poxvirus-vectored vaccines, even interference from preexisting immunity against the vector virus could be completely overcome by two doses of vaccine (11, 14). Our results here also showed that a second dose of rL-RVG, received 3 weeks after the first dose, induced significant boost responses in cats and dogs. The third dose,
received 3 weeks after the second vaccination, did not induce boost responses to either RVG or NDV, indicating that the second vaccination induced a sufficient immune response to prevent the replication of rL-RVG in the animals (data not shown). However, the third dose, received one year later, induced significant immune responses to both RVG and NDV, again indicating that the immunity to the NDV vector would not be strong enough to restrict the replication of recombinant viruses one year later. Moreover, when we used rL-RVG to immunize dogs that had received traditional live rabies vaccine or inactivated rabies vaccine one year earlier, or used traditional live rabies vaccine or inactivated rabies vaccine to immunize the dogs that had received rL-RVG one year earlier, the RV VNAs always induced significant boost responses in all tested animals (data not shown).

rL-RVG grows to similar titers as rL in chicken eggs, which indicates that its manufacture is technically and economically feasible in developing countries. A dosage as low as $10^{8.3}\text{EID}_{50}$, i.e., almost a thirty-fold dilution of 1 ml of egg allantoic fluid, induced an acceptable and durable neutralization antibody response in dogs and provided complete protection from RV street virus challenge after one year. This is in contrast to some other vector systems, such as those based on replication-deficient adenoviruses, which require costly helper cell lines and high-dose immunizations.

NDV-based vaccines are generally administered via the respiratory tract when used to vaccinate against respiratory diseases of poultry (5). In non-human primates, the potency of an NDV-vectored vaccine has been demonstrated via the respiratory tract route (10, 17, 18, 20). Potency in bovines via both the respiratory tract and intramuscular routes has also been demonstrated (34, 36). It is interesting that the intratracheal, but not intranasal route is
essential for induction of humoral immunity in non-human primates (19). This phenomenon might be explained by the fact that NDV replicates poorly in the upper respiratory tract of mammals due to the lower temperature there compared with this site in birds (19). Although the intratracheal route is more likely to induce mucosal immune responses, in this study, we only test the NDV-vectored vaccine via the intramuscular route, since this route should be more practicable than the intratracheal route in dogs and cats for mass application.

Acknowledgements

We thank Susan Watson for editing the manuscript, Dr. Bernard Moss for providing the modified vaccinia strain Ankara expressing the T7 RNA polymerase. This work was supported by Chinese National S&T Plan (2009ZX10004-214), by the grant from Chinese Ministry of Agriculture (200803014) and by the GHI program of Emory University.
References


10. Bukreyev, A., Z. Huang, L. Yang, S. Elankumaran, M. St Claire, B. R. Murphy, S.


range-restricted virus, as a vaccine vector for intranasal immunization against emerging pathogens. Proc Natl Acad Sci U S A 104:9788-9793.


2006. Protection against respiratory syncytial virus by a recombinant Newcastle

McCoy, K., N. Tatsis, B. Korioth-Schmitz, M. O. Lasaro, S. E. Hensley, S. W. Lin,
2007. Effect of preexisting immunity to adenovirus human serotype 5 antigens on the
immune responses of nonhuman primates to vaccine regimens based on human- or

Afzal, S. Chrolavicius, S. S. Jolly, P. Widimsky, A. Avezum, H. J. Rupprecht, J.
360:2165-2175.

Garcia-Sastre, and P. Palese. 2001. Recombinant Newcastle disease virus as a

Nakaya, Y., T. Nakaya, M. S. Park, J. Cros, J. Imanishi, P. Palese, and A.
Garcia-Sastre. 2004. Induction of cellular immune responses to simian
immunodeficiency virus gag by two recombinant negative-strand RNA virus vectors. J
Virol 78:9366-9375.

Neumann, G., M. A. Whitt, and Y. Kawaoka. 2002. A decade after the generation of
a negative-sense RNA virus from cloned cDNA - what have we learned? J Gen Virol
83:2635-2662.


Figure legends.

Fig. 1. Generation of recombinant NDV expressing the RVG gene. (A) Schematic representation of the rL genome with the PmeI site introduced between the P and M genes and RVG inserted at the PmeI site. (B) Immunofluorescence analysis of RVG protein expression. Confluent BHK-21 cells were infected with rL or rL-RVG at an MOI of 0.5. The infected cells were fixed and probed with chicken serum against NDV and mouse serum against RV and then incubated with an FITC-conjugated rabbit anti-chicken antibody or a TRITC-conjugated goat anti-mouse antibody. Cell nuclei were stained with DAPI. Cells were analyzed by using a confocal laser microscope. (C) Western blot analyses of recombinant NDV expressing RVG. Lysates of BHK-21 cells infected with rL or rL-RVG was incubated with chicken serum against NDV, mouse serum against RV, or an anti-β-actin monoclonal antibody. Binding was visualized with 3, 3-diaminobenzidine reagent after incubation with peroxidase-conjugated secondary antibodies. The locations of marker proteins are indicated on the left and the anti-serum or antibody used is indicated on the right. (D) Growth properties of recombinant viruses in embryonated eggs. rL and rL-RVG (0.1 ml of 100 EID$_{50}$) were inoculated into the allantoic cavities of 10-day-old embryonated eggs, and the allantoic fluid of six eggs from each group was harvested at the time points indicated and pooled for EID$_{50}$ determination in eggs. The data shown were mean of three experiments; the error bar indicated the standard deviation (S.D.).

Fig. 2. Cell-to-cell spread of recombinant NDV and vector in BHK-21 cells. (A) Monolayers of BHK-21 cells were infected with either rL or rL-RVG at an MOI of 0.05. After five washes, 1 h post-infection, the infected cells were incubated at 37°C. The infected
cells were then examined at indicated hours post-infection by using IFA with chicken serum against NDV as described in the materials and methods. (B) The percentage of infected cells after infection with rL and rL-RVG at different times was calculated based on the results of the IFA. (C) Cell-to-cell spread inhibition assay for recombinant virus in BHK-21 and NA cells. Monolayers of BHK-21 (I) and NA cells (II) were infected with either rL or rL-RVG at an MOI of 0.05. After five washes, 1 h post-infection, the cells were incubated with culture medium containing 100-fold diluted mouse serum against NDV, mouse serum against RV, or naïve mouse serum. infected cells were examined 48 h post-infection by using IFA with chicken serum against NDV.

**Fig. 3. Kinetics of infectious virus replication in BHK-21 cells.** Monolayers of BHK-21 cells were infected with either egg propagated rL or rL-RVG at an MOI of 0.01 (A, B) or an MOI of 5 (C, D), respectively. After five washes, 1 h post-infection, the cells were incubated with 100-fold diluted mouse serum against NDV for 30 minutes to neutralize the residual of viruses in the supernatants. After replacing the medium with fresh medium, the infected cells were incubated at 37°C in the presence (A, C) or absence (B, D) of TPCK trypsin (1µg/ml). The culture supernatants were collected at different times and their virus titers were determined as EID50s in 10-day-old embryonated eggs. The data shown were mean titers plus S.D. of five independent experiments. Significant differences between rL and rL-RVG were assessed by using the t test, **P < 0.01.

**Fig. 4. RVG incorporation into recombinant virus particles.** (A) Electron microscopy of recombinant and vector virus particles. rL or rL-RVG propagated in eggs was prepared and partially purified by centrifugation through 20% sucrose. Viruses were stained with IgG
purified from mouse naïve serum, mouse serum against NDV, or mouse serum against RV and with goat anti-mouse IgG conjugated with colloidal gold, and then by negatively stained. (B) SDS-PAGE of recombinant and vector virus particles. rL and rL-RVG were propagated in eggs and purified by differential centrifugation and sedimentation through 40% to 60% (wt/vol) sucrose gradients. Viral proteins were analyzed by using SDS-PAGE with 10% gels under reducing conditions (I) and were subjected to Western blot analyses with mouse serum against RV (II).

Fig. 5. Analysis of the trypsin-dependent infectivity of recombinant and vector viruses in BHK-21 and NA cells. rL and rL-RVG, propagated in BHK-21 cells in the presence (TPCK+) or absence (TPCK-) of trypsin, were used to infect BHK-21 (A) or NA cells (B) at a dose of 200 EID$_{50}$. The infections of rL and rL-RVG were examined by using IFA with chicken serum against NDV at 24 and 48 h post-infection, respectively.

Fig. 6. Neutralization assay for the infectivity of recombinant and vector viruses. rL and rL-RVG (104 EID50) were mixed with serially diluted mouse serum against NDV, mouse serum against RV, or naïve mouse serum, and incubated for 1 h at 37°C. The residual infectious viruses were quantified by titration in chicken eggs. The neutralization titers of anti-sera were defined as the dilution for 50% inhibition of infection. The data shown were mean titers plus S.D. of five independent experiments.

Fig. 7. Interferon induction and inhibition in a human cell line. (A) Type I interferon induced in A549 cells after infection with rL and rL-RVG. The inhibition units (InU) were defined as the final dilution to give 50% inhibition of infection. (B) The sensitivity of rL and rL-RVG to type I interferon in A549 cells. The minimal InUs to inhibit 50% infection of rL
and rL-RVG were determined in A549 cells. The data shown were mean plus S.D. of five independent experiments.

Fig. 8. Pathogenicity evaluation in chickens and mice. (A) Pathogenicity assay in SPF eggs and chickens. The MDT, ICPI, and IVPI were determined as described in the materials and methods. (B) Weight changes of mice inoculated with recombinant and vector viruses. Groups of five mice were inoculated i.m. at thigh muscle of hind leg (I) or i.c. (II) with $10^{8.3}$EID$_{50}$ (in 30 µl) of rL or rL-RVG, and observed and weighed daily for 14 days. All mice survived the duration of the experiment. Body weight changes for each group are shown as ratios of the body weight at day 0, which was set as 100.

Fig. 9. Immunogenicity and protective efficacy in mice. (A) Immunization assay. Groups of ten 4-week-old mice were injected with $10^{8.3}$, $10^{7.3}$, and $10^{6.3}$ EID$_{50}$ of rL-RVG, and with $10^{8.3}$ EID$_{50}$ of rL as a control. Three weeks after vaccination, blood samples were collected to detect HI antibodies to NDV (I) and VNA to RV (II) as described in the Materials and Methods. The data shown are mean plus S.D. Significant differences between groups were assessed by using the t test, ** $P < 0.01$; * $P < 0.05$. (B) Mouse challenge. Mice were challenged i.m. with 50 MLD$_{50}$ of the RV street virus GX/09 at 3 weeks post-vaccination and observed for 3 weeks. Percentage survival at different days post-challenge was recorded.

Fig. 10. Immunogenicity evaluation in dogs and cats. Three-month-old Beagle dogs were placed in four groups (6 or 10 each). Three groups were administrated 1 ml of $10^{9.8}$EID$_{50}$ (n=6), $10^{9.3}$EID$_{50}$ (n=10), and $10^{8.3}$EID$_{50}$ (n=10) of rL-RVG, respectively. A fourth group was inoculated with 1 ml of $10^{6}$ FFU of ERA (n=6). Two groups of five cats each were given three doses of $10^{9.8}$EID$_{50}$ of rL-RVG and $10^{6.0}$ FFU of ERA, respectively. At 3 and 60 weeks post
initial vaccination, the dogs and cats received second and third doses, respectively. The serum samples from dogs (A, B) and cats (C, D) were collected at different times after vaccination and the RV VNA titers (A, C) and NDV HI antibody titers (B, D) were detected. The antibody titers for each group of animal was indicated as mean plus S.D. Statistically significant differences were determined by using the $t$ test, $* P<0.05$.

**Fig. 11. Challenge study in dogs.** Dogs received $10^{8.3}\text{EID}_{50}$ of rL-RVG (n=5), $10^{6.0}$ FFU of ERA (n=3), $10^{9.8}\text{EID}_{50}$ of rL (n=3), or PBS (n=3) and were then challenged with $2\times10^{5.5}\text{MLD}_{50}$ of rabies street virus GX/09 at 57 weeks after the second dose. The RV VNA titer for each animal was determined before challenge (A), and each bar shows the RV VNA titer of an individual dog. After viral challenge, the dogs were observed daily for clinical signs of rabies for 12 weeks. The percentage survival for the different groups at different times post-challenge was recorded (B).
### A

<table>
<thead>
<tr>
<th>Viruses</th>
<th>Pathogenicity in eggs and chickens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MDT (h)</td>
</tr>
<tr>
<td>rL</td>
<td>&gt;120</td>
</tr>
<tr>
<td>rL-RVG</td>
<td>&gt;120</td>
</tr>
</tbody>
</table>

### B

#### I

- rL
- rL-RVG
- PBS

#### II

- rL
- rL-RVG
- PBS

Bar charts showing % body weight over time post-inoculation (days) for different treatments.